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Photodynamic Inactivation of *Klebsiella pneumoniae* Biofilms and Planktonic cells by 5-Aminolevulinic Acid and 5-Aminolevulinic Acid Methyl Ester
--Manuscript Draft--

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Abstract:	<p>The treatment of <i>Klebsiella pneumoniae</i>, particularly extended-spectrum β-lactamase (ESBL)-producing <i>K. pneumoniae</i>, is currently a great challenge. Photodynamic antimicrobial chemotherapy is a promising approach for killing antibiotic-resistant bacteria. The aim of this study was to evaluate the capacity of 5-aminolevulinic acid (5-ALA) and its derivative 5-ALA methyl ester (MAL) in the presence of white light to cause photodynamic inactivation (PDI) of <i>K. pneumoniae</i> planktonic and biofilm cells. In the presence of white light 5-ALA and MAL inactivated planktonic cells in a concentration-dependent manner. Biofilms were also sensitive to 5-ALA and MAL-mediated PDI. The mechanisms by which 5-ALA and MAL caused PDI of ESBL-producing <i>K. pneumoniae</i> were also investigated. Exposure of <i>K. pneumoniae</i> to light in the presence of either 5-ALA or MAL induced cleavage of genomic DNA and the rapid release of intracellular biopolymers. Intensely denatured cytoplasmic contents and</p>	

aggregated ribosomes were also detected by transmission electron microscopy. Scanning electron microscopy showed that PDI of biofilms caused aggregated bacteria to detach and that the bacterial cell envelope was damaged. This study provides insights into 5-ALA and MAL-mediated PDI of ESBL-producing *K. pneumoniae*.

Photodynamic Inactivation of *Klebsiella pneumoniae* Biofilms and Planktonic cells by 5-Aminolevulinic Acid and 5-Aminolevulinic Acid Methyl Ester

Abstract The treatment of *Klebsiella pneumoniae*, particularly extended-spectrum β -lactamase (ESBL)-producing *K. pneumoniae*, is currently a great challenge. Photodynamic antimicrobial chemotherapy is a promising approach for killing antibiotic-resistant bacteria. The aim of this study was to evaluate the capacity of 5-aminolevulinic acid (5-ALA) and its derivative 5-ALA methyl ester (MAL) in the presence of white light to cause photodynamic inactivation (PDI) of *K. pneumoniae* planktonic and biofilm cells. In the presence of white light 5-ALA and MAL inactivated planktonic cells in a concentration-dependent manner. Biofilms were also sensitive to 5-ALA and MAL-mediated PDI. The mechanisms by which 5-ALA and MAL caused PDI of ESBL-producing *K. pneumoniae* were also investigated. Exposure of *K. pneumoniae* to light in the presence of either 5-ALA or MAL induced cleavage of genomic DNA and the rapid release of intracellular biopolymers. Intensely denatured cytoplasmic contents and aggregated ribosomes were also detected by transmission electron microscopy. Scanning electron microscopy showed that PDI of biofilms caused aggregated bacteria to detach and that the bacterial cell envelope was damaged. This study provides insights into 5-ALA and MAL-mediated PDI of ESBL-producing *K. pneumoniae*.

Keywords PACT, *Klebsiella pneumoniae*, Biofilms, 5-ALA, MAL

Introduction

Klebsiella pneumoniae can cause serious infections such as liver or splenic abscess, pneumonia, empyema, and endophthalmitis [1]. Due to the extensive use of antibiotics, extended-spectrum β -lactamase (ESBL)-producing *K. pneumoniae* is now

1 a serious public health concern [2]. Since ESBLs can hydrolyse many β -lactam
2 antibiotics, such as penicillins and expanded-spectrum cephalosporins, carbapenems
3 which are resistant to hydrolysis, are often used to treat patients who have in cases
4 serious infections with ESBL-producing *K. pneumonia* [3]. However, the increasing
5 rate of carbapenem resistance in recent years necessitates the development of
6 alternative approaches for treating ESBL-producing *K. pneumoniae* infections [4].
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10 One promising approach against antibiotic-resistant bacteria is photodynamic
11 antimicrobial chemotherapy (PACT). This involves the use of a photosensitizer (PS)
12 which upon illumination with light of an appropriate wavelength is excited from a
13 ground state to a triplet state. The triplet state PS reacts with oxygen present in and
14 around the bacteria, resulting in the formation of singlet oxygen or other
15 reactive-oxygen species (ROS) [5]. The ROS can destroy the plasma membrane and
16 other biopolymers, thus resulting in non-specific killing of bacteria [6, 7]. The
17 non-specific nature of the inactivation of bacteria by singlet oxygen and ROS makes it
18 unlikely that bacteria will develop resistance and thus photodynamic therapy is seen
19 as a promising alternative to current antimicrobial agents [8]. Previous studies have
20 reported that *K. pneumoniae* is sensitive to lethal photodynamic inactivation (PDI)
21 mediated by a variety of PSs, such as toluidine blue O [9], methylene blue [10], and
22 rose bengal [11].
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40 In addition to PSs, other molecules such as 5-aminolevulinic acid (5-ALA),
41 which are not in their own right PSs, but are precursors for PSs, are gaining interest.
42 5-ALA is a metabolic intermediate produced by a rate limiting step in the tetrapyrrole
43 biosynthetic pathway which is essential for production of porphyrins such as haem.
44 When provided exogenously to cells 5-ALA uptake results in the over production and
45 accumulation of photoactive porphyrins since the pathway is no longer rate limited.
46 Uptake of 5-ALA and subsequent synthesis and accumulation of porphyrins is highest
47 in cells that are rapidly dividing, with high metabolic activity, which results in
48 selectivity towards bacteria over normal host cells of the cell [12]. Recently,
49 5-ALA-mediated PACT has been investigated to inactivate several important
50 antibiotic-resistant bacteria, including methicillin-resistant *Staphylococcus aureus*
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1 (MRSA) [13, 14] and multidrug-resistant (MDR) *Escherichia coli* [14, 15]. A
2 previous study reported by Yow *et al.* demonstrated that 5-ALA was not effective at
3 mediating PDI of *K. pneumoniae* [16]. However the methodology used by these
4 authors was not optimal because 5-ALA is not a PS, it is a precursor used by cells to
5 generate endogenous PS. Therefore in this study we have reexamined the potential of
6 5-ALA and its derivative 5-ALA methyl ester (MAL) to mediate the PDI of three *K.*
7 *pneumoniae* strains, either grown as biofilms or as planktonic cultures. The
8 mechanisms by which PDI of ESBL-producing *K. pneumoniae* occurred were also
9 investigated.

10 **Materials and methods**

11 **Bacterial strains and culture conditions**

12 Three *K. pneumoniae* strains were obtained from the First Affiliated Hospital of Xi'an
13 Jiaotong University, Xi'an, China: ATCC 700603, a clinical isolate of
14 non-ESBL-producing *K. pneumoniae*, and a clinical isolate of ESBL-producing *K.*
15 *pneumoniae*. Tryptone Soy Agar (TSA) and Tryptone Soy Broth (TSB) were obtained
16 from Shanghai Biotech, China, and Qingdao Rishui Biotech, China, respectively. For
17 the experiments, a single colony from TSA plates was inoculated into 10 mL TSB and
18 incubated at 37°C for 24 h. A 100- μ L aliquot of the culture was transferred into 10 mL
19 of fresh TSB and incubated at 37°C to attain log-phase growth. The pellets were
20 harvested by centrifugation (4000 rpm for 10 min, Thermo Fisher D-37520,
21 Germany), followed by washing three times with phosphate-buffered saline (PBS, pH
22 7.4). The pellets were resuspended and diluted with PBS or TSB to a density of $1 \times$
23 10^7 colony forming units (CFU)/mL prior to the experiments.

24 **Chemicals and light source**

25 5-ALA and MAL were purchased from Sigma-Aldrich, China, and TCI, China,
26 respectively. Stock solutions (10 mM) were freshly prepared by dissolving 5-ALA or
27 MAL in PBS or TSB and were stored in the dark. The solutions were filtered through
28 a 0.22- μ m filter disk before use. All illuminations were performed with white light

1 from a 150 W xenon lamp (Ceaulight CEL-HXF300, China). A wavelength range
2 between 400 and 780 nm was selected by optical filters. To avoid heating the samples,
3 the light was passed through a 1-cm water filter. The fluence rate at the level of the
4 samples was 100 mW cm^{-2} , as measured with a power meter (Ceaulight CEL-NP2000,
5 China).
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10 PDI on bacteria

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12 Samples of the bacterial suspension (1 mL , $1 \times 10^7 \text{ CFU/mL}$) were centrifuged at
13 4000 rpm for 10 min. The pellets were resuspended in 1 mL of ALA or MAL
14 solutions of varying concentrations and incubated in the dark at 37°C for 4 h in a
15 shaking incubator (100 rpm). The samples were transferred to sterile 35-mm
16 polystyrene culture dishes and irradiated for 60 min. After irradiation, bacterial
17 suspensions were centrifuged at 4000 rpm for 10 min. The pellets were resuspended
18 and serially diluted 10-fold with s PBS, and $20 \mu\text{L}$ of each dilution was spread in
19 triplicate on TSA. Colonies were counted after 24 h incubation at 37°C . The fraction
20 of surviving bacteria was calculated as the CFU/mL after exposure to light divided by
21 the CFU/mL prior to light exposure.
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34 Biofilm formation

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36 The tissue culture plate method [17] was used to screen for biofilm formation.
37 Bacterial suspensions were diluted to $1 \times 10^6 \text{ CFU/mL}$ in TSB and $200 \mu\text{L}$ of the
38 diluted suspension was inoculated into the wells of a 96-well polystyrene microplate
39 (Corning, USA). After incubation at 37°C for 24h the medium was removed and the
40 biofilms were washed twice with PBS. The biofilm was fixed with 95% ethanol for 10
41 min and stained with $200 \mu\text{L}$ of a 0.1% (w/v) aqueous solution of crystal violet for 15
42 min at room temperature. The wells were washed twice with PBS to remove excess
43 stain. After drying at 37°C for 2 h, biofilm formation was quantified by solubilisation
44 of the crystal violet stain in $200 \mu\text{L}$ of 30% (w/v) glacial acetic acid for 10 min with
45 shaking at 200 rpm, and absorbance was read on a multidetection microplate reader
46 (Thermo Fisher 1510, Finland) at 492 nm ($A_{492\text{nm}}$). $A_{492\text{nm}} > 0.240$ was indicative of
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1 biofilm formation.

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3 PDI of the bacterial biofilm

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6 A bacterial suspension (2 mL; 1×10^6 CFU/mL) was inoculated into the wells of a
7 sterile 24-well polystyrene microplate (Corning, USA) that contained sterile glass
8 coverslips and incubated at 37°C for 24 h. The culture medium was removed and the
9 biofilms on coverslips were gently washed twice with PBS to remove loosely
10 adherent bacteria. Then, the coverslips were removed with sterilized forceps and
11 placed into the wells of a new 24-well microplate containing 2 mL 10 mM 5-ALA or
12 MAL. The microplate was incubated at 37°C for 4 h in the dark. After irradiation for
13 60 min, the coverslips were gently washed twice with PBS and placed into the wells
14 of another 24-well microplate. The biofilms were resuspended in 2 mL of PBS and
15 dislodged by ultrasonication (Hangzhou Front Ultrasoni FRQ-1002T, China) for 10
16 min, followed by rapid vortexing with a vortex mixer (Haimen Qilinbeier QL-901,
17 China) for 1 min. Bacterial suspensions were serially 10-fold diluted with PBS, and
18 20 μ L of each dilution was plated in triplicate on TSA. Colonies were counted after 24
19 h incubation at 37°C.
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35 Genomic DNA purification and electrophoresis

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38 To determine if DNA damage was one of the mechanisms behind PDI of
39 ESBL-producing *K. pneumonia*, genomic DNA was extracted and analysed by
40 agarose gel electrophoresis. After irradiation, genomic DNA was immediately
41 extracted from the bacteria using a Genomic DNA Purification Kit (Promega, USA).
42 DNA samples were gently mixed with 6X loading-buffer (0.25% w/v bromophenol
43 blue, 40% w/v sucrose, 1.15% acetic acid, 40 mM Tris, 1 mM EDTA), and were
44 analysed by electrophoresis in a 1% agarose gel in Tris/Borate/EDTA buffer (TBE, 90
45 mM Tris-HCl, 90 mM boric acid, and 2 mM EDTA, pH 8) at 2.9 V cm^{-1} for 1.5 h.
46 Ethidium bromide (1 μ g/mL) was incorporated into the agarose gel. A Lambda
47 DNA/HindIII digest marker (Promega, USA) was used as the molecular weight
48 marker, with DNA fragments between 125 to 23,130 bp.
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Integrity of cell membrane

The integrity of the cell membrane was examined by measuring the release of materials absorbing at 260 nm [18-21]. After irradiation, ESBL-producing *K. pneumoniae* was centrifuged at 4000 rpm for 10 min. The pellets were discarded and the material released into the supernatant was measured by scanning UV-Visible spectroscopy (Agilent 8453, USA) at room temperature.

Transmission electron microscopy (TEM)

TEM samples were prepared according to a previously described method [21, 22]. After irradiation, ESBL-producing *K. pneumoniae* was centrifuged at 4000 rpm for 10 min and fixed in 2.5% glutaraldehyde (SCRC, China) at 4°C for 2 h. The pellets were washed with PBS three times and fixed in 1% osmium tetroxide (Johnson Matthey, England) at 4°C for 2 h. The pellets were dehydrated with gradients of ethanol and embedded in Epon 812 epoxy resin (SPI-Chem, USA) at 60°C for 24 h. Thin-section samples of 50–70 nm were prepared using a LKB-V ultratome (LKB, Sweden). The samples were stained with uranyl acetate and lead citrate for 15 min. Finally, the samples were viewed and digitally photographed using a TEM (Hitachi H-7650, Japan).

Scanning electron microscopy (SEM) analysis of biofilms

SEM was used to observe morphologic alterations in bacterial biofilms. Biofilms of ESBL-producing *K. pneumoniae* on coverslips were washed with PBS three times and fixed in 2.5% glutaraldehyde at 4°C for 2 h. Then, the coverslips were washed with PBS three times and fixed in 1% osmium tetroxide at 4°C for 2 h. After dehydration with gradients of ethanol, the samples were freeze-dried, sputter-coated with gold, and observed using a SEM (Hitachi TM-1000, Japan).

Results

PDI of planktonic cells

5-ALA did not exhibit obvious dark toxicity for the three *K. pneumoniae* strains at the

1 concentrations tested (Fig. 1). In contrast, the irradiated groups showed reduced
2 bacterial survival with increasing concentrations of 5-ALA. When a concentration of
3 10 mM 5-ALA was used, 3.68, 3.17, and 3.20 log₁₀ reductions in the surviving
4 fraction were observed for ATCC 700603, the non-ESBL-producing clinical isolate,
5 and the ESBL-producing clinical isolate, respectively. MAL induced some dark
6 toxicity in a concentration dependent manner. In the dark 10 mM MAL caused 1.04,
7 0.92, and 0.96 log₁₀ reductions in the surviving fraction of ATCC 700603, the
8 non-ESBL-producing clinical isolate, and the ESBL-producing isolate, respectively.
9 After irradiation of MAL treated cells, there were reductions in bacterial survival of
10 4.80, 4.32, and 4.52 log₁₀ for ATCC 700603, the non-ESBL-producing clinical isolate,
11 and the ESBL-producing isolate, respectively. All of the strains were also irradiated
12 without 5-ALA or MAL treatment and this did not cause any change in bacterial
13 survival.
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28 PDI of biofilms

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30 In the 96 well tissue culture plate analysis of biofilm formation A_{492nm} values of 0.73,
31 2.07, and 1.39 were obtained for ATCC 700603, the non-ESBL-producing clinical
32 isolate, and the ESBL-producing clinical isolate, respectively. These values exceeded
33 the lower A_{492nm} limit of 0.240, which is indicative of biofilm-producing bacterial
34 strains. As shown in Fig. 2, no significant difference in the surviving fraction was
35 found for the biofilms treated with 5-ALA or MAL in the dark compared to untreated
36 biofilms (*P* > 0.05). After treatment with 10 mM 5-ALA and irradiation for 60 min,
37 3.09, 1.92, and 2.28 log₁₀ reductions in the surviving fraction were achieved for the
38 biofilms of ATCC 700603, the non-ESBL-producing clinical isolate, and the
39 ESBL-producing clinical isolate, respectively. Greater reductions in the surviving
40 fraction of bacteria in biofilms were seen after treatment with 10 mM MAL and
41 irradiation for 60 min, with 4.25, 3.49, and 3.91 log₁₀ reductions observed for ATCC
42 700603, the non-ESBL-producing clinical isolate, and the ESBL-producing clinical
43 isolate, respectively. These results demonstrate that biofilms formed by the tested
44 strains are sensitive to the 5-ALA and MAL-mediated PDI.
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Photodynamic effect on genomic DNA

As shown in Fig. 3, the genomic DNA isolated from the ESBL-producing *K. pneumoniae* strain incubated with 5-ALA or MAL in the dark migrated the same distance as DNA from untreated cells suggesting that these molecules do not cause macroscale DNA damage. However after irradiation of 5-ALA or MAL treated cells degradation of genomic DNA was observed indicating that 5-ALA and MAL-mediated PDI induced DNA damage in ESBL-producing *K. pneumoniae*.

Leakage of intracellular biopolymers

As an indication of membrane damage the release of intracellular components was monitored by scanning spectroscopy. The results of scanning UV-Vis spectroscopy are shown in Fig. 4. The absorbance at 260nm of the supernatant from the untreated bacteria was the same as the supernatants from bacteria incubated with 5-ALA or MAL in the dark (the inner plot). A slight increase in the absorption at 260 nm of the supernatant obtained from bacteria which had been sonicated for 15min was observed. The supernatants from bacteria which had been treated with 10 mM 5-ALA or MAL and irradiated for 60 min exhibited a large increase in absorption at 260 nm, suggesting that the functionality of the membrane had been impaired allowing the release of intracellular biopolymers.

Photodynamic effect on cellular structure

The ESBL-producing *K. pneumoniae* clinical isolate was analysed by TEM to determine if the photodynamic effect mediated by 5-ALA and MAL caused any cell structure changes at the morphological level. Representative results are shown in Fig. 5. The normal rod-shaped structure was observed for bacteria which had not been treated (Fig. 5 A) and for cells treated with 5-ALA or MAL in the dark (Fig. 5 B and Fig. 5 C). In these images the cell wall and plasma membrane are well differentiated. The ribosomes are dispersed in the cytoplasm, dark particles, with nucleic acid differentiated as a clear zone. Fig. 5 D and 5 E show images of bacteria treated with 10 mM 5-ALA or MAL and irradiated for 60 min, respectively. Damage to the

1 bacterial cells can be seen in these images with condensation of ribosomes resulting in
2 a low-density area in the centre of the bacterial cells and in the case of Fig 5 E
3 filamentous structures in the cell centre.
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7 SEM results

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10 Biofilms of the ESBL-producing *K. pneumoniae* clinical isolate were analysed by
11 SEM to determine if the photodynamic effect mediated by 5-ALA and MAL caused
12 structural changes in the biofilm community. As shown in Fig. 6 A-C, large cellular
13 aggregates surrounded by extracellular matrix were observed for the biofilms without
14 any treatment and for the biofilms incubated with 5-ALA or MAL in the dark.
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16 Treatment of biofilms with 10 mM 5-ALA or MAL and irradiation for 60 min caused
17 biofilm disruption with only small clusters of cells or individually adherent cells
18 remaining (Fig. 6 D and 6 E). In addition some changes in the appearance of these
19 bacteria could be observed. As shown in Fig. 6 D flaws (white arrow) and concavities
20 giving different cell shapes (black arrow) could be observed in individual bacteria. In
21 Fig. 6 E, hollow cracks (white arrows) could be observed on the surface of some
22 bacteria. These results indicated that the cell envelope was significantly damaged
23 during PDI.
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37 Discussion

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40 The biosynthesis of 5-ALA is a rate limiting step downstream of haem biosynthesis
41 and exists in nearly all cells. Unlike eukaryotes in which ALA is synthesized by the
42 enzyme aminolevulinate synthetase by condensation of glycine and succinyl coenzyme
43 A, in most bacteria ALA is synthesized via the C5-pathway utilizing glutamate as a
44 precursor. The next steps in the biosynthesis of porphyrins involve the condensation
45 of two molecules of 5-ALA to form porphobilinogen, in a reaction catalysed by
46 5-ALA dehydratase, followed by the condensation of four molecules of
47 porphobilinogen to form a linear tetrapyrrole, which cyclizes to form
48 uroporphyrinogens I and III. Bacteria can use uroporphyrinogen III as an intermediate
49 in the synthesis of various photoactive porphyrins such as uroporphyrin III,
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1 coproporphyrin III, and protoporphyrin IX. Because the biosynthesis of 5-ALA is the
2 rate limiting step in these biosynthetic pathways, when an excessive amount of this
3 molecule is provided exogenously and taken up by cells, photoactive porphyrins
4 accumulate, particularly in rapidly growing cells such as bacteria thereby giving a
5 degree of selective targeting [12].
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10 One issue with the use of 5-ALA is that it does not transverse cell membranes
11 and other biological barriers efficiently, and to counter this several more lipophilic
12 derivatives have been developed to improve bioavailability [23]. Fotinos *et al.*
13 investigated the capacity of 5-ALA and 5-ALA esters, with increasing chain lengths,
14 from methyl ester to octyl ester and hence increasing lipophilicities, to cause the
15 accumulation of photoactive porphyrins in bacteria and to mediate PDI. They found
16 that the dark toxicities of the 5-ALA derivatives increased with their lipophilicity [14].
17 5-ALA pentyl ester, hexyl ester, and octyl ester did not induce photoactive porphyrin
18 accumulation nor did they mediate effective PDI, the authors attributed this finding to
19 the strong dark toxicity of these molecules. In the case of MAL these authors only
20 observed dark toxicity at higher concentrations, which is in accordance with our
21 findings reported here. Fotinos *et al* also investigated the capacity of 5-ALA and
22 MAL to mediated PDI of different bacterial species and strains, and found that the
23 PDI varied with the bacterial strain. The gram-positive strains appeared to be
24 significantly more sensitive to 5-ALA-mediated PDI than the gram-negative strains
25 [14]. Yow *et al.* compared the capacity of methylene blue (MB) and 5-ALA to
26 mediate PDI of *K. pneumoniae* and ESBL-producing *K. pneumoniae* strains [16].
27 They demonstrated a 5.9 log₁₀ reduction in the viability of an ESBL-nonproducing
28 clinical isolate of *K. pneumoniae* and 4.8 log₁₀ reduction of an ESBL-producing *K.*
29 *pneumoniae* with 10 μM MB. However they found that ALA was ineffective at
30 mediating PDI of *K. pneumoniae* at the highest concentration tested, 6 μM. In contrast,
31 here we report that *K. pneumonia* is in fact sensitive to ALA mediated PDI. The
32 difference between the findings of Yow *et al.* and the results we present here are
33 probably due to the fact that they used very low concentrations of 5-ALA. Another
34 difference between the two studies is that the bacterial cell density we used was lower
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1 than that used by Yow *et al* and it has been previously been reported that this can
2 affect the efficiency of PDI, albeit for different bacteria [24]. Our data and that of
3 Fotinos *et al.*, where they treated two *E. coli* strains (K12 and a uropathogenic isolate)
4 with 0.1 and 1.0 mM 5-ALA and upon irradiation with 120 J cm⁻² of white light
5 achieved 3.31 and 4.30 log₁₀ reductions in the survival [14], suggest that *K.*
6 *pneumoniae* is less susceptible to 5-ALA mediated PDI than *E. coli*.
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12 Bacterial biofilms are communities of cells, attached to a surface and embedded
13 in extracellular polymeric substances (EPS) [25]. Bacteria in biofilms are more
14 resistant to antibiotics and host immune defenses than their planktonic counter parts,
15 thus hampering treatment and clearance of infections [26]. In addition to causing
16 significant morbidity and mortality biofilm associated infections are also a significant
17 economic burden on healthcare services since they are recalcitrant to antimicrobial
18 therapy and therefore also require the removal of colonized indwelling devices. A
19 previous study has shown that biofilms formed by MRSA are sensitive to
20 5-ALA-mediated PDI treatment [13]. Lee *et al* reported that no viable bacteria were
21 detected when *Pseudomonas aeruginosa* biofilms were treated with 20 mM 5-ALA
22 and exposed to 120 J cm⁻² of 630 nm LED light [27]. In the present study PDI of *K.*
23 *pneumoniae* biofilms mediated by 5-ALA and MAL was also assessed. *K.*
24 *pneumoniae* strains were susceptible to PDI when grown as biofilms but the
25 magnitude of inactivation was not as great as with planktonic cells. As mentioned
26 microbial biofilms are generally less susceptible than planktonic cells to antimicrobial
27 agents, such as antimicrobial peptides and antibiotics [28], and less susceptible to PDI.
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29 Although a number of factors account for increased resistance of biofilm cells to
30 antimicrobial agents, including changes in gene expression, the affinity of a PS for the
31 EPS will also affect PS binding, and ultimately the efficiency of PDI [29]. Our data on
32 5-ALA mediated PDI of *K. pneumoniae* biofilms where we find that the greatest
33 biofilm formers are the least susceptible to PDI suggests that EPS may have a
34 protective role. The SEM investigation suggested that 5-ALA and MAL-mediated
35 PDI had another important action beyond killing the bacteria in biofilms. After
36 irradiation, the number of adherent bacteria greatly reduced and only a few colonies
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1 were observed, implying that 5-ALA and MAL-mediated PDI could disrupt the
2 biofilm presumably by destroying EPS which is responsible for bacterial aggregation.
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4 PDI of bacteria has been proposed to occur through inactivation of a number of
5 biomolecules. For example damage to DNA would interfere with chromosome
6 segregation, DNA replication and transcription. PDI of bacteria has been
7 demonstrated to cause breaks in single and double stranded DNA and the
8 disappearance of the supercoiled form of plasmids [30, 31]. However other authors
9 have suggested that although DNA damage occurred, it might not be the prime cause
10 of bacterial death. In support of this Caminos and Choi found that DNA was only
11 slightly degraded after a relatively long period of irradiation, although the bacterial
12 strains were effectively inactivated [22, 32]. In a study by Nitzan *et al.* on the PDI of
13 *Acinetobacter baumannii* and *E. coli* using the cationic PS TMPyP it was found that
14 structural damage to the membrane was induced by the ROS produced upon
15 photosensitization, while DNA breakage appeared only after a long period of
16 irradiation, when the bacterial cells were no longer viable [33]. They concluded that
17 cytoplasmic membrane damage and not DNA breakage was the major cause of
18 bacterial photoinactivation. In the present study, we detected rapid DNA degradation
19 and release of intracellular biopolymers upon PDI treatment, indicating that 60-min
20 irradiation was sufficient to cause damage to the membrane and genomic DNA.
21 Interestingly on a macro-scale examination of bacteria exposed to 5-ALA mediated
22 PDI by TEM, showed damage to the bacterial cells and condensation of cytoplasmic
23 components (Fig. 5 D). However when bacteria exposed to MAL mediated PDI were
24 examined by TEM strikingly different macro-scale changes were observed, with
25 filamentous structures having been formed in low density areas (Fig. 5 E). These
26 results indicate that PDI mediated by 5-ALA and MAL have different effects on
27 cellular structures but this requires further investigation.
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54 **Conclusions**

55 In summary, the present study assessed the efficacy of 5-ALA and MAL to mediate
56 PDI of three *K. pneumoniae* strains in planktonic and biofilm culture. 5-ALA and
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1 MAL mediated inactivation of the planktonic cultures in a concentration-dependent
2 manner upon irradiation. Biofilms were also sensitive to 5-ALA and MAL-mediated
3 PDI. Furthermore SEM demonstrated that 5-ALA and MAL mediated PDI also
4 caused biofilm disruption. After PDI of ESBL-producing *K. pneumoniae*, obvious
5 cleavage of genomic DNA and a rapid release of intracellular biopolymers were
6 detected. The condensation of cytoplasmic components was also observed by TEM.
7 Although cleavage of genomic DNA was detected, PDI of ESBL-producing *K.*
8 *pneumoniae* might be predominantly due to cell envelope injury, intracellular
9 biopolymer leakage, and the denaturation of cytoplasmic components.
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22 at the College of Chemistry and Materials Science, Northwest University, China. This
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24 China Postdoctoral Science Foundation (2014M562424), and the Fundamental
25 Research Funds for the Central Universities.
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33 **Conflict of interest** The authors declare no conflict of interest.
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Antimicrob Chemother doi: 10. 1093/jac/dks301

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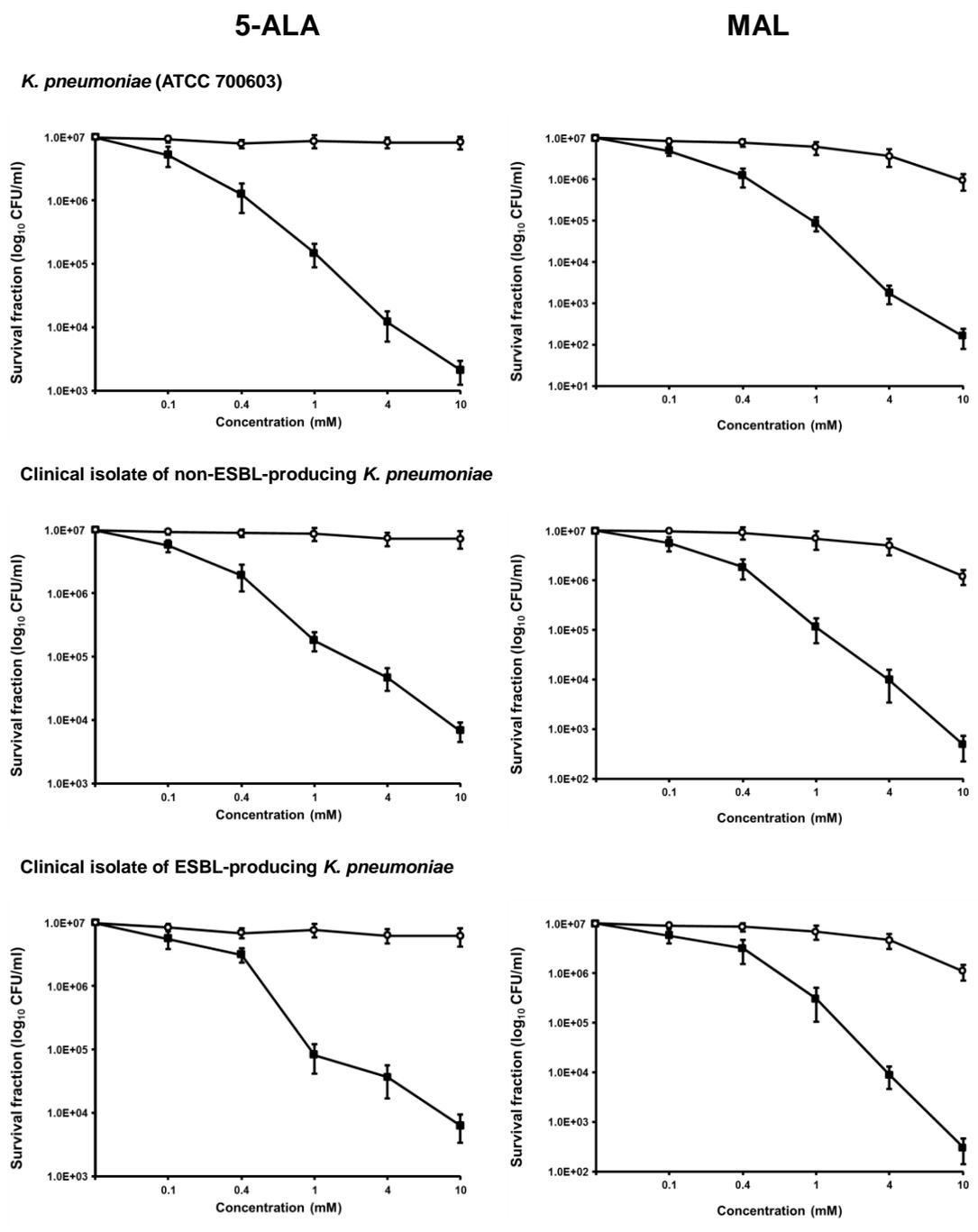


Fig. 1 Bacterial survival curves of *K. pneumoniae* incubated with 5-ALA or MAL at 37°C for 4 h in the dark. Open circles (○) represent the surviving fraction after incubation (dark toxicity), and filled squares (■) represent the surviving fraction after irradiation by 400–780 nm white light for 60 min.

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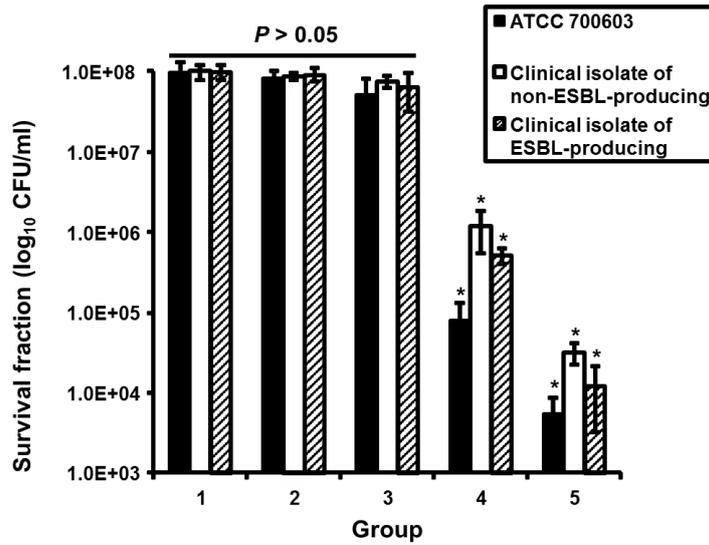


Fig. 2 Surviving fraction of *K. pneumoniae* biofilms after 5-ALA and MAL-mediated PDI treatment. Group 1: without any treatment, group 2: incubated with 10 mM 5-ALA at 37°C for 4 h in the dark, group 3: incubated with 10 mM MAL at 37°C for 4 h in the dark, group 4: incubated with 10 mM 5-ALA and irradiated with 400–780 nm white light for 60 min, group 5: incubated with 10 mM MAL and irradiated for 60 min. * $P < 0.05$ compared with groups 1–3.

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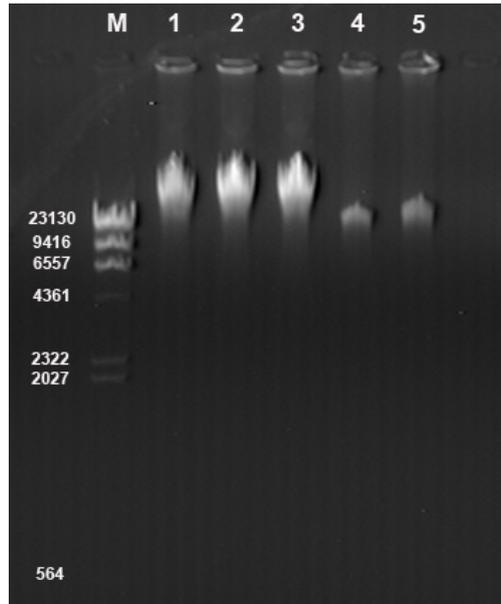


Fig. 3 Agarose gel electrophoresis of genomic DNA samples extracted from ESBL-producing *K. pneumoniae*. Lane M: DNA weight maker, lane 1: without any treatment, lane 2: incubated with 10 mM 5-ALA at 37°C for 4 h in the dark, lane 3: incubated with 10 mM MAL at 37°C for 4 h in the dark, lane 4: incubated with 10 mM 5-ALA and irradiated with 400–780 nm white light for 60 min, lane 5: incubated with 10 mM MAL and irradiated with 400–780 nm white light for 60 min.

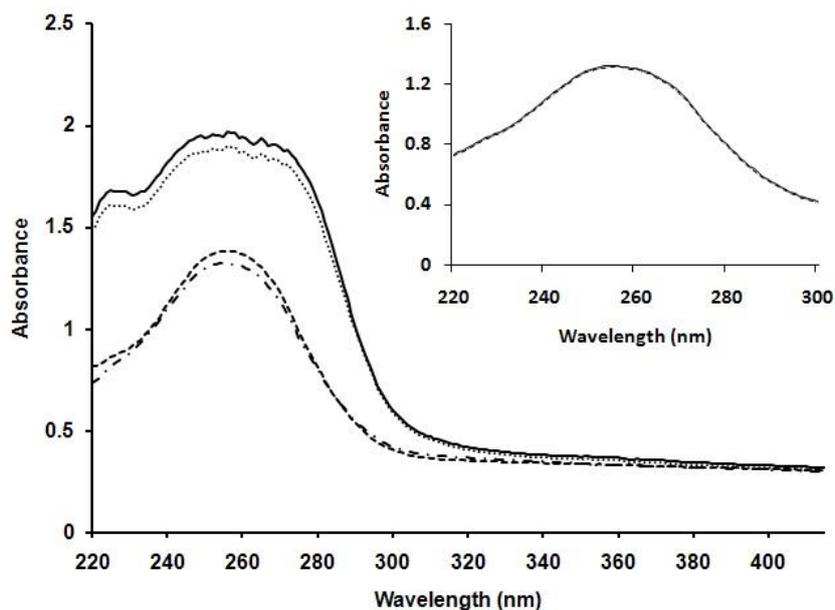


Fig. 4 Release of UV-absorbing material from ESBL-producing *K. pneumoniae*.
 Dot-dashed line: without any treatment, dashed line: sonicated for 15 min, dotted line: incubated with 10 mM 5-ALA and irradiated with 400–780 nm white light for 60 min, solid line: incubated with 10 mM MAL and irradiated with 400–780 nm white light for 60 min. Inner plot: solid line: without any treatment, dotted line: incubated with 10 mM 5-ALA at 37°C for 4 h in the dark, dashed line: incubated with 10 mM MAL at 37°C for 4 h in the dark.

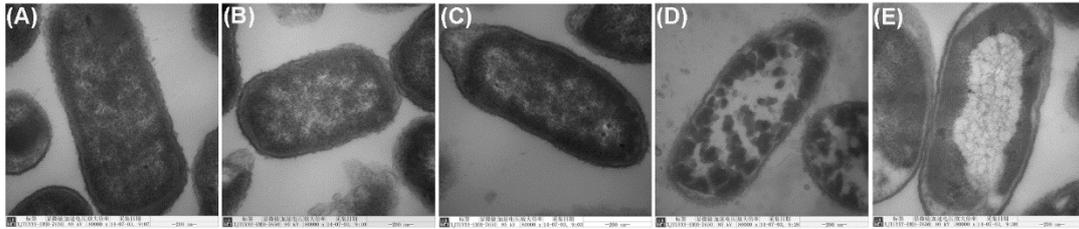


Fig. 5 Transmission electron microscopy images of ESBL-producing *K. pneumoniae*. (A): without any treatment, (B): incubated with 10 mM 5-ALA at 37°C for 4 h in the dark, (C): incubated with 10 mM MAL at 37°C for 4 h in the dark, (D): incubated with 10 mM 5-ALA and irradiated with 400–780 nm white light for 60 min, and (E): incubated with 10 mM MAL and irradiated with 400–780 nm white light for 60 min.

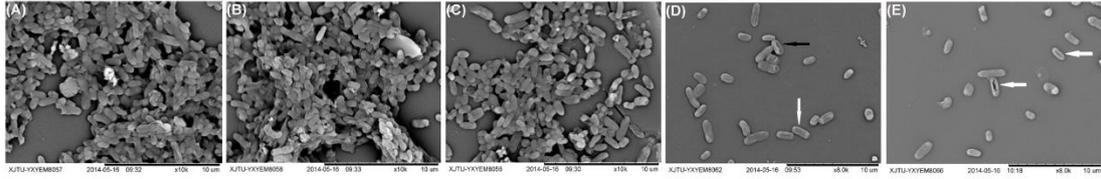


Fig. 6 Scanning electron microscopy images of ESBL-producing *K. pneumoniae*. (A): without any treatment, (B): incubated with 10 mM 5-ALA at 37°C for 4 h in the dark, (C): incubated with 10 mM at 37°C for 4 h MAL in the dark, (D): incubated with 10 mM 5-ALA and irradiated with 400–780 nm white light for 60 min, and (E): incubated with 10 mM MAL and irradiated with 400–780 nm white light for 60 min.

**Photodynamic Inactivation of *Klebsiella pneumoniae* Biofilms
and Planktonic cells by 5-Aminolevulinic Acid and
5-Aminolevulinic Acid Methyl Ester**

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Muhammad Ishaq ¹, Jiru Xu ^{1, *}, Sean P. Nair ^{3, *}

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Dear Prof. Keyvan Nouri and Reviewers:

Thank you very much for your letter and for the reviewer's good comments on our manuscript entitled "Photodynamic Inactivation of *Klebsiella pneumoniae* and Biofilms by 5-Aminolevulinic Acid and 5-Aminolevulinic Acid Methyl Ester" (LIMS-D-14-00456). These comments are all valuable and have been helpful to us for improving the manuscript. We have addressed all of the comments made by the reviewers and incorporated their suggested. The main corrections in the paper and the responses to the reviewer's comments are as follows:

Responses to the reviewers' comments:

Reviewer #2:

1. "It seems to me that this paper reports a well done experimental work. The authors have used good approaches to determine the effect of PDT on *Klebsiella pneumoniae*. Moreover most of the initial criticisms have been answered by the authors. Now my main concern is derived from the fact that *Klebsiella pneumoniae* is a close microorganism to *Escherichia coli*. Thus, it should be expected an identical (or highly similar) behavior when treated with an identical PS and light, as the authors found. In other words the description of the effect of 5-ala mediated PDT on the different Enterobacteriaceae species could initiate a long long series of "salami" papers just determining their quantitative effect in each species. Enterobacteriaceae includes more than 30 genera and more than one hundred species and probably their susceptibility to PDT is quite similar. I believe the authors should highlight where is the novelty of this paper, or a stronger rationale. Alternatively they can compare the effect on different species and discuss... but I think this is not the case."

R: We thank the reviewer for the important comment on the close evolutionary relationship between *K. pneumoniae* and *E. coli* which might lead one to conclude

that any study of PDI of *K. pneumoniae* would be similar to studies on PDI of *E. coli*. We should have made it clearer in the original manuscript that there is one study looking at 5-ALA mediated PDI of *K. pneumoniae* which showed that it was not effective. In addition the literature shows that there are differences in the susceptibility of different *E. coli* strains to 5-ALA mediated PDI. We have modified the text of the manuscript to emphasize this in the introduction and discussion. Our results on 5-ALA mediated PDI of *K. pneumoniae* also suggest that this bacterium is less susceptible to PDI than *E. coli* and we mention this in the discussion as well.

Our study is also novel because the capacity of 5-ALA to mediate PDI of biofilms of these organisms has not been reported. We show that 5-ALA mediated PDI of biofilms depends on the capacity of the bacterium to form a biofilm and that this treatment not only kills the bacteria but also disrupts the biofilm.

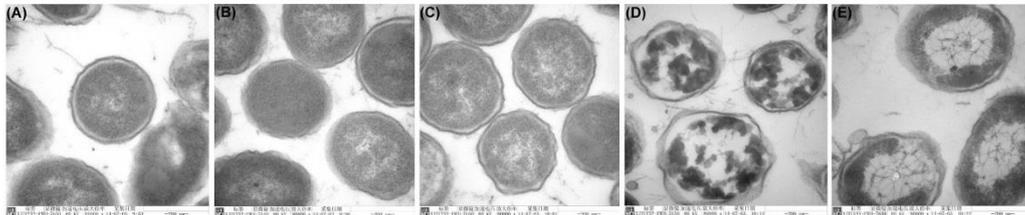
Electron microscopy.

2. “I cannot see injuries in the wall in (E). If the authors do they should include a sentence to describe. It would be better to include pictures with more than one single cell. I've some experience in TEM of bacteria and there is certain heterogeneity; thus, images are suggestive when one can see various individuals or when statistical analysis is done (this is time consuming and actually unnecessary). On the other hand ultrathin sections should be observed taking into account that one can visualize sections in which the genome cannot be seen because it was below the section. In my opinion the unique image reported by authors that gives significant information is (D) because the condensation of cytoplasmic components.”

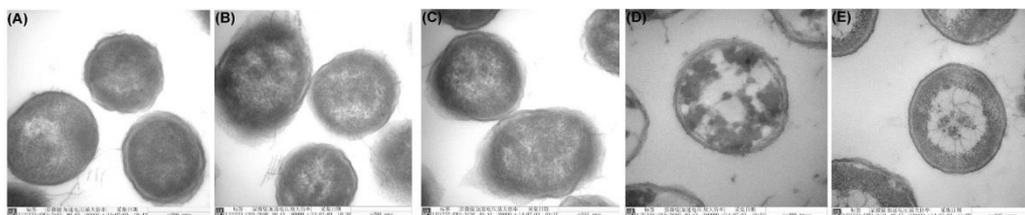
R: We have rewritten this part of the results section as requested by the reviewer. When we were performing this experiment, most cells seen in different fields were very similar to those shown in image (E). The cellular structure differences in image (D) and (E) have been described in the text of the results section and the discussion. We have also investigated the capacity of 5-ALA and MAL to mediate PDI of

vancomycin-resistant *Enterococci* (VRE), and seen similar cell structure changes. We do not know the reason for these differences.

Clinical isolate of VRE



E. faecalis (ATCC 51299)



TEM images of the clinical isolate of VRE and *E. faecalis* (ATCC 51299). (A): without any treatment, (B): incubated with 10 mM 5-ALA at 37 °C for 4 h in the dark, (C): incubated with 10 mM MAL at 37 °C for 4 h in the dark, (D): treated with 10 mM 5-ALA and irradiated for 60 min, (E): treated with 10 mM MAL and irradiated for 60 min.

Scanning microscopy

3. “Images of SEM are also difficult to be interpreted. I assume that initially all biofilms had the same age before treatment. Whereas in A B and C one can observe "normal biofilms" in D and E there is not biofilm , but only a few individuals; this means that biofilm has been physically removed. This is much more relevant than one cell/electronmicrograph exhibiting a certain abnormal shape. But I cannot understand by which mechanism biofilm was removed. Authors should add an explanation.”

R: We agree SEM images can be difficult to be interpreted. The biofilms are all of the same age. The reviewer’s interpretation of the SEMs showing that biofilms have been

disrupted is correct and that this is an important finding has been emphasized in the revised manuscript.

4. “Despite my native tongue is not English I believe the English style can be improved a little. Some sentences remain unclear.”

R: The manuscript has been revised throughout to improve the English language and style.

Reviewer #3:

1. “I like the article, it has interesting useful results for researchers in the field. I would recommend however for future experiments not to perform all experiments in buffer, but in more realistic protein rich environments. Additionally, I am not sure whether UV absorbance is the best way to estimate membrane injuries.”

R: Special thanks to you for the good comments and suggestions. In future work we will perform the PDI experiments in more protein rich environments. However given that in 5-ALA mediated PDI, the active PS is endogenous and located within the cytoplasm of the bacterium such an environment may not affect the outcome very much. The question raised by the reviewer as to the use of UV absorbance being the best method to estimate membrane injury is valid. It would not be the best method to accurately quantify the amount of membrane damage but it is easy to perform and useful for estimating/ establishing that membrane damage is occurring. It is a method that is used in studies such as ours. For example Spesia *et al.* used UV absorbance at 260 nm to estimate membrane injuries in the photodynamic inactivation of *Escherichia coli* and *Streptococcus mitis* [1, 2]. Chen and Je also used the method to detect membrane injuries of bacteria treated with dendrimer biocides and chitin derivative [3, 4].

1. Spesia MB et al (2009) Mechanistic insight of the photodynamic inactivation of *Escherichia coli* by a tetracationic zinc(II) phthalocyanine derivative. *Photodiagn Photodyn Ther* 6 (1): 52–61
2. Spesia MB, Duraniti EN (2013) Photodynamic inactivation mechanism of *Streptococcus mitis* sensitized by zinc (II) 2, 9, 16, 23-tetrakis [2-(*N, N, N*-trimethylamino) ethoxy] phthalocyanine. *J Photochem Photobiol B* 125 (5): 179–187
3. Chen CZ, Cooper SL (2002) Interactions between dendrimer biocides and bacterial membranes. *Biomaterials* 23 (16): 3359–3368
4. Je JY, Kim SK (2006) Antimicrobial action of novel chitin derivative. *Biochim Biophys Acta* 1760 (1): 104–109

We appreciate for your and the reviewers' work on critically reading our manuscript and offering us constructive feedback which we believe has improved what we have written. We hope that the corrections will meet with approval.

Once again, thank you very much for your comments and suggestions.

Thank you and best regards!

Yours sincerely,

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