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1 ***Staphylococcus epidermidis* biofilms control by N-**

2 **acetylcysteine and rifampicin**

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4 *Bruna Leite^{a,b}, MSc, Fernanda Gomes^a, BSc, Pilar Teixeira^a, PhD, Clovis*
5 *Souza^b, PhD, Elisabeth Pizzolitto^{b,c}, PhD, Rosário Oliveira^a, PhD**
6

7 ^aIBB-Institute for Biotechnology and Bioengineering, University of Minho,
8 Campus of Gualtar, Braga, Portugal.

9 ^bDepartment of Biotechnology, Federal University of São Carlos, São
10 Carlos, Brazil.

11 ^cFaculty of Pharmaceutical Sciences, São Paulo State University,
12 Araraquara, Brazil.

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15 *Correspondence to: Rosário Oliveira, IBB-Institute for Biotechnology and
16 Bioengineering, Centre of Biological Engineering, University of Minho,
17 Campus de Gualtar, 4710-057, Braga, Portugal; Tel: +351253604409;
18 Fax: +351253678986; E-mail: roliveira@deb.uminho.pt

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21 Running title: *N*-acetylcysteine as therapeutic adjuvant
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4
5 **Abstract**

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7 26 Medical device-associated infections caused by *Staphylococcus*
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10 27 *epidermidis* usually involve biofilm formation and its eradication is
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12 28 particularly challenging. Although rifampicin has been proving to be one of
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14 29 the most effective antibiotics against *S. epidermidis* biofilms its use as a
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16 30 single agent can lead to the acquisition of resistance. Therefore, we
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18 31 assessed the combined effect of rifampicin with *N*-acetylcysteine (NAC)
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20 32 known by its mucolytic effect, in the control of *S. epidermidis* biofilms.
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22 33 Biofilms of two *S. epidermidis* strains (9142 and 1457) were treated with
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24 34 1xMIC (4 mg/mL) and 10xMIC (40 mg/mL) of NAC and 10 mg/L (peak
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26 35 serum) of rifampicin alone and in combination. NAC at 40 mg/L alone or in
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28 36 combination with rifampicin (10 mg/L) significantly reduced (4 log₁₀) the
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30 37 number of biofilm cells. Considering their different modes of action, the
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32 38 association of NAC with rifampicin constitutes a promising therapeutic
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34 39 strategy in the treatment of infections associated to *S. epidermidis*
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36 40 biofilms.
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45 42
46 43 **Key words:** *Staphylococcus epidermidis*, biofilm, planktonic cells, *N*-
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48 44 acetylcysteine, rifampicin.
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5 48 **INTRODUCTION**
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7 49 *Staphylococcus epidermidis* and other coagulase-negative
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10 50 staphylococci (CoNS) produce extracellular matrix, which is an important
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12 51 virulence factor. This polymeric matrix promotes bacterial adhesion and
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14 52 produces a biofilm that makes the eradication of microorganisms more
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16 53 difficult (1). *Staphylococcus epidermidis*, the most frequently isolated
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18 54 coagulase-negative staphylococcus, is the leading cause of infection
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20 55 related to implanted medical devices (IMDs). This is directly related to its
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22 56 capability to establish multilayered, highly structured biofilms on artificial
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24 57 surfaces. Bacterial biofilms are difficult to detect in routine diagnostics and
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26 58 are inherently tolerant to host defenses and conventional antibiotic
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28 59 therapies. Thus, device-related infections are notoriously difficult to treat
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30 60 and bacteria within biofilm communities on the surface of IMDs frequently
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32 61 outlive treatment, and removal of the medical device is often required for
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34 62 successful therapy (2). Additionally, the emergence of antibiotic-resistant
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36 63 bacteria and the slow progress in identifying new classes of antimicrobial
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38 64 agents have encouraged research into novel therapeutic strategies (3).
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46 65 *N*-acetylcysteine (NAC) is a non-antibiotic drug that has antibacterial
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48 66 properties. It is a mucolytic agent that disrupts disulphide bonds in mucus
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50 67 and reduces the viscosity of secretions (4). NAC is commonly used in
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52 68 medical treatment of chronic bronchitis and cancer (5, 6) and is one of the
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54 69 smallest drug molecules in use (7). NAC affects several processes that are
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2 70 important for bacterial biofilm formation on stainless steel surfaces,
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4 71 including a drastic reduction in extracellular polysaccharide production,
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7 72 and thus acts as an antibiofilm substance (8).
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10 73 Previous results have demonstrated that rifampicin is the most
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12 74 effective of traditional antibiotics against *S. epidermidis* cells in biofilms (9-
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14 75 12).
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17 76 Accordingly, the aim of this work was to determine the *in vitro* effect
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19 77 of NAC alone but specifically in combination with rifampicin on biofilms of
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22 78 two different *S. epidermidis* strains.
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5 94 **MATERIALS AND METHODS**

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7 95 **Bacterial strains and growth conditions**

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10 96 Two *Staphylococcus epidermidis* clinical isolate strains (9142 and
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12 1457, good biofilm-producers) were used in this study. Both strains were
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15 98 provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine,
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17 99 Brigham and Women's Hospital, Harvard Medical School, Boston, USA.

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20 100 The culture media used tryptic soy broth (TSB) and tryptic soy agar
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22 101 (TSA), were prepared according to the manufacturer's instructions. All
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25 102 strains were inoculated into 15 mL of TSB from TSA plates not older than
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27 103 2 days and grown for 24 (\pm 2) h at 37°C in an orbital shaker at 130 rpm.
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29 104 Cells were harvested by centrifugation (for 5 min at 9500g and 4°C), and
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31
32 105 resuspended in TSB adjusted to an optical density (640 nm) equivalent to
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34 106 1×10^6 cells/mL and then used in the subsequent assays.

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37 107 Solutions of NAC 4 mg/mL (1x MIC) and 40 mg/mL (10x MIC) (13)
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39 108 were prepared in TSB and stock solution of rifampicin (10 mg/L - peak
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41 109 serum concentration) (9, 14) was prepared in methanol.

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46 111 **Minimum inhibitory concentration (MIC)**

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49 112 MIC determination of the tested agent *N*-acetylcysteine and for each
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51 113 *S. epidermidis* strain (9142 and 1457) was carried with dilution range of
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54 114 0.5-64 mg/mL.

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2 115 The MIC was determined in 96 well tissue culture plates (Sarstedt,
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4 116 Newton, NC, USA) containing 100 μ L of a stock solution of NAC (64
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7 117 mg/mL) and the dilution was realized with TSB, adding at the end 100 μ L
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9 118 of a *S. epidermidis* cell suspension (1×10^6 cells/mL). Plates were
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11
12 119 incubated at 37°C with orbital shaking at 130 rpm for 24 h. After
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14 120 incubation the minimum inhibitory concentration was determined with the
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16 121 lowest concentration able to inhibit bacterial growth.

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19 122 The controls were not exposed to the antimicrobial agent tested. All
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22 123 experiments were carried out in triplicate and repeated three times.
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25 26 125 **Effect of NAC and rifampicin on biofilm cells**

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29 126 Biofilms were formed in 96 well tissue culture plates (Sarstedt,
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31 127 Newton, NC, USA) containing 200 μ L of a *S. epidermidis* cell suspension
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33 128 (1×10^6 cells/mL) in TSB supplemented with 0.25% of glucose per well to
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35 129 promote biofilm formation. Plates were incubated at 37°C with orbital
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37 130 shaking at 130 rpm for 24 h. At the end, planktonic cells were removed
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39 131 carefully, and the biofilm was washed twice with 200 μ L of saline solution
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41 132 (0.9% NaCl; Merck). The biofilms were incubated for 24 h in fresh nutrient
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43 133 medium containing NAC (4 mg/mL and 40 mg/mL) or rifampicin (10 mg/L)
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45 134 alone or a combination of both NAC concentrations tested with rifampicin.

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51 135 Crystal violet (CV) staining was used as indicator of total biofilm
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53 136 biomass. After exposure to the treatment agents, biofilms were washed
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55 137 with 200 μ L of saline solution, then 250 μ L of methanol was added and
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2 138 allowed to act for 15 min. Afterwards methanol was removed and crystal
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4 139 violet was added (5 min). The wells were washed with water and finally,
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7 140 acetic acid 33% (v/v) was added. The absorbance was measured at 570
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9 141 nm.

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12 142 Another colorimetric method based on the reduction of XTT ({2,3-
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14 143 bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phe-nylamino)carbonyl]-2H-
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17 144 tetrazolium hydroxide}); Sigma, St Louis, USA) was applied to determine
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19 145 cell activity (XTT is converted to a colored formazan salt in the presence of
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21 146 metabolic activity) (15). After exposure to antimicrobial agents, biofilms
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24 147 were washed with 200 μ L of saline solution, then 200 μ L of a solution
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26 148 containing 200 mg/L of XTT and 20 mg/L of phenazine methosulphate
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28 149 (PMS; Sigma, St Louis, USA) was added to each well. The microtiter
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31 150 plates were incubated for 3 h at 37°C in the dark. The absorbance was
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34 151 measured at 490 nm.

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36 152 To assess the number of viable cells after treatment, 200 μ L of saline
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38 153 solution were added to each well before removing the biofilm by
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41 154 scrapping. An aliquot of 1 mL of each sample was centrifuged (for 10 min
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43 155 at 9500g and 4°C) and the pellet resuspended in 1 mL of saline solution.
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46 156 Next, the suspension was sonicated (20 s with 22% of amplitude;
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48 157 Ultrasonic Processor, Cole-Parmer, Illinois, USA) to promote biofilm
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51 158 disruption. The number of colony forming units (CFU) in biofilm for each *S.*
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53 159 *epidermidis* strain was determined by performing 10-fold serial dilutions in
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56 160 saline solution and plating in TSA in triplicate and incubating for 24–48 h.

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2 161 Biofilm controls were not exposed to any antimicrobial agent tested.

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5 162 All experiments were carried out in triplicate and repeated three times.

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7 163 **Effect of NAC and rifampicin on planktonic cells**

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9 164 For each strain, 200 μ L of a cell suspension adjusted to 1×10^9
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12 165 cells/mL and 1.5 mL of that suspension was added to 15 mL of TSB until a
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14 166 cell density of 1×10^8 cells/mL was reached and were then dispensed in
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17 167 test tubes followed by the addition of the treating agents alone, NAC (4
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19 168 mg/mL and 40 mg/mL) or rifampicin (10 mg/L), and in combination. All the
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22 169 tubes were incubated at 37°C with shaking at 130 rpm.

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24 170 Cell susceptibility to the treatment agents was determined by the
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27 171 XTT colorimetric method. For that, 1 mL of each sample was collected and
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29 172 centrifuged (for 10 min at 9500g and 4°C) and the pellet was
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32 173 resuspended in 1 mL of saline solution (0.9% NaCl; Merck). From each
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34 174 suspension, 200 μ L were transferred to individual wells of a 96-well
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36 175 microtiter plate. Then, 50 μ L of a solution containing 200 mg/L of XTT and
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39 176 20 mg/L of PMS were added to each well and the microtiter plates were
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41 177 incubated for 3 h at 37°C in the dark. The absorbance was measured at
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44 178 490 nm.

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46 179 For CFU determination, 1 mL of each sample was collected and
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49 180 centrifuged (for 10 min at 9500g and 4°C) and the pellet resuspended in
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51 181 1 mL of saline solution. The suspension was sonicated (20 s with 22% of
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53 182 amplitude; Ultrasonic Processor, Cole-Parmer, Illinois, USA) and the
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56 183 viable planktonic cells were determined by performing 10-fold serial

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2 184 dilutions in saline solution and plating in TSA. Colonies were counted after
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5 185 24-48 h at 37°C.

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7 186 Controls were planktonic cells not exposed to any treatment agent
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10 187 tested. All experiments were carried out in triplicate and repeated three
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12 188 times.

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16 190 **Extraction of the exopolymeric matrix**

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19 191 Biofilms were formed in 6 well tissue culture plates (Sarstedt,
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22 192 Newton, NC, USA) containing 4 mL of a *S. epidermidis* cell suspension (1
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24 193 x 10⁶ cells/mL) in TSB supplemented with 0.25% of glucose per well to
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27 194 promote biofilm formation. Plates were incubated at 37°C with orbital
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29 195 shaking at 130 rpm for 24 h. At the end, planktonic cells were removed
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31 196 carefully, and the biofilm was washed twice with 4 mL of saline solution.

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34 197 The biofilms were incubated in fresh nutrient medium containing NAC (4
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36 198 mg/mL and 40 mg/mL), rifampicin (10 mg/L) alone and NAC in
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39 199 combination with rifampicin. After 24 h of incubation, the biofilm was
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41 200 washed twice with 4 mL of saline solution. Next, 4 mL of phosphate
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44 201 buffered saline (pH 7.0) were added and the biofilm formed was removed
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46 202 from the well by scrapping with a cell scraper (zellschaber/24 cm). The
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49 203 samples were centrifuged for 5 min at 9500g and 4°C, and resuspended
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51 204 in 5 mL of extraction buffer (2 mM Na₃PO₄; 4 mM NaH₂PO₄; 9 mM NaCl
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53 205 and 1 mM KCl, pH 7.0) and 1 g of resin (Dowex/50X8, Na⁺ form, 20-50
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56 206 mesh, Aldrich-Fluka 44445) was added. The samples were incubated at -

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2 207 5°C for 2 h. After the incubation the samples were centrifuged for 20 min
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5 208 at 9500g and 4°C.

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7 209 The polysaccharide content in the biofilm matrix was quantified by
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10 210 the method of Dubois et al. (16). The protein content of the biofilm matrix
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12 211 was measured using the BCA[™] Protein Assay Kit (Bicinchoninic Acid,
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14 212 Sigma-Aldrich, St Louis, USA).

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17 213 Controls were biofilms not exposed to any treatment agent tested. All
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19 214 experiments were carried out in triplicate and repeated three times.

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23 24 216 **Scanning electron microscopy (SEM)**

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26 217 Biofilms formed in 12 well tissue culture plates (Sarstedt, Newton,
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28 218 NC, USA) were dehydrated by 15 min immersion in increasing ethanol
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30 219 concentrations (70, 95 and 100% [v/v]), and then placed in a sealed
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34 220 desiccator. The samples were mounted on aluminum stubs with carbon
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36 221 tape, sputter coated with gold and observed with a Leica Cambridge S-
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39 222 360 scanning electron microscope (Leo, Cambridge, UK).

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41 223 Controls were biofilms not exposed to any treatment agent tested. All
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43 224 experiments were carried out in triplicate.

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47 48 226 **Statistical Analysis**

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51 227 The data from all assays were compared using one-way analysis of
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53 228 variance (ANOVA) by applying Tukey's test with all calculations carried out
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56 229 using SPSS software (Statistical Package for the Social Sciences).

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2 230 Differences achieving a confidence level of 95% were considered
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5 231 significant.

6 7 232 **RESULTS**

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10 233 Figure 1 presents the number of viable cells of the two *S. epidermidis*
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12 234 strains tested, expressed as log CFU, remaining after the treatment of
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15 235 either biofilm or planktonic cells. It can be observed that NAC alone at 4
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17 236 mg/mL (1x MIC) had a slight inhibitory effect on planktonic cells but almost
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19 237 no effect on biofilms, while at 10x MIC it showed a notorious killing effect
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21 238 on planktonic cells (almost total eradication) and a significant bactericidal
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23 239 effect on biofilms, in this case promoting CFU reductions of about 4 log₁₀
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25 240 (p<0.05). Rifampicin alone, showed an effect on biofilm cells promoting
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27 241 CFU reduction of about 3 log₁₀ (p<0.05). Although the reduction promoted
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29 242 by rifampicin in planktonic cells was less than 3 log. The NAC-rifampicin
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31 243 combination consistently decreased the number of viable biofilm
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33 244 associated bacteria by 3-4 log₁₀ independently of NAC concentration used.
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35 245 In fact, NAC at 40 mg/ml (10x MIC), rifampicin alone or NAC-rifampicin
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37 246 combinations showed significant but similar bactericidal effect against
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39 247 biofilms (p<0.05). The combination of NAC (10x MIC) with rifampicin in
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41 248 planktonic cells promoted a killing effect (p<0.05).

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44 249 The results expressing the decrease in metabolic activity measured
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46 250 by the XTT reduction assay after treatment with the tested agents are
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49 251 showed in figure 2.

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2 252 In addition, total biofilm biomass, assessed by CV staining, also
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5 253 confirms the effect of the agents tested as can be seen in figure 3.
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7 254 The matrix composition showed a generally significant increase
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9 255 ($p < 0.05$) in the amount of proteins and polysaccharides after treatment,
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11 256 presented in figure 4. The amount of proteins was very high after
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13 257 treatment with the combination NAC 40 mg/mL with rifampicin ($p < 0.05$).
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15 258 However, the amount of polysaccharides was high after treatment with
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17 259 NAC (10x MIC) alone or the combination NAC-rifampicin, independently of
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19 260 NAC concentration used ($p < 0.05$).
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24 261 Scanning electron microscope images of the biofilms are presented
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26 262 in figure 5. The SEM shows representative images of *S. epidermidis*
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28 263 (strain 1457) biofilms after treatment with the agents tested alone and in
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30 264 combination. As can be seen (Figure 5C and F), after treatment with NAC
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32 265 at 40 mg/mL alone or combined with rifampicin the amount of matrix
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34 266 present is very small and this might be due to an easy removal of the
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36 267 matrix during the desiccation procedure by successively washing with
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38 268 increasing ethanol concentrations.
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8 276 **DISCUSSION**

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11 277 Modern medicine is facing the challenge to control the increasing
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13 278 incidence of biofilm infections and this situation has boosted the search of
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15 279 new therapeutic strategies able to evade the intrinsic tolerance of biofilms
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18 280 to antimicrobial agents. Accordingly, the rationale of this study stems from
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21 281 the reported effect of NAC in disrupting mature biofilms (4) and the high
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23 282 efficacy of rifampicin against *S. epidermidis* biofilms when compared with
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25 283 other common antibiotics (9-12). Thus, we hypothesized that in
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28 284 combination they could have a synergistic effect due to their different
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30 285 modes of action.

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33 286 The great efficacy of NAC against planktonic cells was also
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35 287 confirmed in experiments with other gram-positive and gram-negative
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37 288 strains, showing that NAC did indeed reduce the growth of all strains
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40 289 tested (8). Rifampicin alone, as we could expect from previous studies (9-
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42 290 11), showed higher efficacy against biofilm cells than planktonic cells. As
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45 291 demonstrated by viable cells reduction (Figure 1) and in opposition to our
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47 292 hypothesis the combinations tested were not synergistic or additive in
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50 293 controlling *S. epidermidis* biofilms. In a previous study (13), reported a
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52 294 synergistic effect of NAC in combination with tigecycline on *S. epidermidis*
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55 295 biofilms using NAC at 20x MIC (80 mg/mL) and tigecycline at 1000x MIC
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57 296 (1 mg/mL) with the MIC values reporting to planktonic cells. Nevertheless,
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2 297 their results in viable cells reduction of this species were less than 3 log₁₀.
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4 298 Regarding the NAC-rifampicin combination tested herein the values
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7 299 obtained were higher, corresponding to a bactericidal action, even if not
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10 300 corresponding to a synergy between the two agents.

11
12 301 In this work, the biofilm formation capability was as well evaluated
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14 302 through crystal violet, allowing the quantification of the total biomass. The
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17 303 metabolic activity of biofilms was evaluated through XTT reduction assays.
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19 304 The results shown are in very good agreement with those obtained in
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22 305 terms of cell viability.

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24 306 Interestingly, the matrix composition showed a generally significant
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27 307 increase in the amount of proteins and polysaccharides after treatment
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29 308 (Figure 4). Notoriously, the amount of proteins was very high after
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32 309 treatment, which is consistent with a higher degree of cell lysis. The
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34 310 increase in polysaccharides content is probably due in greater extent to
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36
37 311 the loosening effect of NAC on the matrix structure and thus making its
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39 312 extraction more efficacious. As shown previously the strain 1457 produces
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41 313 more extracellular polysaccharides than 9142 (17). Moreover, NAC is a
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44 314 thiol containing molecule that is described to disrupt disulfide bonds in
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46 315 mucus (18). In fact, SEM observations support this hypothesis.

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48 316 It should be stressed that it is now generally accepted that a
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51 317 combination of antimicrobial agents is a strategy to evade the
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54 318 development of bacterial resistance to antibiotics. In fact, it is not
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56 319 advisable to use rifampicin as a single agent to treat infections because of
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2 320 the rapid selection of resistant mutants (19). Zheng and Stewart (20)
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4 321 detected rifampicin-resistant mutants, when colony biofilms were exposed
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7 322 to rifampicin for periods longer than 48 h. In the present case, the
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10 323 combination 40 mg/mL NAC- 10 mg/L rifampicin showed a higher and real
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12 324 bactericidal effect on biofilms (above 3 log₁₀), which probably opens the
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14 325 way for an efficient action of the immune system in the eradication of *in*
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16 326 *vivo* biofilms.

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7 436 **LEGEND TO FIGURES**

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12 438 Figure 1. Number of viable cells (expressed as log₁₀ CFU) of *S.*
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14 439 *epidermidis* recovered from biofilm cells (A) and planktonic cells (B) after
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17 440 treatment with NAC and rifampicin (RIF) alone or in combination.

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22 442 Figure 2. Cellular activity expressed as XTT absorbance of *S.*
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24 443 *epidermidis* biofilm cells (A) and planktonic cells (B) after treatment with
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27 444 NAC and rifampicin (RIF) alone or in combination.

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32 446 Figure 3. *S. epidermidis* total biofilm biomass expressed as crystal violet
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34 447 absorbance after treatment with NAC and rifampicin (RIF) alone or in
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37 448 combination on biofilm cells.

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42 450 Figure 4. Quantification of protein (A) and polysaccharides (B) of the
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44 451 exopolymeric matrix of *S. epidermidis* (9142 and 1457) biofilms.

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49 453 Figure 5. Scanning electron microscopy photographs of 24 h *S.*
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51 454 *epidermidis* (strain 1457) biofilms: control (A), NAC 4 mg/mL (B), NAC 40
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54 455 mg/mL (C), rifampicin (D), NAC (4 mg/mL) with rifampicin (E) and NAC

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456 (40 mg/mL) with rifampicin (F). The bar in the images corresponds to
457 5µm. Magnification x 5000.