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31 **Antimicrobial and antibiofilm efficacy of graphene oxide against chronic wound**
32 **microorganisms**
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57 **Running title: Effect of graphene oxide against wound microorganisms**
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61 **Keywords:** chronic wound microorganisms, graphene oxide, antibacterial activity, antibiofilm
62 activity.
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67 **ABSTRACT** Chronic wounds represent an increasing problem worldwide. Graphene oxide
68 (GO) has been reported to exhibit strong antibacterial activity towards both Gram-positive and
69 Gram-negative bacteria. The aim of this work was to investigate the *in vitro* antimicrobial and
70 antibiofilm efficacy of GO against wound pathogens. *Staphylococcus aureus* PECHA 10,
71 *Pseudomonas aeruginosa* PECHA 4 and *Candida albicans* X3 clinical isolates were incubated
72 with 50 mg/L of GO for 2 and 24 h to evaluate the antimicrobial effect. Optical and Atomic
73 force microscopy images were performed to visualize the GO effect on microbial cells.
74 Moreover, the antibiofilm effect of GO was tested on biofilms both in formation and mature.
75 When compared to the respective time controls, GO significantly reduced the *S. aureus* growth
76 both at 2 and 24 h in a time dependent way, and displayed a bacteriostatic effect in respect to the
77 GO t=0; an immediate (after 2 h) slowdown of bacterial growth was detected for *P. aeruginosa*
78 whereas a tardive effect (after 24 h) was recorded for *C. albicans*. Atomic force microscopy
79 images showed the complete wrapping of *S. aureus* and *C. albicans* with GO sheets that
80 explains its antimicrobial activity. Moreover, significant inhibition of biofilm formation and a
81 reduction of mature biofilm were recorded for each detected microorganism. The antibacterial
82 and antibiofilm properties of GO against chronic wound microrganisms make it an interesting
83 candidate to incorporate into wound bandages to treat and/or prevent microbial infections.
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85 Chronic wounds represent an increasing problem worldwide and are difficult to heal,
86 becoming an important challenge for the healthcare system (1). Chronic wounds, such as
87 diabetic foot ulcers, venous ulcers and surgical non-healing wounds cause poor quality of life
88 and discomfort in patients, in addition to high healthcare costs (2). As known, wounds become
89 chronic for the prolonged inflammation and persistent microbial infections, and often for the
90 presence of multispecies drug-resistant microbial biofilms (3, 4).
91 *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most prevalent bacteria
92 isolated from wounds and frequently found together forming mixed clusters; *S. aureus* is located
93 on the wound surfaces, whereas *P. aeruginosa* is in the deeper region of the chronic wound bed
94 (5, 6). Also the opportunistic pathogen, *Candida albicans,* is known to be associated with the
95 mixed microbiota of wounds and contributes to form dense synergistic complex with bacteria
96 (7, 8).
97 A number of studies have shown the presence of microbial biofilms in chronic wounds.
98 Biofilm represents an important challenge in infectious diseases due to the difficulty in
99 treatment that interferes seriously with healing processes (6).
100 The antibiotic tolerance of microbial biofilm together with the presence of drugs resistant
101 strains make the eradication of bacteria difficult, mainly due to the limited penetration of
102 antibiotics or their ineffectiveness against resistant strains (9). For these reasons, new strategies
103 should be conceived in order to treat chronic wounds.
104 Graphene has emerged in the last fifteen years as an exceptional material able to conduct
105 electrons and heat and possess an extremely high mechanical strength associated to a high
106 elasticity. These properties are enclosed in a 2D material with a very high aspect ratio and
107 therefore a low weight/superficial area value (10). All of these features, gathered in one
108 material, confer to graphene characteristics of high interest for meaningful applications.
109 Graphene has therefore been exploited in different fields and applications ranging from
110 optoelectronics and high-energy physics to material science and medicine (11–14). One
111 drawback of graphene is its low solubility in both organic and aqueous solvents. For this reason,
112 mostly for biomedical applications, hydrophilic graphene derivatives have been prevailingly

113 used and tested. One among them is graphene oxide (GO). This hydrophilic graphene derivative
114 has been particularly investigated due to its hydrophilicity, low tendency to form aggregates and
115 therefore high capacity to homogeneously disperse in water, easily produced from cheap
116 graphite via Hummer's oxidative exfoliation (15).

117 Recently, GO has been reported to exhibit strong antibacterial activity towards both
118 Gram-positive and Gram-negative bacteria (16).

119 To the best of our knowledge, no report is currently available concerning GO effects on
120 biofilms both in formation and mature of clinical chronic wound microbial isolates.

121 Hence, the aim of this work was to investigate the *in vitro* antimicrobial and antibiofilm
122 efficacy of GO against representative wound pathogens, namely *S. aureus, P. aeruginosa* and *C.*
123 *albicans*.

124

125 **RESULTS**

126 Figure 1 shows the effect of GO at 50 mg/L on planktonic growth of clinical isolates from
127 chronic wounds: *S. aureus* PECHA 10, *P. aeruginosa* PECHA 4 and *C. albicans* X3. For *S.*
128 *aureus*, GO significantly reduced the bacterial growth after 2 and 24 h when compared to the
129 respective time controls; no evident ($P > 0.05$) growth increase at 2 and 24 h in respect to the
130 GO t=0 detection was recorded, displaying a clear bacteriostatic effect. For *P. aeruginosa*, after
131 a significant slowdown in the bacterial growth at 2 h of treatment, no significant reduction was
132 recorded at 24 h when compared to the respective time controls. The bacteriostatic effect of GO
133 was detected for *P. aeruginosa* after 2 h when compared to the GO t=0 value. *Candida albicans*
134 showed a significant growth reduction after 24 h of treatment with GO in respect to the time
135 control.

136 Figure 2A and B reports the anti-biofilm effect of GO on biofilm both in formation and
137 mature, respectively. On Biofilm formation, a significant reduction of biomass on polystyrene
138 surfaces was recorded for each detected microorganism in the presence of GO (Fig. 2A, upper).
139 The representative images in Fig. 2A show the correspondent wells of Control and GO,
140 highlighting the visible and clear inhibition to form biofilm of each strain, in presence of GO.

141 This effect was confirmed by the enumeration of the viable and cultivable cells compared to the
142 untreated samples (Fig. 2A, lower). The most significant effect was detected for *S. aureus* with
143 a reduction from 8.63 ± 0.09 to 6.89 ± 0.09 Log_{10} CFU/mL. A combined effect of GO can be
144 hypothesized: the inhibition of microbial growth and the interference with the microbial
145 adhesion.

146 On mature biofilm, GO was able to significantly reduce the produced biomass for all
147 strains, inducing the detachment of cells from the wells as detectable from the corresponding
148 images (Fig. 2B, upper). An important reduction of the Log_{10} CFU/mL count of mature biofilms
149 was observed after treatment with GO (Fig. 2B, lower). With respect to the controls, all
150 produced biofilms undergo a general detachment from the wells, suggesting a possible GO
151 capability to penetrate into the polymeric matrix of biofilm and to destroy its tridimensional
152 structure.

153 Subsequently, microscope observations were employed to evaluate cellular changes after
154 treatment with GO.

155 Figure 3 shows representative images after Gram staining by optical microscopy and
156 images recorded by using AFM. After treatment with GO for 24 h, Gram staining showed the
157 GO capability to trap *S. aureus* and *C. albicans* (Fig. 3, arrows). This effect was confirmed by
158 AFM images where isolated and barely flattened cells wrapped by a thin layer of GO (Fig. 3,
159 arrows) were detected. The trapping effect of GO was not detected for *P. aeruginosa* (Fig. 3,
160 asterisks).

161 Figure 4 demonstrates that all the investigated microrganisms are characterized by a
162 relatively negative zeta potential. The particularly high value for *S. aureus* could be related to
163 the presence, in the outer surface of the bacterium, of teichoic acid. Zeta potential measurements
164 were included to show that the interaction between GO and bacteria is not only electrostatic but
165 they are linked to stronger covalent interactions.

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169 **DISCUSSION**

170 Graphene is a material with potential applications in many fields (17–19). In particular
171 GO has received increasing attention in biomedical fields for its antimicrobial effect (20–23).

172 Lot of studies investigated the GO effect after 2 h of contact (23, 24), in this work we
173 wanted to evaluate the effect of GO during the time to study both immediate and tardive effects
174 on microbial cells (after 2 and 24 h). Results showed an immediate and tardive effect against *S.*
175 *aureus* and only an immediate effect against *P. aeruginosa* with a significant reduction of
176 planktonic growth compared to the respective time controls. In presence of GO, no increase of
177 *S. aureus* planktonic population was recorded, at each detected time, whereas for *P. aeruginosa*
178 this bacteriostatic effect was observed only at 2 h.

179 As previously reported for some Gram-negative and Gram-positive bacteria (25–29), we
180 observed a weak antibacterial activity against *P. aeruginosa* and a greater loss of viability in
181 time for *S. aureus*. The recorded inhibition of microbial growth is probably due to the complete
182 wrapping of single bacterial cell by GO and the consequent interruption of its metabolic activity
183 (i.e. discontinuance of nutrient metabolism). As evidenced by optical microscopy and AFM
184 measurements, the cells still maintained their spherical shape and cell integrity, no sign of
185 membrane damage can be envisaged, the deflation of the cells observed by measuring their
186 thickness by AFM (Fig. 3, AFM images evidenced that the high of the cells reduces of ca. 20%
187 in the presence of GO) is not statistically significant. It is very likely that GO wraps the cells up
188 by interacting with the peptidoglycan layer. As a matter of fact, the peptidoglycan consists of
189 sugars, formed of alternating residues of β-(1-4) linked N-acetylglucosamine (NAG) and N-
190 acetylmuramic acid (NAM), and amino acids and the free amino groups of these molecules can
191 easily form amide linkages with GO (30). The consequent isolation of the bacterium from the
192 growth medium reduces simply its duplication rate. It may appear anomalous that GO,
193 characterized by a relatively negative zeta potential, interacts with the highly negative surface
194 charge of Gram-positive. Nevertheless, true covalent bonds among carboxylic groups and
195 reactive epoxide moieties of GO and amine of peptidoglycan and aminoacids as well as
196 hydrogen bond interactions between carbonyl and hydroxyl groups abundant in peptidoglycan,

197 teichoic acid and GO and π–π interactions between the amide bonds in peptidoglycan and
198 graphenic domains in GO, do easily counterbalance electrostatic interactions (30, 31). These
199 interactions are confirmed by AFM analyses that evidenced how *S. aureus* is overlaid by GO.
200 On the other hand, the interactions of GO with *P. aeruginosa* are strongly depressed and AFM
201 showed the scarce tendency of *P. aeruginosa* to establish a contact with the GO flake. This
202 evidence is in perfect agreement with recently published force spectroscopy measurements that
203 demonstrated physical interactions between GO and *Escherichia coli* are prevailingly repulsive
204 (31). Indeed, Akhavan et al. (16) suggested that the outer cell membrane of Gram-negative
205 protects them from GO damage.

206 The suggested effect of GO on the present investigated bacteria is thus different from that
207 described in literature, where flattened and completely disrupted cells were evidenced (28), but
208 is in line with previous evidences (32) where bacteria were demonstrated to be trapped within
209 the aggregated sheets of graphene and could not proliferate in the culture medium. Probably this
210 difference is due to the different size of GO flakes. The ones investigated in the present paper
211 are micrometer-sized GO (DLS measurements evidenced a diameter of 830±50 nm, thus
212 confirming the producer characterization) and they are recognized to be too big in order to enter
213 the membrane and puncture it (16, 29).

214 For *C. albicans*, no relevant effect was recorded at 2 h of treatment, but a significant
215 reduction was observed after 24 h of incubation. To our knowledge, no information about GO
216 effect on yeasts are available. For the first time, we tested the GO against *C. albicans* isolated
217 from wound chronic ulcers. Probably, the tardive effect of GO is due to the slower growth rate
218 of the yeast. The same mechanisms observed for *S. aureus* can be hypothesized for *C. albicans*:
219 GO entraps yeasts within the sheets and disconnects them from the environment as shown by
220 AFM images. Indeed, the cell wall of *C. albicans* is in part formed by 2-4% of chitin (NAG)
221 and 80-90% of glucans and mannans.

222 *C. albicans* is a dimorphic opportunistic fungus able to invade wounds contributing to
223 interfere with the normal wound healing (8). The yeast form plays a key role in the
224 dissemination and systemic infection (33), the hyphal morphology is important for the biofilm

225 formation and is involved in the establishment and maintenance of the infection. In this work,
226 we observed that the addition of GO induces a sustained hyphal morphology. Despite the fact
227 that much remains unclear and additional studies are needed, it is plausible that GO affects the
228 cell separation process inducing the polarized growth of hyphae as shown by microscopic
229 observations.

230 Chronic wounds are an increasing problem characterized by a not normal wound healing
231 process also due to the microbial biofilm formation. Fazli et al. (5), demonstrated a nonrandom
232 distribution of *P. aeruginosa* and *S. aureus* in wounds. Bacteria are present as large aggregates
233 in the wound bed and *S. aureus* aggregates are located at the wound surface whereas the *P.*
234 *aeruginosa* clusters are in the deeper part of the wound bed. The GO capability to affect the
235 superficial staphylococcal barrier could allow antimicrobials to reach the deeper sites and affect
236 the basal microbial part.

237 Microbial biofilm mode of growth allows microbes to protect themselves against host
238 immune system and antimicrobial agents making biofilm related infections difficult to treat and
239 eradicate. We tested the GO effect on the biofilm formation and mature biofilm highlighting the
240 GO capability to hinder microbial adhesion and penetrate the biofilm matrix.

241 This effect is likely due to the strong capacity of GO to solubilize in different polymeric
242 environments thought to specific interactions of polymers and proteins given its wide and
243 carbon rich, but hydrophilic, surface (18, 34, 35); i.e. hydrogen bond interactions between
244 carbonyl and hydroxyl groups abundant in polysaccharide and GO and π–π interactions between
245 the DNA and RNA bases and graphenic domains in GO. These interactions disfavor
246 microorganisms aggregation (29) and adhesion on the surfaces (36). In particular, the
247 antibiofilm effect is sustained by the synergic effects of the reduction of microbial growth and
248 the inhibition of the adhesion of microrganisms to each other and on the surfaces.

249 The antibacterial and anti-biofilm properties of GO against chronic wound
250 microorganisms make it an interesting candidate to incorporate into wound bandages to treat
251 and/or prevent microbial infections.

252

253 ## MATERIALS AND METHODS

254 **Microbial cultures.** *Staphylococcus aureus* PECHA 10, *P. aeruginosa* PECHA 4 and *C.*
255 *albicans* X3 clinical isolates, obtained from the private collection of Bacteriological
256 Laboratories of the Dep. Pharmacy, University "G. d'Annunzio" Chieti-Pescara were used in
257 this study. Microorganisms were isolated from the wounds of patients with chronic venous leg
258 ulcer and cultured on Mannitol Salt agar, Cetrimide agar and Sabouraud dextrose (SAB; Oxoid,
259 Milan, Italy) agar, respectively. For the experiments, bacteria were cultured in Trypticase Soy
260 Broth (TSB; Oxoid) and incubated at 37 °C overnight in aerobic condition. Cultures were
261 refreshed for 2 h at 37 °C in an orbital shaker in aerobic condition and standardized at $\sim 5 \times 10^7$
262 CFU/mL in TSB diluted 1:5 in phosphate-buffered saline (PBS). Previous analysis
263 demonstrated that TSB 1:5 did not interfere with GO characterization (data not shown). Fresh
264 colonies of *C. albicans*, grown on SAB agar, were used to obtain a broth-culture at $\sim 5 \times 10^5$
265 CFU/mL in RPMI 1640 (Sigma-Aldrich, Milan, Italy) plus 2% glucose (37).

266 **Preparation of GO aqueous dispersion.** An aqueous solution of 4 g/L GO
267 (GRAPHENEA, Donostia-San Sebastian, Spain) was diluted in PBS at the elected
268 concentration, bath ultrasonicated for 10 minutes (Elmasonic P60H, 37 kHz, 180 W) and
269 sterilized for 2 h under UV lamp (Spectronics Spectroline EF 160/C FE, 6 W, 50 Hz, 0.17 A).
270 The concentration of GO was checked by UV-vis spectrophotometry at λ_{max} 230 nm.
271 Dimensions of GO flakes were measured by using Dynamic Laser Light Scattering (DLS;
272 90Plus/BI-MAS ZetaPlus multi angle particle size analyzer, Brookhaven Instruments Corp.)
273 (21).

274 **Antimicrobial activity.** The standardized broth-cultures of *S. aureus* PECHA 10, *P.*
275 *aeruginosa* PECHA 4 and *C. albicans* X3, prepared as indicated above, were incubated with
276 GO at a final concentration of 50 mg/L in TSB (final dilution 1:10 in PBS) for bacteria, and in
277 RPMI 1640 plus 2% glucose (final diluted 1:2 in PBS) for *C. albicans,* at 37 °C for 2 and 24 h
278 to evaluate both an immediate and tardive effect. As controls, bacteria were incubated with fresh
279 diluted TSB (1:10 in PBS) and *C. albicans* with diluted RPMI 1640 plus 2% glucose (1:2 in

280 PBS). The microbial viability was evaluated at t=0, 2 h and 24 h by counting the colony forming
281 units (CFUs) through the Colony Counter Star-Count STC 1000 (VWR International PBI Srl,
282 Via San Giusto, Milan, ITALY). Briefly, series of 10-fold cell dilutions (100 µL) were spread
283 on TSA plates for bacteria and on SAB agar for *C. albicans* and incubated for 24-48 h at 37 °C.
284 The cell growth inhibition was detected comparing the colony counts between GO vs. the
285 respective time Control and between GO vs. the GO t=0 detection (38).

286 **Graphene oxide effect on biofilm formation.** The effect of GO on bacterial and *C.*
287 *albicans* biofilm-forming ability was tested on polystyrene flat-bottomed microtiter plates for: i)
288 biofilm biomass evaluation by safranin staining method (39) and ii) the CFU count for the
289 quantification of cultivable cells (38).

290 Briefly, bacterial cultures were grown overnight in TSB, refreshed and standardized as above
291 described in TSB 1:5 plus 0.5% (v/v) glucose. 100 µL were dispensed into each well of 96-well
292 polystyrene flat-bottomed microtiter plates treated with collagen, in the presence of 100 µL of
293 GO at final concentration of 50 mg/L or 100 µL sterile PBS (Control). *Candida albicans*,
294 standardized in RPMI 1640 plus 2% glucose as above described, was dispensed (100 µL) into
295 each well of 96-well polystyrene flat-bottomed microtiter plates in the presence of 100 µL of
296 GO at final concentration of 50 mg/L or 100 µL PBS (Control).

297 After incubation for 24 h at 37 °C: i) for the biofilm biomass evaluation, each well was
298 washed twice with sterile PBS, dried, stained for 1 min with 0.1% safranin and eluted in 200 µL
299 ethanol and OD_{492} was measured by spectrophotometry using an ELISA microplate reader
300 (SAFAS, Munich, Germany); ii) for the quantification of cultivable cells, each well was washed
301 twice with sterile PBS to remove non adherent cells, and scraped. The bacterial suspensions
302 were sonicated by ultrasonic bath (160 W, 220/240 V, 50-60 Hz; FALC Ultrasonic cleaning
303 instrument, Treviglio, Italy) for 6 min, *C. albicans* was vortexed 3 min with glass beads to
304 disgregate microbial aggregates. Microscopic observations in Live/Dead staining (Invitrogen,
305 Milan, Italy), prior to plating, confirmed that the microbial suspension consisted of a mixture of
306 single viable microbial cells (data not shown).

307 After treatments, each sample was serially diluted (1:10) in PBS and plated on TSA for
308 bacteria and on SAB for *C. albicans* and incubated for 24-48 h at 37°C. The cell concentration
309 was calculated as a mean value of all detections and reported as Log_{10} CFU/ml.

310 **Graphene oxide effect on mature biofilm.** The effect of GO on bacterial and *C.*
311 *albicans* mature biofilm was tested on polystyrene flat-bottomed microtiter plates for ~~by using~~:
312 i) biofilm biomass evaluation by safranin staining method (39) and ii) the CFU count for the
313 quantification of cultivable cells (38).

314 The overnight bacterial cultures, as above described, were refreshed for 2 h at 37 °C in an
315 orbital shaker in aerobic condition and standardized at ~5 × 10^7 CFU/mL in TSB 0.5% (v/v)
316 glucose. Fresh colonies of *C. albicans,* grown on SAB agar, were used to obtain a broth-culture
317 at ~5 × 10^5 CFU/mL in RPMI 1640 (Sigma-Aldrich, Milan, Italy) plus 2% glucose. Microbial
318 standardized cultures, were incubated on 96 flat-bottomed microtiter plates at 37 °C for 24 h.
319 Subsequently, the planktonic cells were gently removed and the wells were washed with sterile
320 PBS and filled with 200 µL of GO solution (50 mg/L) or PBS for the Control. After incubation
321 for 24 h at 37 °C, each well was washed twice with sterile PBS, and subject to i) biofilm
322 biomass evaluation by safranin method and ii) quantification of cultivable cells, as above
323 described.

324 **Preparation of microbial suspension for zeta potential measurements.** Microbial
325 suspensions were prepared as above described. The obtained suspensions were centrifuged
326 (8000 rpm, 30 min) to pellet the microbial cells before further analysis. For zeta potential
327 measurements, the microbial cell suspensions were prepared by re-suspending the cell pellets in
328 milliQ water. Zeta potential data was obtained by using a Zeta PALS, Zeta Potential Analyzer,
329 Brookhaven Instruments Corp. This measurement was performed in order to investigate how
330 GO interacts with microrganisms (40).

331 **Optical microscopic and Atomic Force microscopic observation.** For the microscopic
332 observations, *S. aureus* PECHA 10, *P. aeruginosa* PECHA 4 and *C. albicans* X3 were treated
333 with GO (50 mg/L), in TSB 1:10 in PBS for bacteria and in RPMI 1640 plus 2% glucose (1:2 in
334 PBS) for *C. albicans* and incubated for 24 h at 37 °C. For the Controls, bacteria were incubated

with diluted TSB (1:10 in PBS) and *C. albicans* with diluted RPMI 1640 plus 2% glucose (1:2 in PBS). From each broth-culture, 10 µL was Gram-stained and observed microscopically under a Leica 4000 DM microscope (Leica microsystems Spa, Milan, Italy).

Atomic Force microscopic (AFM) measurements were performed by using a Multimode 8 Bruker AFM microscope with Nanoscope V controller (Bruker, Billerica, MA, USA). Silicon cantilever and a RTESPA-150 tip (spring constant = 5 N/m and resonant frequency 150 kHz) were used in a tapping in air mode. Each broth-culture (10 µL), as above prepared, was placed on sterile glass square (0.5 × 0.5 cm), air dried and observed under AFM (41).

Statistical analysis. The statistical significance of differences between controls and experimental groups was evaluated using Student's *t* test. Probability levels of <0·05 were considered statistically significant. All data was obtained from three independent experiments performed at least in triplicate.

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465

466 **FIGURE LEGENDS**

467

468 **FIG 1** Effect of Graphene oxide (GO) 50 mg/L, on planktonic growth of *Staphylococcus aureus*
469 PECHA 10, *Pseudomonas aeruginosa* PECHA 4 and *Candida albicans* X3 in time. Asterisks
470 indicate the significance ($P<0.01$) between the samples treated with GO and the respective time
471 controls.

472 **FIG 2** Effect of Graphene oxide (GO) 50 mg/L on sessile phase of *Staphylococcus aureus* PECHA
473 10, *Pseudomonas aeruginosa* PECHA 4 and *Candida albicans* X3. (A) Activity of GO on biofilm
474 in formation. (B) Activity of GO on Mature Biofilm. A and B, upper: Biomass quantification and
475 representative images before safranin solubilization compared to the respective controls. A and B,
476 lower: Colony Forming Unit Counts (Log_{10} CFU/mL) compared to the respective controls.

477 **FIG 3** Effect of Graphene oxide (GO) 50 mg/L on planktonic phase of *Staphylococcus aureus*
478 PECHA 10, *Pseudomonas aeruginosa* PECHA 4 and *Candida albicans* X3. Representative images,
479 Control and treated with GO, obtained with Gram staining (columns on the left) and AFM
480 (columns on the right). Arrows indicate GO wrapping microganisms. Asterisks indicate the GO
481 layer.

482 **FIG 4** Zeta Potential values for *Staphylococcus aureus* PECHA 10, *Pseudomonas aeruginosa*
483 PECHA 4, *Candida albicans* X3 and GO in milliQ water.