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1 **The key lethal effect existed in the antibacterial behavior of short, medium, and**
2 **long chain fatty acid monoglycerides on *Escherichia coli***
3
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22 **Abstract:** Monoglyceride is an amphiphilic molecule with promising antimicrobial
23 activity for bacteria; however, the key lethal effect in its antibacterial behavior was still
24 unknown. In the study, monobutyrate (MB), monolaurate (ML), monomyristate (MM)
25 were selected to represent the short, medium, and long chain monoglycerides to compare
26 their inhibitory effect against *Escherichia coli*, and a new dose-dependent inhibitory
27 mechanism was proposed by the key lethal effect. The minimal inhibitory concentration
28 and antibacterial curve showed a huge diversity existed in biology activity of MB, ML
29 and MM. The results in scanning electron microscopy and flow cytometry assay
30 indicated that the interference level of MB on cell membrane was obviously weaker than
31 that of ML and MM, while the latter two had similar performance in increasing cell
32 permeability at low doses. The results presented in UV-Vis spectroscopy, cell cycle and
33 biomacromolecules synthesis inhibition assay showed that the cell cycle of *Escherichia*
34 *coli* was obviously affected by three monoglycerides at doses near MIC, which was
35 therefore regarded as the key lethal effect. The reason for the better biological activity of
36 MM than ML was the stronger interference ability on bacterial cell cycle. In addition, an
37 expanded antibacterial mode was raised that cell permeability increase at low doses was
38 antimicrobial basis, cell cycle arrest at medium doses played the key lethal effect, and
39 cell lysis at high doses was the result of combined action.

40

41 **Keywords**: different chain length; monoglyceride; *Escherichia coli*; key lethal effect;
42 DNA interference; cell cycle arrest

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43 **1. Introduction**

44 *Escherichia coli O157:H7 (E. coli O157:H7)*, a common foodborne pathogenic
45 bacterium, causes a series of infectious diseases in human body such as hemorrhagic
46 diarrhea and enteritis, hemolytic uremic syndrome and thrombotic thrombocytopenic
47 purpura (1-3). This bacteria has a strong pathogenicity and can cause obvious symptom
48 at a very low infection dose which is lower than 50—100 cells (4). It was firstly
49 discovered and isolated from the feces of food poisoning patients consuming
50 contaminated hamburger in America, and subsequently appeared some large-scale
51 outbreak cases in Japan, Canada, Australia, and some Nordic countries (4-8). The most
52 serious disease outbreak occurred in Japan, and the infected population was up to tens of
53 thousands of people, causing 11 of death (9). According to the report from The Centers
54 for Disease Control, *E. coli O157:H7* infection caused 20000 disease cases and
55 200—500 death each year with estimated annually medical expenses of 405 million
56 dollars in the United States (10, 11). This toxin-producing pathogenic *E. coli* has become
57 the second common intestinal pathogen following Salmonella, and *E. coli O157:H7*
58 infection accounted for 91.4% in pathogenic *E. coli* diseases (12).

59 Microbial contamination has become a major source of safety risks in food industry,
60 and various physical treatments and chemical additives have been used to eliminate
61 potential pathogens (13, 14). Owing to the concern about safeness of chemicals,
62 consumers prefer natural preservatives or bactericides isolated from some animal and
63 plant materials instead of synthetic chemicals (15). Fatty acid monoglycerides are a class

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64 of promising antibacterial agents, naturally occurred in American pusa, animal milk and
65 some other foods (16, 17). The esters have broad antibacterial activity toward
66 gram-negative, gram-positive bacteria and fungi including yeasts and moulds, and
67 inhibition effects are little affected by pH (18). In addition, monoglycerides can be
68 resolved in the gastrointestinal tract and do not show any toxicity to human body (19). In
69 the past decade, the studies on antibacterial activity of monoglycerides have been limited
70 to some medium carbon chain glycerides ranged from 8 to 12 carbons (20-23). Recently,
71 some literatures reported that the antimicrobial activity of long carbon chain fatty acid
72 monoglycerides, including monomyrisate and monopalmitate (24, 25). Clelia Altieri
73 revealed that the inhibition indexes of monomyrisate and monopalmitate were obviously
74 higher than that of monolaurin on *E. coli O157:H7* at 20 ppm after 10 h of incubation,
75 and the inhibitory effectiveness was in dose-dependent for monolaurin, but the two else
76 were exactly the opposite (26). However, the systematic studies on the antibacterial effect
77 of short, medium and long carbon chain fatty acids monoglycerides are still lacking,
78 more comparative studies are therefore necessary if selecting suitable monoglycerides for
79 the inhibition of different microorganisms.
80 The action mechanisms in most monoglycerides antimicrobial studies focused on
81 membrane damage, which could increase cell permeability and even lead to the leakage
82 of cellular contents (17, 21, 27). Hyldgaard, M. visualized membrane disruption caused
83 by monocaprylate using atomic force microscopy, indicating that cell membrane was a
84 important action site in antibacterial test (28). Although it is known that monoglyceride

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85 play the inhibitory effect by membrane damage, the complete antimicrobial mechanism
86 is still not fully understood. Interestingly, the cell wall of the bacteria with incomplete
87 cell membrane gradually split in the late stage of antibacterial test, suggesting that the
88 cell wall lysis was obviously later than the membrane disruption, which was more like an
89 antibacterial result than a action mode (29). Recently, scientists also found some cationic
90 antibacterial peptides not only formed holes on membranes, but also bound to DNA and
91 interfered normal cell metabolism, indicating that intracellular action was a
92 non-negligible process after antimicrobial agents penetrating through cell membrane
93 (30-32). Therefore, the potential intracellular action goals, especially for DNA, should be
94 incorporated into research scope in antibacterial test in the future.

95 In present study, the inhibitory effect of short chain (2－6 carbons), medium chain (8
96 －12 carbons), and long chain (14－18 carbons) fatty acid monoglycerides on *E. coli*
97 *O157:H7* were compared by minimal inhibitory concentration (MIC) and antibacterial
98 curves. The effects of monoglycerides with different chain lengths on cell surface and
99 membrane permeability were evaluated by scanning electron microscopy (SEM) and
100 flow cytometry. In addition, the interaction between monoglyceride and genomic DNA
101 were studied by UV-visible spectrum and cell cycle. Moreover, intracellular DNA, RNA
102 and protein detection was employed to explain the possible relation between DNA
103 double helix disruption and cell division inhibition. This study was aimed at seeking the
104 key lethal effect in monoglyceride antibacterial test and to explain the reason for the
105 difference in the antibacterial effect of short, medium, and long chain monoglycerides.

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106 **2. Materials and methods**

107 2.1 Materials

108 Monoacetate (MA), monobutyrate (MB), monocaprylate (MC), monolaurate (ML),
109 monomyristate (MM) and Monopalmitate (MP) were obtained from Molbase Chemical
110 Co. (Shanghai, China) with purity≥99.0%. A series of stock solutions of the six
111 monoglycerides were made in ethanol to obtain the concentrations of 0.01, 0.02, 0.04,
112 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5, and 10 mg/mL, respectively. Propidium iodide (PI,
113 purity≥94.0% in HPLC) was purchased from Sigma-Aldrich Co. (Santa Clara, USA). A
114 PI stock solution was prepared to achieve the concentration of 10 mg/mL in phosphate
115 buffer solution (PBS, 0.1 M, pH 7.5, Sigma-Aldrich Co., Santa Clara, USA) and stored at
116 2-8°C. Hoechst 33342 (HO, purity≥98%) and fluorescein isothiocyanate (FITC,
117 purity≥90%) were purchased from Yuanye Biological Technology Co. (Shanghai, China),
118 while Pyronin Y (PY，high purity biological stain) was obtained from Acros Co.
119 (Belgium). Their stock solutions were prepared in PBS to harvest 500 μg/mL of HO, 2
120 mg/mL of PY, and 100 μg/mL of FITC. An ezup column bacteria genomic DNA
121 purification kit was bought from Sangon Biotech Co. (Shanghai, China). Tris-HCl buffer
122 (0.05 M, pH 7.4) was obtained from Yuanye Biological Technology Co. (Shanghai,
123 China). The water used in the study was purified by Milli-Q device (Merck Millipore Co.,
124 Massachusetts, USA). Glutaraldehyde solution (25% in H_2O) and osmium tetroxide
125 (purity≥98%) were bought from Sigma-Aldrich Co. (Santa Clara, USA). 2.5% (v/v)
126 glutaraldehyde work solution and 2% (w/v) osmium tetroxide work solution were

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127 prepared in PBS for later use.

128 2.2 Experimental strain

129 A gram-negative bacterium, *E. coli O157:H7* was purchased from Guangdong Culture
130 Collection Center (Guangzhou, China). The strain powder was activitied by dissolving
131 with 1ml sterile PBS and then transferred to tryptone soy agar (TSA, Shuoheng
132 Biotechnology Co., Guangzhou, China) solid plate to culture for 24 h or longer at 37°C.
133 Subsequently, a single colony on solid medium was hooked onto the TSA slope medium
134 with multiple scribing operations and cultured for 24—48 h before storing at 2—8°C.

135 2.3 Antibacterial activity assays

136 The antibacterial effects of short, medium and long carbon chain fatty acid
137 monoglycerides on *E. coli* were characterized by detecting MIC and inhibition curves (33,
138 34). The refrigerated strain was cultured in 100 mL tryptic soy broth (TSB, Shuoheng
139 Biotechnology Co., Guangzhou, China) medium with 120 rpm shaking in 37°C until
140 mid-logarithmic growth phase and centrifugated at 3000 rpm for 5 min. The cell pellets
141 were firstly washed with PBS and then resuspended into diluent with cell concentration
142 of approximate 10^5 CFU/mL in TSB, which was corresponding to optical density of 0.3
143 at 600 nm (OD_{600}≈0.3). 20 μL of different concentrations of MA, MB, MC, ML, MM
144 and MP stock solutions were blended with 180 μL of *E. coli* diluent to achieve final
145 concentrations of 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, and 1000 μg/mL in 96 well plates.
146 The controls were adding the same volume of ethanol, and each concentration was
147 repeatly performed three times. The plates were cultured at 37°C for 24 h with 120 rpm

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148 shaking. The OD_{600} values were recorded at 0 h and 24 h by a microplate reader
149 (SpectraMax i3x, Molecular Devices Co., San Jose, USA). The MIC was defined as the
150 lowest concentration in which the OD_{600} increase was smaller than 0.05 during 24 h.

151 The inhibition curves reflected the vitality of bacterial cells in monoglyceride
152 treatment and were measured by plate counting method. According to the results of MIC
153 assay, MB, ML and MM were selected for representing short, medium and long chain
154 fatty acid monoglycerides to complete the research. 50 μL of MB, ML, and MM
155 solutions were mixed with 950 μL of *E. coli* diluent in sterile 1 mL centrifuge tubes to
156 reach final concentrations of 8, 16, 32, 64, 125, 250, 500, and 1000 μg/mL, respectively.
157 The controls were adding 50 μL of ethanol and all samples were conducted in triplicate.
158 The cells were cultivated for 1 h at 37°C with 120 rpm shaking. Then 10-fold serially
159 dilutions were performed for all *E. coli* samples and cultivating for 24 h at 37°C prior to
160 colony counting.

161 2.4 Membrane integrity test

162 2.4.1 SEM assay

163 According to the reports of previous literatures (35, 36), the influence of MB, ML and
164 MM on the *E. coli* surface was assessed by SEM. 50 μL of three monoglycerides
165 solutions were mixed with 950 μL of cell diluent prepared as the above method in sterile
166 centrifuge tubes to obtain final concentrations of 1/2 MIC, 1 MIC, and 2 MIC,
167 respectively. The controls were adding the same volume of ethanol. The cell samples
168 were cultured for 1 h at 37°C with orbital shaking at 120 rpm and collected by

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169 centrifugation at 3000 rpm for 5 min. Then cell pellets were washed twice with sterile
170 PBS and fixed with 0.5 mL of 2.5% (v/v) glutaraldehyde work solution for 12 h at 4°C.
171 On the second day, the *E. coli* cells were post fixed in 0.1 mL of 2% (w/v) osmium
172 tetroxide work solution at room temperature for 2 h. Subsequently, the immobilized cells
173 were washed with sterile PBS and orderly dehydrated with 30%, 50%, 60%, 70%, 80%,
174 90%, and 100% ethanol solution for 10 min. Then a drop of cell solution in ethanol was
175 added onto the coverslip to stand until the cells had settled down. After freeze-drying in
176 vacuum for 24h or longer, a cold field scanning electron microscopy (UHR FE-SEM
177 SU8220, Hitachi Ltd., Tokyo, Japan) was allowed to record the change of membrane
178 morphology.

179 2.4.2 Cell permeability measurement

180 The changes of *E. coli* membrane permeabilities after adding MB, ML and MM were
181 investigated by the method of Hyldgaard, M. et al. with some modification (37). The *E.*
182 *coli* cells were harvested in mid-log growth phase by centrifugation and wash, and
183 resuspended to cell density of 10^5 CFU/mL with sterile TSB. 950 μL of aliquot cell
184 diluents were combined with 50 μL of three monoglycerides solutions to obtain final
185 concentrations of 0, 2, 4, 8, 16, 32, 64, 125, 250, and 500 μg/mL. Each concentration was
186 performed three times in parallel. All groups were cultured at 37°C for 1 h except of
187 85°C heating for 1 h in positive controls, which provided maximum detection boundary
188 (MDB). Subsequently, the cells were washed with sterile PBS and resuspended in 1 mL
189 PBS before staining with 50 μL of 100 μg/mL PI work solution for 20 min in dark

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190 condition. Finally, the cell samples were immediately allowed to fluorescence analysis by
191 a flow cytometer (CytoFLEX，Beckman Coulter Co., California, USA). The excitation
192 and emission wavelength of PI were located at 488 nm and 610 nm, respectively (38).
193 The sample flow rate was controlled as 100—500 cells/s, and at least 10000 cells were
194 collected for the following data analysis.

195 2.5 Interaction of monoglycerides with different chain lengths and genomic DNA

196 2.5.1 Impact of different monoglycerides on the structure of genomic DNA

197 To examine the impact for DNA brought by monoglycerides, UV-visible spectroscopy
198 was carried out similar to the method described by Hegde, A. H. et al (39). The genomic
199 DNA was purified from viable *E. coli* cells in mid-log growth phase by an ezup column
200 bacteria genomic DNA purification kit. The ratio of the UV absorbance of genomic DNA
201 at 260 nm and 280 nm was 1.85, suggesting that the purified DNA was free from proteins
202 (40). The concentration of *E. coli* DNA was diluted to 3.6 mM in sterile Tris-HCl buffer,
203 calculating by UV absorbance at 260 nm in 1 cm quartz cell divided by 6600 $M^{-1}cm^{-1}$ of
204 a molar absorption coefficient (32). Subsequently, 25 μL of MB, ML and MM solution
205 were mixed with 475 μL of *E. coli* diluents with cell density of 10^5 CFU/mL to obtain
206 final monoglycerides concentrations of 2, 4, 8, 16, 32, 64, 125, 250, 500, and 1000
207 μg/mL in sterile 1.5 mL centrifuge tubes. The control groups were adding the same
208 volume of ethanol and all samples were allowed to equilibrate for 5 min before UV
209 spectral scanning. To eliminate the adverse effect from background, the baseline was
210 firstly corrected for Tris-HCl buffer signal. The UV spectral was recorded at a

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211 wavelength range from 220 nm to 320 nm and obtained the average of three
212 determinations in parallel.

213 2.5.2 Effect of different monoglycerides on the cell division of *E. coli*

214 Cell cycle is an important indicator of cell division function and is measured by flow
215 cytometry combined with PI staining according to the report from Steen, H. B. et al (41).
216 50 μL of MB, ML and MM solutions were combined with 950 μL of *E. coli* suspensions
217 (10^5 CFU/ml) to achieve final monoglyceride concentrations of 1/4 MIC, 1/2 MIC, 1
218 MIC, 2 MIC, and 4 MIC, respectively. The controls were added 50 μL of ethanol and all
219 groups were repeatly performed three times. The treated cells were cultured at 37°C for 1
220 h with 120 rpm shaking before centrifuge collection and PBS wash. The cell pellets were
221 fixed with 70% (v/v) ice ethanol (pre-cooling at -20°C overnight) for 12 h or longer at
222 4°C. Subsequently, the cells were centrifuged and washed to remove the stationary liquid.
223 Finally, 1 mL of 50 μg/mL PI work solution (containing 1 mg/mL Rnase) was added into
224 the immobilized cells to stain for 20 min at 4°C in the dark condition before detecting by
225 the Beckman flow cytometer. The excitation and emission wavelength of PI-DNA
226 complex were located at 488 nm and 610 nm, respectively. The flow rate of cell solution
227 was turned to 100—200 cells/s, and at least 30000 cells were captured for the subsequent
228 scatter and histogram analysis.

229 2.5.3 Influence of different monoglycerides on the synthesis of intracellular
230 biomacromolecules

231 The synthesis inhibition of MB, ML and MM on DNA, RNA and protein in *E. coli* was

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232 studied by flow cytometry combined with three fluorescence staining (42). Three dyes,
233 HO, PY and FITC, were used to stain intracellular DNA, RNA and protein, respectively.
234 Their relative contents were characterized by measuring light intensity of blue, red and
235 green fluorescence which almost had no overlap in the emission spectrum region. The
236 viable *E. coli* cells were harvested from overnight cultures by centrifugation and
237 resuspended to cell density of 10^5 CFU/mL in broth. 950 μL aliquots of cell suspensions
238 were supplemented with 50 μL of MB, ML and MM solution to reach final concentration
239 of 1 MIC. The controls contained 50 μL of ethanol instead of monoglycerides solutions.
240 The solutions were incubated for 0, 10, 20, 30, 40, 50, and 60 min at 37°C, respectively.
241 After different time periods, the *E. coli* cells were collected by centrifugation.
242 Subsequently, 1 mL of 70% (v/v) ice ethanol (pre-cooled at -20°C for 12h) was added to
243 fix overnight at 4°C. The stationary liquid was removed by centrifugation prior to PBS
244 wash. Finally, 1 mL of mixed dyes solution containing 0.5 μg/mL HO, 2.0 μg/mL PY,
245 and 0.1 μg/mL FITC in PBS was added to stain for 20 min at 4°C in the dark condition
246 before allowing to the analysis of flow cytometry.

247 The semi-automatic device was equipped with three laser excitation flow system.
248 Specifically, the excitation laser wavelengths of DNA, RNA and protein fluorescence
249 were located at 355 nm, 530 nm, and 457 nm respectively. Correspondingly, the emission
250 fluorescence of HO-DNA (blue), PY-RNA (red), and FITC-protein (green) were
251 measured at 450 nm, 580 nm, and 520 nm, respectively. The fluorescence signal was
252 repeatly captured three times for each cell sample before drawing frequency distribution

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253 histograms and calculating phase proportions.

254 2.6 Statistical analysis

255 The averages of colony count of three repetitions were converted into cell density (log
256 CFU/mL). All data in figure were showed as average ± standard deviation (SD) of three
257 replicate measurements. The variance analysis of results was performed by One-Way
258 ANOVA in OriginPro 8.5. A tukey test was used to confirm the significant variance in
259 statistics between controls and samples when $p < 0.05$.

260 **3. Results**

261 3.1 Antibacterial effects of monoglycerides with different chain lengths

262 The antimicrobial activity of short, medium, and long chain monoglycerides was
263 qualitatively compared by MIC and quantitatively characterized by antibacterial curve.
264 The MIC of MB, ML, and MM against *E. coli* were 500 μg/mL, 64 μg/mL, and 32
265 μg/mL, suggesting that ML and MM are more active than MB in terms of inhibitory
266 concentration. Subsequently, the population of *E. coli* cells after three monoglycerides
267 treatment was shown in Figure 1. The cell counts all decreased in a
268 concentration-dependent manner with the larger decline in ML and MM groups. Besides,
269 the cell population declined more than 2 log units when adding MB, ML, and MM at
270 respective MIC, which exceeded the action effect of three monoglycerides at
271 corresponding half maximal inhibitory concentrations. Furthermore, 500 μg/mL of ML
272 and 250 μg/mL of MM played totally bactericidal activity against *E. coli*, which could
273 not achieve for MB even if its concentration increased to 1000 μg/mL, suggesting that

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274 evident variance was existed in the sensitivity of *E. coli* to monoglycerides with different
275 chain lengths.

276 3.2 Membrane integrity assay

277 3.2.1 SEM observation

278 As shown in Figure 2A, the untreated *E. coli* cells had a flat, smooth surface. However,
279 after adding MB, ML, and MM, the cell membrane became uneven, rough, and even
280 concave. Specifically, the *E. coli* cells did not change into rough until MB concentration
281 increased to 2 MIC (Figure 2B-D). After ML treatment, the cell membrane lose smooth
282 and flat surface at 1/2 MIC, and increasing concentration further to 1 MIC and 2 MIC,
283 the cell surface showed further distortion with deeper depression and larger deformation.
284 As for MM treatment at 1/2 MIC, 1 MIC, and 2 MIC, the cells surface sequentially
285 appeared flat, rough, and obvious depression.

286 3.2.2 Membrane permeability measurement

287 The fluorescence signal of PI in *E. coli* cells was often regarded as an indicator of
288 membrane destruction because it was captured after penetrating through damaged cell
289 surface and binding to intracellular DNA (43). The permeability ratios of membranes of
290 *E. coli* exposed to various concentrations of MB, ML, and MM were calculated by
291 dividing the cell population with significant fluorescence signal by the total cell number,
292 and the statistical results were shown in Figure 3. Compared to 25.81±1.50% of
293 permeability ratio of *E. coli* cells in control group, the percent of cell with damaged
294 membrane after three monoglycerides treatment all increased with an increase in

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295 concentration, but all did not exceed the MDB of 93.59±3.37%. Specifically, the
296 monoglycerides concentrations causing above 50% of penetration ratio were 250 μg/mL
297 for MB, 8 μg/mL for ML, and 8 μg/mL for MM, suggesting that the penetration capacity
298 of MB to the cell membrane of *E. coli* was much lower than that of ML and MM which
299 did not show obvious difference in penetration ability. Surprisingly, increasing further
300 ML and MM concentration to 4 MIC led a decrease in membrane permeability, which
301 might be due to the fact that a large part of cell death and membrane lysis appeared in
302 membrane-damaged *E. coli* at high concentration, resulting in the appearance of the
303 decrease trend in Figure 3 (44).

304 3.3 Action of monoglycerides with different chain lengths on *E. coli* DNA

305 3.3.1 Effect of different monoglycerides on DNA structure of *E. coli*

306 The result of UV-visible spectral from the interaction between genomic DNA of *E. coli*
307 and monoglycerides was shown in Figure 4. Before adding monoglyceride, the
308 absorbance peak of *E. coli* DNA appeared at the wavelength of 260 nm. After adding
309 three monoglycerides, the maximum absorbance values increased to varying degrees
310 without obvious shift in wavelength, which was called hyperchromic effect, indicating
311 that the double helix of *E. coli* DNA was destroyed by the foreign monoglycerides.
312 Furthermore, examination the spectrum difference in MB, ML, and MM group revealed
313 that the maximum absorbance value in MM group (0.544 at 250 nm) was greater than
314 that in ML group (0.499 at 250 nm) and MB group (0.52 at 251 nm), suggesting that the
315 destruction level of DNA duplexes by MM was greater than that by MB and ML. In

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316 addition, the monoglyceride concentrations causing the maximum absorption peaks in
317 UV spectra were in great discrepancy with 16 ppm for MB, 8 ppm for ML, and 4 ppm for
318 MM.

319 3.3.2 Cell cycle changes

320 As shown in Figure 5, the flow histogram in control group showed a DNA distribution
321 of a large peak close to a small peak. After adding MB, ML and MM, the two peaks in
322 treated groups both became higher and narrower, indicating that monoglyceride treatment
323 might alter DNA distribution in the cell cycle of *E. coli*. Furthermore, the cell proportion
324 in G1, S, and G2 phases after the treatment of three monoglycerides was recorded in
325 Figure 6. The G1 percents in three treated groups were all increased with different
326 degrees, and only a significant effect was observed at 2 MIC for MB, 1 MIC for ML, and
327 1/2 MIC for MM. Surprisingly, further increasing MM concentration to 4 MIC resulted
328 in a decrease in G1 proportion, which may be attributed to the leakage of DNA in
329 damaged cells, which could be observed in Figure 2I. Comparison the discrepancy of *E.*
330 *coli* cell cycle among MB, ML, and MM treatment showed the most remarkable growth
331 appeared at MM group, followed by ML group, and MB group grew the slowest,
332 indicating that a great discrepancy occurred at the interference action of monoglycerides
333 with different chain lengths on the cell cycle of *E. coli*.

334 3.3.3 Biomacromolecule synthesis inhibition

335 Apart from studying the effect of monoglycerides on structure and function of *E. coli*
336 DNA, the synthesis inhibition of DNA, RNA and protein was shown in Figure 7.

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337 Compared to the steadily growth of DNA, RNA and protein content in control group, the
338 content of the three biomacromolecules in experimental groups showed a totally different
339 growth trend. Specifically, the RNA amount declined immediately without any time
340 delay once adding monoglyceride, different from a surprisingly phenomenon of
341 increasing firstly and then decreasing in DNA and protein content measurement,
342 suggesting that RNA synthesis was firstly affected after adding monoglycerides, and then
343 the DNA and protein synthesis was inhibited after 30min or completing a generation of
344 cell division. Compared to timely interference in RNA synthesis, the hysteresis in the
345 suppression of DNA and protein synthesis also implied that DNA transcription or RNA
346 synthesis, rather than the synthesis process of protein and DNA, was the first action
347 target in genetic central dogma. Examination the growth difference among MB, ML, and
348 MM treatment revealed that the synthesis inhibition of ML and MM on three
349 biomacromolecules was obviously greater than that of MB in the same conditions.

350 4. Discussion

351 4.1 The sensitivity of *E. coli* to the monoglycerides with different chain lengths

352 In general, fatty acid monoglycerides have broad antibacterial spectrum and stable
353 inhibitory activity, which are often used to control foodborne pathogens according to
354 some previous reports (17, 20, 21, 27, 45, 46). They offer a preliminary view on
355 antimicrobial performance of individual monoglyceride in medium or food ingredients.
356 Moreover, our study found that fatty acid chain length markedly affected the antibacterial
357 performance of monoglycerides on *E. coli*, among which the sensitivity to ML and MM

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358 exceeded 8 times of that to MB. The comparative investigation in antibacterial effect of
359 medium carbon chain monoglycerides against *E. coli* has been conducted to select the
360 most active lipid. Clelia Altieri found *E. coli O157:H7* was completely inhibited in PC
361 broth plus 50 μg/mL of monolaurate, whereas monomyristate and monopalmitate at the
362 same concentration did not have the effect, which could be explained by different
363 medium compositions and various culture conditions (26).

364 In this assay, the treatment with ML at 500 μg/mL or MM at 250 μg/mL killed *E. coli*
365 cells to undetectable level, whereas MB bactericidal ability was limited even at the
366 maximum concentration (only resulting in more than 2.2 log reduction in cell population).
367 The huge difference in antibacterial activity among short and medium-long chain
368 monoglycerides may be attributed to the weaker hydrophobic interaction between acyl
369 carbon chain and the lipid membrane (47, 48). On the other hand, the hydrophobic of
370 monoglyceride increased with increasing the length of carbon chain, which reduced its
371 solubility in aqueous solution, thus preventing its transport through *E. coli* cell
372 membrane (49).

373 4.2 Membrane action mechanism

374 At present, many studies on action mechanism of monoglyceride on bacterial cell
375 membrane involve the change in physical morphology and the decrease in the ability to
376 control material enter and exclude cell membrane (28, 50, 51). SEM images revealed that
377 three monoglycerides exposure produced an uneven, rough and wrinkled surface as well
378 as the appearance of depression on the affected *E. coli* cells, which was similar to the

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379 phenomenon reported in some literatures (21, 27, 50, 52, 53). Monoglycerides are a class
380 of non-ionic surfactants with acyl carbon chains and hydroxyl groups, which could
381 interact with the hydrophobic region and disrupt the composition of *E. coli* membrane
382 (29, 54). In addition, the interaction of monoglyceride into cell membrane also increases
383 the fluidity and permeability of the membrane (55, 56). This explanation is further
384 supported by the growth trend of membrane permeability of monoglyceride-treated *E.*
385 *coli* in Figure 3.

386 Review of the relationship among MIC, membrane morphology changing and cell
387 permeability increasing revealed that the bacterial cell membrane was not the only site
388 where monoglycerides work. As shown in Figure 2, the monoglyceride concentrations
389 when depression and breakage appeared on *E. coli* surface were above 2 MIC (1000
390 μg/mL) for MB, 1 MIC (64 μg/mL) for ML, and 2 MIC (64 μg/mL) for MM, all
391 exceeding their respective MIC, which was more like the final result of many
392 antibacterial effects. Furthermore, the results in Figure 3 indicated that the MB, ML, and
393 MM concentrations causing more than 50% of permeability ratio were 250 μg/mL, 8
394 μg/mL, and 8 μg/mL, respectively. However, compared to control groups, the three
395 monoglycerides at the above concentrations did not cause significant decrease in cell
396 population in experimental groups, suggesting that the increase in membrane
397 permeability of *E. coli* had little correlation with the loss of cell viability. On the whole,
398 the above observations showed that monoglyceride increased cell permeability at low
399 concentrations and cause cell damage or even cell lysis at high concentrations, however,

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400 they were both not the key lethal action in the antibacterial test of monoglycerides
401 against *E. coli.*

402 The reason for the difference in sensitivity to short, medium, and long chain
403 monoglycerides may be discrepancy in action for membrane integrity. The MB
404 concentration causing *E. coli* breakage was above 1000 μg/mL, which was far more than
405 that of ML and MM. Similarly, the MB concentration causing above 50% of penetration
406 ratio was 250 μg/mL, which was also much higher than that of ML and MM. The poor
407 action effect of MB on cell membrane might be a suitable explanation for the
408 unsatisfactory antibacterial performance in MB treatment, but was still insufficient to
409 clarify the causes for antibacterial difference between ML and MM treatment if only in
410 terms of the increase level in cell permeability. Considering the finding of the potential
411 intracellular action targets in the antibacterial test of peptides (57-59), more in-depth
412 studies are encouraged to make clear the causes for antibacterial difference of ML and
413 MM on *E. coli*.

414 4.3 Interference of monoglyceride on the structure and function of *E. coli* DNA

415 There are many potential intracellular action objects after monoglyceride penetrating
416 through cell membrane, and genomic DNA is one of the goals that cannot be ignored.
417 According to the reports in some literatures, the modes in which foreign compounds
418 interact with DNA are summarized as three types: intercalation binding between
419 molecular and stacked base pairs in DNA, groove binding between molecular and major
420 or minor grooves in DNA double-helix, and electrostatic binding between charged

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421 molecule and the external DNA backbone (60, 61). UV-visible spectral is a useful tool to
422 study the interaction between *E. coli* DNA and foreign antibacterial agents (62). As we
423 known, the hypochromic effect combined with a red shift in wavelength is observed only
424 when the axial change of DNA conformation occurs, causing by the foreign molecular
425 intercalating into DNA (63). In general, the growth magnitude in absorption spectrum is
426 closely related to its binding force, which means the stronger its binging force, the more
427 evident DNA hypochromic effect (64). However, Figure 4 showed a hyperchromic effect
428 without obvious wavelength shift in UV-visible spectra, which was different from the
429 above law of axial change, suggesting that the interaction mode between monoglyceride
430 and *E. coli* DNA was not the typical intercalation binding. In addition, monoglyceride is
431 an amphiphilic molecule without any charged groups, which excludes the possibility of
432 electrostatic binding. Therefore, it is speculated that groove binding is a suitable and
433 acceptable action mode.

434 The adverse impact of monoglyceride on DNA double helix may affect the DNA
435 function and cell division, and the cell cycle is an important index for assessing whether
436 cell division is normal or not (65). It is known that bacteria are prokaryotic cells that
437 have phases I, R, and D in the cell cycle, corresponding to phases G1, S, and M in
438 eukaryotic cells (66). Unlike eukaryotic cells, bacteria will directly enter into the phase D
439 to begin chromosome separation immediately without going through phase G2. Flow
440 cytometry is a common method to investigate the effect of foreign substances on the cell
441 cycle. Figure 5 showed that peak shape in flow histogram of *E. coli* was obviously

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442 affected by MB, ML, and MM. Specifically, the growth in phase G1, and the decline in
443 phases S and G2 in Figure 6 indicated that monoglyceride firstly disturbed phase G1
444 instead of phases S and G2, causing *E. coli* cells to arrest in phase G1 and therefore were
445 unable to complete normal cell division. The finding does not fully correspond to some
446 previous reports of antibacterial agents (67, 68). PAN Ling Zi also found that phase G1
447 of cell cycle was evidently affected in *GS115* after melittin treatment for 4h (66).
448 Whereas, Jin Cai revealed that the Laminaria japonica extract acted on phase R (or phase
449 S) rather than phase I (or phase G1), causing cell cycle of *C. michiganense* arrest in
450 phase R (69).

451 In order to further study the correlation between DNA double helix disruption and
452 abnormal cell cycle, the synthesis of intracellular DNA, RNA, and protein was evaluated
453 using flow cytometry. The results presented in Figure 7 indicated that DNA, RNA, and
454 protein synthesis are affected after adding monoglyceride, and the time of DNA and
455 protein synthesis receiving inhibition are much later than that of RNA. Similar studies
456 have been conducted to figure out the effect of antimicrobial peptides on bacterial
457 macromolecular synthesis (70-72). Aleksander Patrzykat found that the RNA synthesis in
458 *E. coli CGSC 4908* was affected after adding pleurocidin peptide at MIC within 5 min,
459 followed by the suppression for DNA synthesis, whereas protein synthesis was not
460 obviously affected except for a decrease in growth rate (73). Evan F. Haney also
461 observed a similar growth trend of DNA, RNA, and protein synthesis in *E. coli CGSC*
462 *4908* with three Puro B peptides treatment for 30 min (67). The difference between the

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463 assay and previous reports is due to differences in antibacterial agent types, binding
464 modes with DNA and action time (72, 74, 75). Combination with the action site of
465 monoglyceride on the cell cycle of *E. coli*, it is speculated that DNA double helix is
466 firstly damaged, followed by the inhibition of RNA synthesis, subsequently protein and
467 DNA synthesis is also affected, resulting in DNA replication suppression and cell cycle
468 arrest in phase G1.

469 A new concentration-dependent antibacterial mechanism is summarized in Figure 8.
470 The monoglyceride firstly crosses *E. coli* membrane at concentrations far below the MIC,
471 which is the basis for the antibacterial effect. Then it targets to genomic DNA and
472 damages its double helix, affecting RNA synthesis and subsequent protein and DNA
473 synthesis, causing cell cycle arrest, and finally resulting in cell division disorder. The
474 interference of cell cycle often means the inhibition of cell division which occurred at
475 concentrations close to MIC; therefore, we consider this type of intracellular action as the
476 key lethal effect in the antibacterial test of monoglycerides on *E. coli*. And the membrane
477 lysis and cell disruption appear when adding monoglycerides at 2 MIC or more, which is
478 more like the final result of a combination of various antimicrobial modes.

479 The differentiated performance of ML and MM treatment in UV-visible spectra and
480 cell cycle assay may be used for explaining the causes for different sensitivity of *E. coli*
481 to two monoglycerides. The saturation action concentration of ML in hyperchromic
482 effect was 8 μg/mL, which was higher than 4 μg/mL of that in MM treatment.
483 Furthermore, a significant growth in phase G1 after adding ML was observed at 64

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484 μg/mL, much larger than 4 μg/mL of concentration observed in MM treatment. These
485 data revealed that the saturated action concentration of MM to DNA double helix and cell
486 cycle in *E. coli* was obviously smaller than that of ML, indicating that the inhibitory
487 effect of MM to *E. coli* DNA was greater than that of ML at doses near MIC. As for the
488 reason for the different affinity between two esters and *E. coli* DNA, we are still not clear,
489 which may need to be further studied by the interaction force including hydrogen bond,
490 van der Waal's force, and hydrophobic interaction (76, 77).

491 In conclusion, the study presented here explained the cause for the divergence in the
492 antibacterial activity of short, medium and long chain monoglycerides on *E. coli*, and
493 identified DNA interference and cell cycle arrest as the key lethal effect in the
494 antibacterial behavior of monoglyceride, and proposed a new dose-dependent inhibitory
495 mechanism. In three monoglycerides, MB has the worst antibacterial effect due to its
496 poorest membrane permeability, and the superiority of MM in comparison with ML is
497 mainly due to its stronger DNA destroying ability and higher interference effect on cell
498 cycle, which also means better inhibition ability on cell division. As for the inhibitory
499 modes, the increase of membrane permeability at low doses is the basis for playing the
500 antibacterial effect, then the arrest of cell cycle at doses near MIC is the key lethal effect
501 in antibacterial test, and the final cell lysis at high doses is considered as the result of
502 synergistic action of various antibacterial effects. In addition, a possible route was
503 proposed to explain the correlation between DNA double helix disruption and cell cycle
504 arrest.

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512 **Conflict of interest**

513 The authors declare no competing financial interest.

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526 **References**

527 1. Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL. 2005.
528 Epidemiology of Escherichia coli O157 : H7 outbreaks, United States, 1982-2002.
529 Emerging Infectious Diseases 11:603-609.
530 2. Lee GY, Jang HI, Hwang IG, Rhee MS. 2009. Prevalence and classification of
531 pathogenic Escherichia coli isolated from fresh beef, poultry, and pork in Korea.
532 International Journal of Food Microbiology 134:196-200.
533 3. Scholl D, Cooley M, Williams SR, Gebhart D, Martin D, Bates A, Mandrell R.
534 2009. An Engineered R-Type Pyocin Is a Highly Specific and Sensitive
535 Bactericidal Agent for the Food-Borne Pathogen Escherichia coli O157:H7.
536 Antimicrobial Agents and Chemotherapy 53:3074-3080.
537 4. Kaper JB, Nataro JP, Mobley HLT. 2004. Pathogenic Escherichia coli. Nature
538 Reviews Microbiology 2:123-140.
539 5. Doyle MP, Schoeni JL. 1987. Isolation of Escherichia coli O157:H7 from retail
540 fresh meats and poultry. Applied and environmental microbiology 53:2394-6.
541 6. Mead PS, Griffin PM. 1998. Escherichia coli O157 : H7. Lancet 352:1207-1212.
542 7. Tsuboi I, Ida H, Yoshikawa E, Hiyoshi S, Yamaji E, Nakayama I, Nonomiya T,
543 Shigenobu F, Shimizu M, O'Hara K, Sawai T, Mizuoka K. 1998. Antibiotic
544 susceptibility of enterohemorrhagic Escherichia coli O157 : H7 isolated from an
545 outbreak in Japan in 1996. Antimicrobial Agents and Chemotherapy 42:431-432.
546 8. Rhee MS, Lee SY, Dougherty RH, Kang DH. 2003. Antimicrobial effects of

bioRxiv preprint doi: https://doi.org/10.1101/339309; this version posted June 6, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

547 mustard flour and acetic acid against Escherichia coli O157 : H7, Listeria
548 monocytogenes, and Salmonella enterica serovar typhimurium. Applied and
549 Environmental Microbiology 69:2959-2963.
550 9. Kim SA, Rhee MS. 2015. Predictive model and optimization of a combined
551 treatment of caprylic acid and citric acid for the reduction of Escherichia coli
552 O157:H7 using the response surface methodology. International Journal of Food
553 Microbiology 197:9-14.
554 10. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones
555 JL, Griffin PM. 2011. Foodborne Illness Acquired in the United States-Major
556 Pathogens. Emerging Infectious Diseases 17:7-15.
557 11. Frenzen PD, Drake A, Angulo FJ, Program EI. 2005. Economic cost of illness
558 due to Escherichia coli O157 infections in the United States. Journal of Food
559 Protection 68:2623-2630.
560 12. Gould LH, Mungai EA, Johnson SD, Richardson LC, Williams IT, Griffin PM,
561 Cole DJ, Hall AJ. 2013. Surveillance for Foodborne Disease Outbreaks - United
562 States, 2009-2010. Mmwr-Morbidity and Mortality Weekly Report 62.
563 13. Bae YY, Lee HJ, Kim SA, Rhee MS. 2009. Inactivation of Alicyclobacillus
564 acidoterrestris spores in apple juice by supercritical carbon dioxide. International
565 Journal of Food Microbiology 136:95-100.
566 14. Kim SA, Kim OY, Rhee MS. 2010. Direct application of supercritical carbon
567 dioxide for the reduction of Cronobacter spp. (Enterobacter sakazakii) in end

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568 products of dehydrated powdered infant formula. Journal of Dairy Science
569 93:1854-1860.
570 15. Kim SA, Lee MK, Park TH, Rhee MS. 2013. A combined intervention using
571 fermented ethanol and supercritical carbon dioxide to control Bacillus cereus and
572 Bacillus subtilis in rice. Food Control 32:93-98.
573 16. Takeuchi H, Noguchi O, Sekine S, Kobayashi A, Aoyama T. 2006. Lower weight
574 gain and higher expression and blood levels of adiponectin in rats fed
575 medium-chain TAG compared with long-chain TAG. Lipids 41:207-212.
576 17. Luo CY, Zeng ZL, Gong DM, Zhao CY, Liang QF, Zeng C. 2014. Evaluation of
577 monolaurin from camphor tree seeds for controlling food spoilage fungi. Food
578 Control 46:488-494.
579 18. Schlievert PM, Peterson ML. 2012. Glycerol Monolaurate Antibacterial Activity
580 in Broth and Biofilm Cultures. Plos One 7.
581 19. Zhang H, Xu YQ, Wu LJ, Zheng XD, Zhu SM, Feng FQ, Shen LR. 2010.
582 Anti-yeast activity of a food-grade dilution-stable microemulsion. Applied
583 Microbiology and Biotechnology 87:1101-1108.
584 20. Bunkova L, Bunka F, Janis R, Krejci J, Dolezalkova I, Pospisil Z, Ruzicka J,
585 Tremlova B. 2011. Comparison of antibacterial effect of seven 1-monoglycerides
586 on food-borne pathogens or spoilage bacteria. Acta Veterinaria Brno 80:29-39.
587 21. Marounek M, Putthana V, Benada O, Lukesova D. 2012. Antimicrobial Activities
588 of Medium-chain Fatty Acids and Monoacylglycerols on Cronobacter sakazakii

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589 DBM 3157(T) and Cronobacter malonaticus DBM 3148. Czech Journal of Food
590 Sciences 30:573-580.
591 22. Sevcikova P, Kasparkova V, Hauerlandova I, Humpolicek P, Kucekova Z,
592 Bunkova L. 2014. Formulation, antibacterial activity, and cytotoxicity of
593 1-monoacylglycerol microemulsions. European Journal of Lipid Science and
594 Technology 116:448-457.
595 23. Bunkova L, Krejci J, Janis R, Kasparkova V, Vltavska P, Kulendova L, Bunka F.
596 2010. Influence of monoacylglycerols on growth inhibition of micromycetes in
597 vitro and on bread. European Journal of Lipid Science and Technology
598 112:173-179.
599 24. Altieri C, Bevilacqua A, Cardillo D, Sinigaglia M. 2009. Antifungal activity of
600 fatty acids and their monoglycerides against Fusarium spp. in a laboratory
601 medium. International Journal of Food Science and Technology 44:242-245.
602 25. Altieri C, Cardillo D, Bevilacqua A, Sinigaglia M. 2007. Inhibition of Aspergillus
603 spp. and Penicillium spp. by fatty acids and their monoglycerides. Journal of
604 Food Protection 70:1206-1212.
605 26. Altieri C, Bevilacqua A, Cardillo D, Sinigaglia M. 2009. Effectiveness of fatty
606 acids and their monoglycerides against gram-negative pathogens. International
607 Journal of Food Science and Technology 44:359-366.
608 27. Kim SA, Rhee MS. 2013. Marked Synergistic Bactericidal Effects and Mode of
609 Action of Medium-Chain Fatty Acids in Combination with Organic Acids against

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610 Escherichia coli O157:H7. Applied and Environmental Microbiology
611 79:6552-6560.
612 28. Hyldgaard M, Sutherland DS, Sundh M, Mygind T, Meyer RL. 2012.
613 Antimicrobial Mechanism of Monocaprylate. Applied and Environmental
614 Microbiology 78:2957-2965.
615 29. Bergsson G, Arnfinnsson J, Steingrimsson O, Thormar H. 2001. Killing of
616 Gram-positive cocci by fatty acids and monoglycerides. Apmis 109:670-678.
617 30. Hsu CH, Chen CP, Jou ML, Lee AYL, Lin YC, Yu YP, Huang WT, Wu SH. 2005.
618 Structural and DNA-binding studies on the bovine antimicrobial peptide,
619 indolicidin: evidence for multiple conformations involved in binding to
620 membranes and DNA. Nucleic Acids Research 33:4053-4064.
621 31. Lan Y, Ye Y, Kozlowska J, Lam JKW, Drake AF, Mason AJ. 2010. Structural
622 contributions to the intracellular targeting strategies of antimicrobial peptides.
623 Biochimica Et Biophysica Acta-Biomembranes 1798:1934-1943.
624 32. Liu X, Li X, Zhang Z, Dong Y, Liu P, Zhang C. 2013. Studies on Antibacterial
625 Mechanisms of Copper Complexes with 1,10-phenanthroline and Amino Acid on
626 Escherichia coli. Biological Trace Element Research 154:150-155.
627 33. Branen JK, Davidson PM. 2004. Enhancement of nisin, lysozyme, and
628 monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and
629 lactoferrin. International Journal of Food Microbiology 90:63-74.
630 34. Jang HI, Rhee MS. 2009. Inhibitory effect of caprylic acid and mild heat on

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631 Cronobacter spp. (Enterobacter sakazakii) in reconstituted infant formula and
632 determination of injury by flow cytometry. International Journal of Food
633 Microbiology 133:113-120.
634 35. Jung SW, Thamphiwatana S, Zhang LF, Obonyo M. 2015. Mechanism of
635 Antibacterial Activity of Liposomal Linolenic Acid against Helicobacter pylori.
636 Plos One 10.
637 36. Obonyo M, Zhang L, Thamphiwatana S, Pornpattananangkul D, Fu V, Zhang LF.
638 2012. Antibacterial Activities of Liposomal Linolenic Acids against
639 Antibiotic-Resistant Helicobacter pylori. Molecular Pharmaceutics 9:2677-2685.
640 37. Hyldgaard M, Mygind T, Piotrowska R, Foss M, Meyer RL. 2015. Isoeugenol has
641 a non-disruptive detergent-like mechanism of action. Frontiers in Microbiology 6.
642 38. Cuestas ML, Sosnik A, Mathet VL. 2011. Poloxamines Display a Multiple
643 Inhibitory Activity of ATP-Binding Cassette (ABC) Transporters in Cancer Cell
644 Lines. Molecular Pharmaceutics 8:1152-1164.
645 39. Hegde AH, Prashanth SN, Seetharamappa J. 2012. Interaction of antioxidant
646 flavonoids with calf thymus DNA analyzed by spectroscopic and electrochemical
647 methods. Journal of Pharmaceutical and Biomedical Analysis 63:40-46.
648 40. Zhang GW, Wang LH, Zhou XY, Li Y, Gong D. 2014. Binding Characteristics of
649 Sodium Saccharin with Calf Thymus DNA in Vitro. Journal of Agricultural and
650 Food Chemistry 62:991-1000.
651 41. Steen HB, Boye E. 1980. Bacterial growth studied by flow cytometry. Cytometry

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652 1:32-6.

653 42. Crissman HA, Darzynkiewicz Z, Tobey RA, Steinkamp JA. 1985. Normal and
654 perturbed Chinese hamster ovary cells: correlation of DNA, RNA, and protein
655 content by flow cytometry. The Journal of cell biology 101:141-7.

656 43. Sachidanandham R, Gin KYH, Poh CL. 2005. Monitoring of active but
657 non-culturable bacterial cells by flow cytometry. Biotechnology and
658 Bioengineering 89:24-31.

659 44. Kim SA, Rhee MS. 2016. Highly enhanced bactericidal effects of medium chain
660 fatty acids (caprylic, capric, and lauric acid) combined with edible plant essential
661 oils (carvacrol, eugenol, beta-resorcylic acid, trans-cinnamaldehyde, thymol, and
662 vanillin) against Escherichia coli O157:H7. Food Control 60:447-454.

663 45. Thormar H, Hilmarsson H, Bergsson G. 2006. Stable concentrated emulsions of
664 the 1-monoglyceride of capric acid (Monocaprin) with microbicidal activities
665 against the food-borne bacteria Campylobacter jejuni, Salmonella spp., and
666 Escherichia coli. Applied and Environmental Microbiology 72:522-526.

667 46. Vltavska P, Kasparkova V, Janis R, Bunkova L. 2012. Antifungal and
668 antibacterial effects of 1-monocaprylin on textile materials. European Journal of
669 Lipid Science and Technology 114:849-856.

670 47. Wang LL, Johnson EA. 1992. Inhibition of Listeria monocytogenes by fatty acids
671 and monoglycerides. Applied and environmental microbiology 58:624-9.

672 48. Skrivanova E, Marounek M, Dlouha G, Kanka J. 2005. Susceptibility of

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673 Clostridium perfringens to C-2-C-18 fatty acids. Letters in Applied Microbiology
674 41:77-81.
675 49. Ouattara B, Simard RE, Holley RA, Piette GJP, Begin A. 1997. Antibacterial
676 activity of selected fatty acids and essential oils against six meat spoilage
677 organisms. International Journal of Food Microbiology 37:155-162.
678 50. Bergsson G, Arnfinnsson J, Steingrimsson O, Thormar H. 2001. In vitro killing of
679 Candida albicans by fatty acids and monoglycerides. Antimicrobial Agents and
680 Chemotherapy 45:3209-3212.
681 51. Zhang H, Taxipalati M, Yu LY, Que F, Feng FQ. 2013. Structure-Activity
682 Relationship of a U-Type Antimicrobial Microemulsion System. Plos One 8.
683 52. Valipe SR, Nadeau JA, Annamali T, Venkitanarayanan K, Hoagland T. 2011. In
684 vitro antimicrobial properties of caprylic acid, monocaprylin, and sodium
685 caprylate against Dermatophilus congolensis. American Journal of Veterinary
686 Research 72:331-335.
687 53. Thakur S, Karak N. 2013. Castor oil-based hyperbranched polyurethanes as
688 advanced surface coating materials. Progress in Organic Coatings 76:157-164.
689 54. Sun CQ, O'Connor CJ, Roberton AM. 2003. Antibacterial actions of fatty acids
690 and monoglycerides against Helicobacter pylori. Fems Immunology and Medical
691 Microbiology 36:9-17.
692 55. Glover RE, Smith RR, Jones MV, Jackson SK, Rowlands CC. 1999. An EPR
693 investigation of surfactant action on bacterial membranes. Fems Microbiology

bioRxiv preprint doi: https://doi.org/10.1101/339309; this version posted June 6, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

694 Letters 177:57-62.
695 56. Madler B, Binder H, Klose G. 1998. Compound complex formation in
696 phospholipid membranes induced by a nonionic surfactant of the oligo(ethylene
697 oxide) alkyl ether type: A comparative DSC and FTIR study. Journal of Colloid
698 and Interface Science 202:124-138.
699 57. Hancock REW, Chapple DS. 1999. Peptide antibiotics. Antimicrobial Agents and
700 Chemotherapy 43:1317-1323.
701 58. Manhong Wu, Elke Maier, Roland Benz A, ‡ REWH. 1999. Mechanism of
702 Interaction of Different Classes of Cationic Antimicrobial Peptides with Planar
703 Bilayers and with the Cytoplasmic Membrane of Escherichia coli†. Biochemistry
704 38:7235-7242.
705 59. Wu MH, Hancock REW. 1999. Interaction of the cyclic antimicrobial cationic
706 peptide bactenecin with the outer and cytoplasmic membrane. Journal of
707 Biological Chemistry 274:29-35.
708 60. Shi Y, Guo CL, Sun YJ, Liu ZL, Xu FG, Zhang Y, Wen ZW, Li ZA. 2011.
709 Interaction between DNA and Microcystin-LR Studied by Spectra Analysis and
710 Atomic Force Microscopy. Biomacromolecules 12:797-803.
711 61. Geng S, Wu Q, Shi L, Cui F. 2013. Spectroscopic study one thiosemicarbazone
712 derivative with ctDNA using ethidium bromide as a fluorescence probe.
713 International Journal of Biological Macromolecules 60:288-294.
714 62. Qiao M, Li C, Shi Y, Liu S, Liu Z, Hu X. 2015. Study on interactions of

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715 aminoglycoside antibiotics with calf thymus DNA and determination of calf
716 thymus DNA via the resonance Rayleigh scattering technique. Luminescence the
717 Journal of Biological & Chemical Luminescence 30:1159.
718 63. Kashanian S, Khodaei MM, Pakravan P. 2010. Spectroscopic Studies on the
719 Interaction of Isatin with Calf Thymus DNA. DNA and Cell Biology 29:639-646.
720 64. Long XF, Miao Q, Bi SP, Li DS, Zhang CH, Zhao H. 2004. Resonance Rayleigh
721 scattering method for the recognition and determination of double-stranded DNA
722 using amikacin. Talanta 64:366-372.
723 65. Inácio ÂS, Domingues NS, Nunes A, Martins PT, Moreno MJ, Estronca LM,
724 Fernandes R, Moreno AJ, Borrego MJ, Gomes JP. 2015. Quaternary ammonium
725 surfactant structure determines selective toxicity towards bacteria: mechanisms of
726 action and clinical implications in antibacterial prophylaxis. Journal of
727 Antimicrobial Chemotherapy 44:103-114.
728 66. Pan LZ, Na J, Xing Z, Fang HJ, Wang GL. 2007. Inhibiting effect of melittin on
729 pathogens of crops. Chinese Science Bulletin 52:639-644.
730 67. Haney EF, Petersen AP, Lau CK, Jing WG, Storey DG, Vogel HJ. 2013.
731 Mechanism of action of puroindoline derived tryptophan-rich antimicrobial
732 peptides. Biochimica Et Biophysica Acta-Biomembranes 1828:1802-1813.
733 68. Efthimiadou EK, Karaliota A, Psomas G. 2010. Metal complexes of the
734 third-generation quinolone antimicrobial drug sparfloxacin: Structure and
735 biological evaluation. Journal of Inorganic Biochemistry 104:455-466.

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736 69. Cai J, Feng J, Xie S, Wang F, Xu Q. 2014. Laminaria japonica Extract, an
737 Inhibitor of Clavibater michiganense Subsp. Sepedonicum. Plos One 9:e94329.
738 70. Ulvatne H, Samuelsen Ø, Haukland HH, Krämer M, Vorland LH. 2004.
739 Lactoferricin B inhibits bacterial macromolecular synthesis in Escherichia coli
740 and Bacillus subtilis. Fems Microbiology Letters 237:377-384.
741 71. Subbalakshmi C, Sitaram N. 2010. Mechanism of antimicrobial action of
742 indolicidin. Fems Microbiology Letters 160:91-96.
743 72. Pellegrini A, Thomas U, Wild P, Schraner E, Von FR. 2000. Effect of lysozyme or
744 modified lysozyme fragments on DNA and RNA synthesis and membrane
745 permeability of Escherichia coli. Microbiological Research 155:69-77.
746 73. Patrzykat A, Friedrich CL, Zhang LJ, Mendoza V, Hancock REW. 2002.
747 Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit
748 macromolecular synthesis in Escherichia coli. Antimicrobial Agents and
749 Chemotherapy 46:605-614.
750 74. Jr OL, I O, Rogers ME, Consolvo PJ, Condie BA, Lovas S, Bulet P,
751 Blaszczykthurin M. 2000. Interaction between heat shock proteins and
752 antimicrobial peptides. Biochemistry 39:14150-14159.
753 75. Chan BP, Kim HS, Sun CK. 1998. Mechanism of Action of the Antimicrobial
754 Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell
755 Membrane and Inhibiting Cellular Functions. Biochemical & Biophysical
756 Research Communications 244:253-257.

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757 76. Tong C, Xiang G, Bai Y. 2010. Interaction of paraquat with calf thymus DNA: a
758 terbium(III) luminescent probe and multispectral study. Journal of Agricultural &
759 Food Chemistry 58:5257.
760 77. Zhong-Qin LI, Xiao-Ping XU, Meng C, Shi XA, Guo YH. 2004. The
761 Determination of Polybasic Ionization Constants of Kanamycin A and B. Journal
762 of Chemical Engineering of Chinese Universities.

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778 **Figure captions**

779 Figure 1. Counts of viable *E. coli* cells after treatment with MB, ML, and MM at 8, 16,
780 32, 64, 125, 250, 500, and 1000 μg/mL for 1 h. Error bar was represented for standard
781 deviations (SD) of three repeatly determinations (n=3).

782

783 Figure 2. SEM images of *E. coli* after cultivation with short, medium, and long chain
784 fatty acid monoglycerides at 37°C for 1 h. The control group (A) was not added any
785 monoglycerides. The *E. coli* cells were treated with MB (B to D), ML (E to G), and MM
786 (H to J) at 1/2 MIC (B, E, and H), 1 MIC (C, F, and I), and 2 MIC (D, G, and J),
787 respectively.

788

789 Figure 3. The changes in permeability ratio of membrane of *E. coli* treated with MB, ML,
790 and MM. The statistical results of the control groups (adding the same volume of ethanol
791 and cultivating at 37°C for 1 h), the heated treat groups (adding the same volume of
792 ethanol and cultivating at 85°C for 1 h), and the test groups (adding MB, ML, and MM at
793 2, 4, 8, 16, 32, 64, 125, 250, and 500 μg/mL respectively and cultivating at 37°C for 1 h)
794 were shown above. Error bar was stood for SD (n=3).

795

796 Figure 4. UV-visible spectrum of *E. coli* genomic DNA treated with MB (A), ML (B),
797 and MM (C) at concentrations of 2, 4, 8, 16, 32, 64, 125, 250, 500, and 1000 μg/mL. The
798 spectra scanning was recorded at a wavelength range from 220 nm to 320 nm, and each

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799 curve was stood for the average of three repeatly determinations

800 Figure 5. Flow histograms of *E. coli* treated with MB (A0-A5), ML (B0-B5), *and* MM
801 (C0-C5) at 0 (A0, B0, and C0), 1/4 MIC (A1, B1, and C1), 1/2 MIC (A2, B2, and C2), 1
802 MIC (A3, B3, and C3), 2 MIC (A4, B4, and C4), and 4 MIC (A5, B5, and C5).

803

804 Figure 6. Ratio changes of G1, S, and G2 phase in *E. coli* cell cycle after treatment with
805 MB, ML, and MM at 0, 1/4 MIC, 1/2 MIC, 1 MIC, 2 MIC, and 4 MIC. "*, § and #"
806 indicated statistical significant difference from the control group in G1, S, and G2 phase
807 respectively. Error bar was represented for SD (n=3).

808

809 Figure 7. Changes of DNA (A), RNA (B) and protein (C) content in *E. coli* cell after
810 treatment with MB, ML, and MM at MIC for 0, 10, 20, 30, 40, 50, and 60 min
811 respectively. The control groups were added with the same volume of ethanol instead of
812 monoglyceride solution. Error bar was represented for SD (n=3).

813

814 Figure 8. Explanation of the overall inhibitory mechanisms of glycerol monoglyceride on
815 *E. coli* with monolaurate as an example. ML firstly crossed the cell membrane and
816 interfered with the normal function of the DNA, eventually leading to cell lysis. The
817 action site of ML on DNA was identified as the process of DNA transcription, causing
818 the reduction in the synthesis of RNA and protein, resulting in cell cycle arrest and
819 ultimately cell division inhibition.

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820 Table of contents graphics

821 The main content of the manuscript is summarized in a figure. Glycerol monoglyceride
822 with different chain lengths shows different biological activity against *Escherichia coli*
823 and their antibacterial mechanism can be summarized as the following: the antibacterial
824 basis is the increase of membrane permeability at low concentration, the key action is the
825 inhibition of cell division in medium concentration near MIC, and the cell lysis at high
826 concentration can be regarded as the result of combination action of multiple
827 antibacterial effects.

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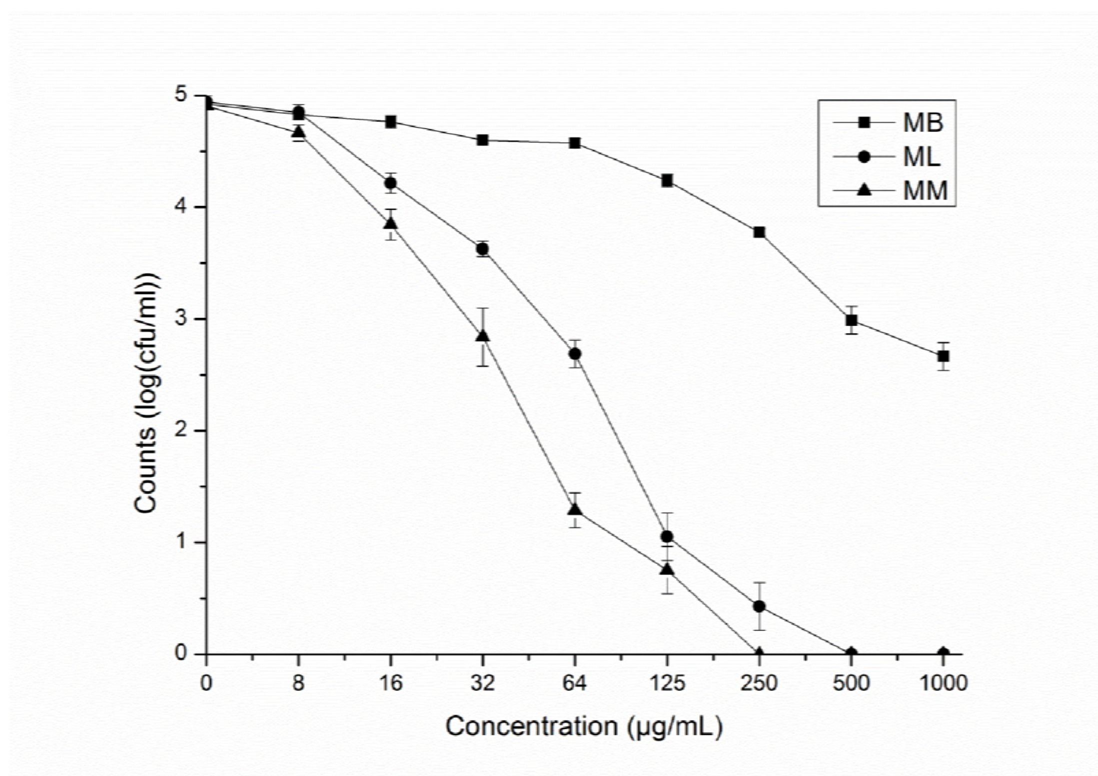
841 Figure 1



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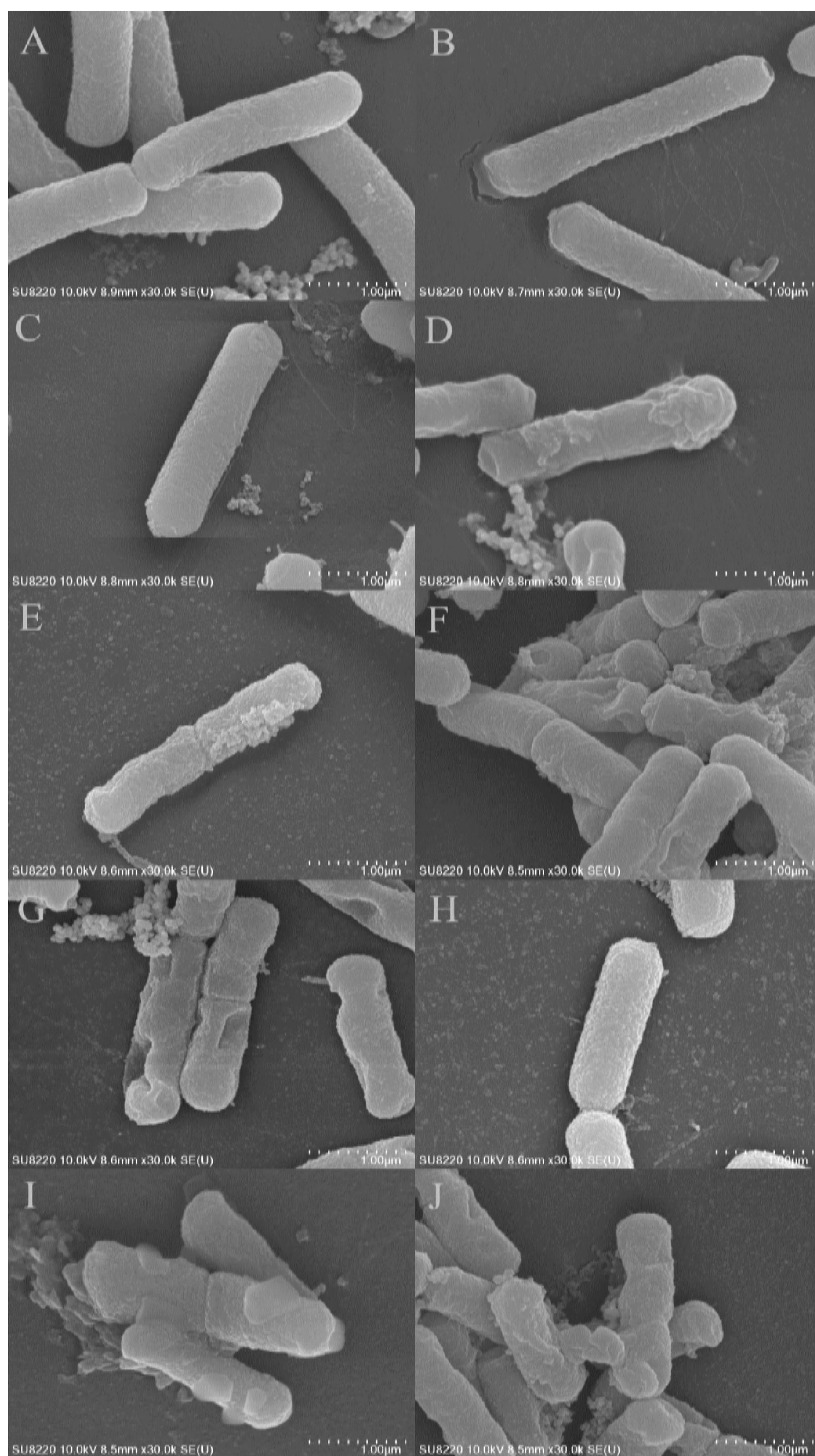
853 Figure 2



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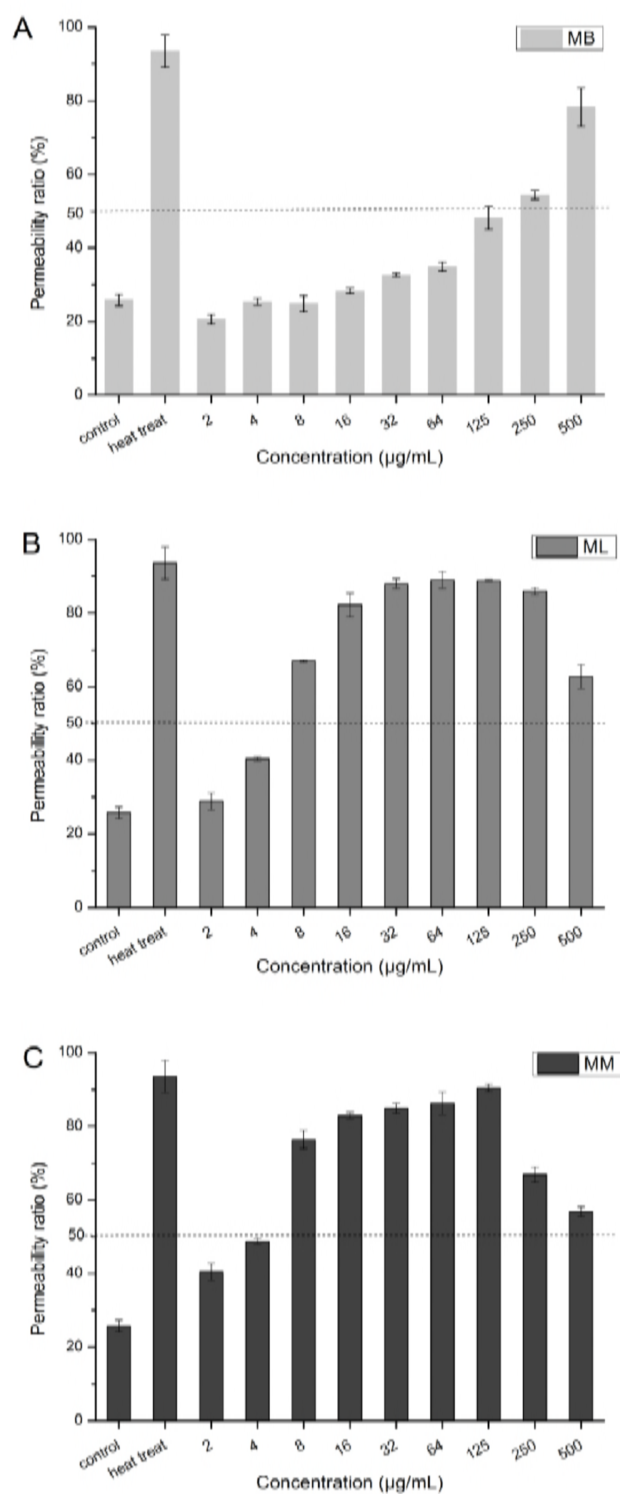

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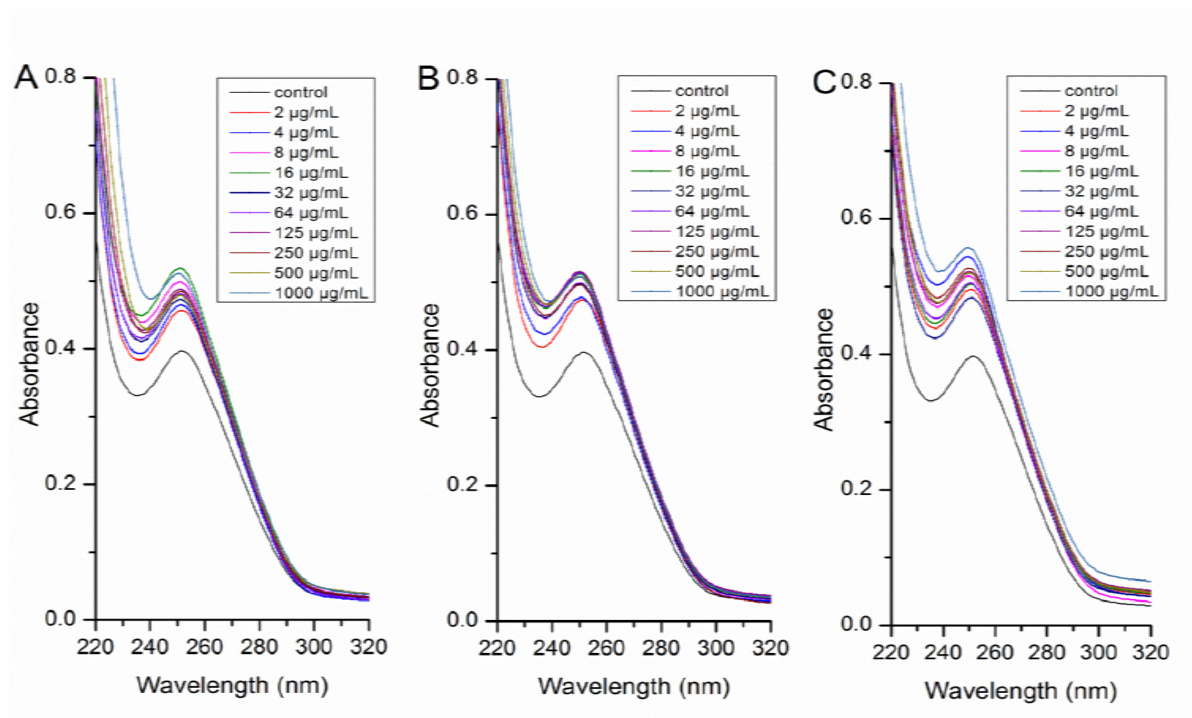

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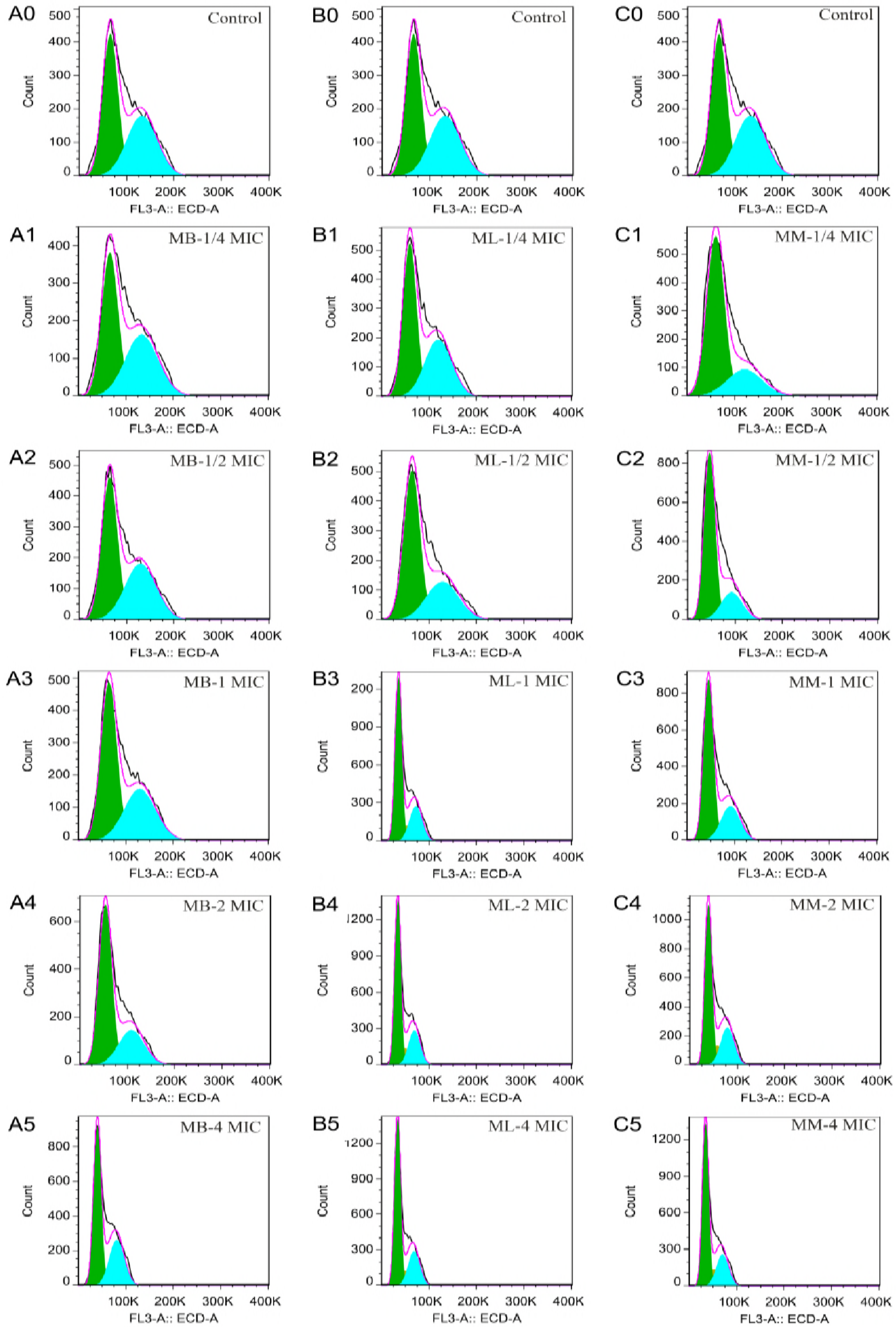
861 Figure 5



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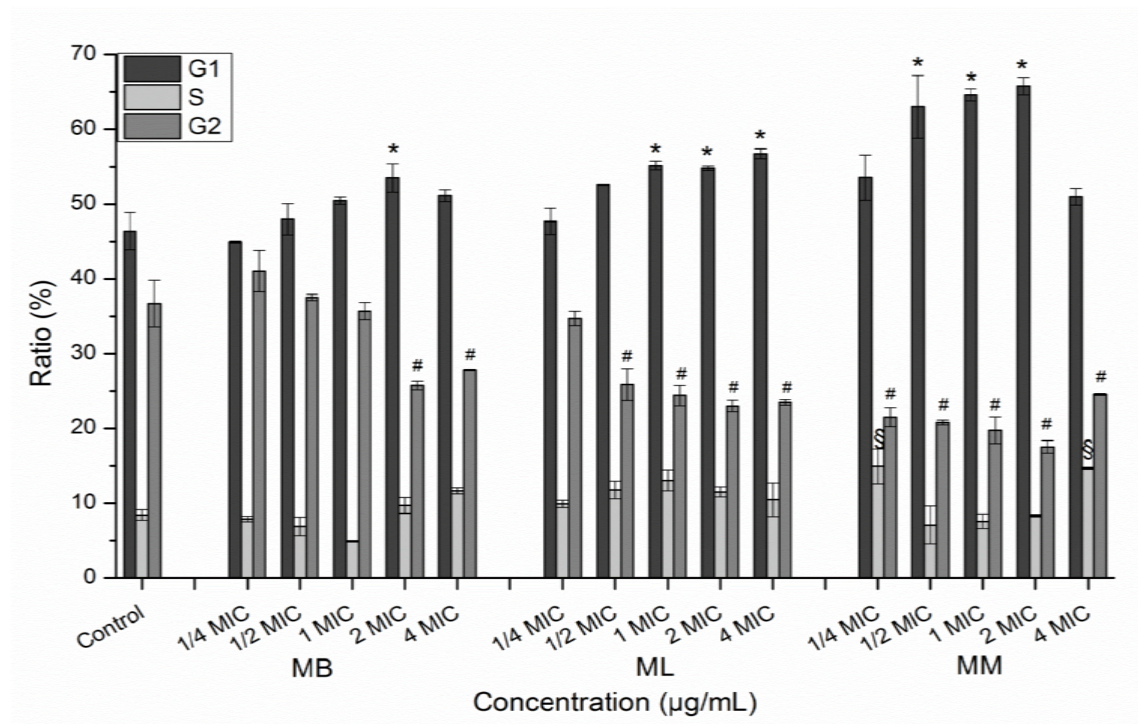
864 Figure 6



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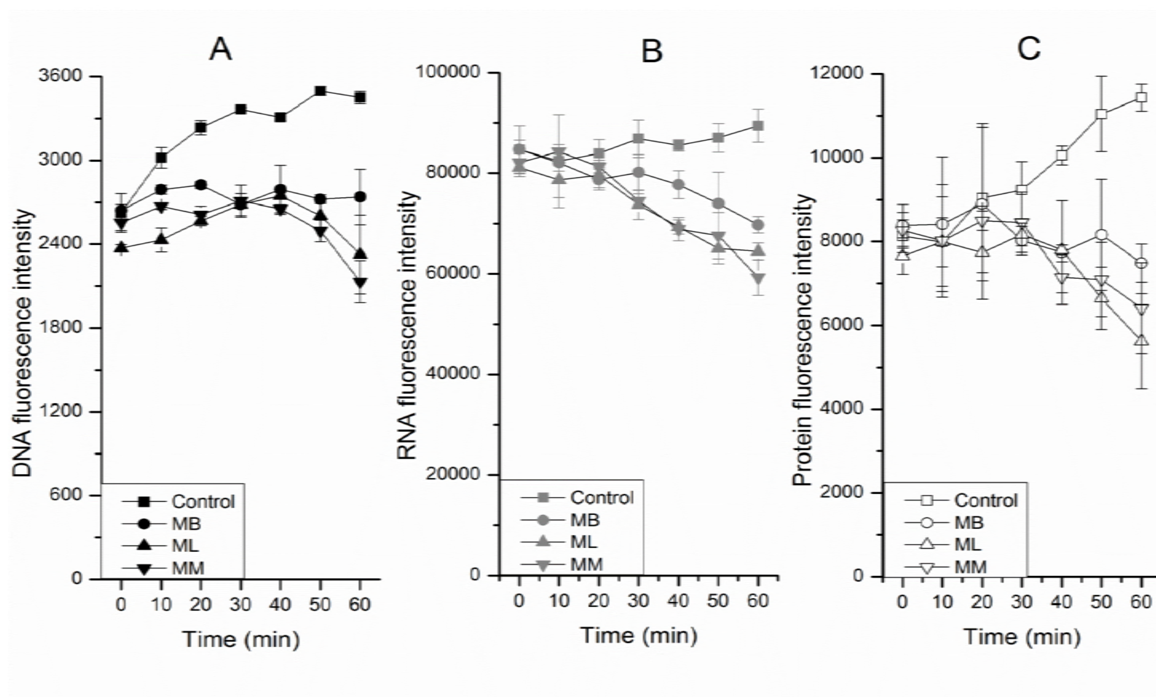
877 Figure 7



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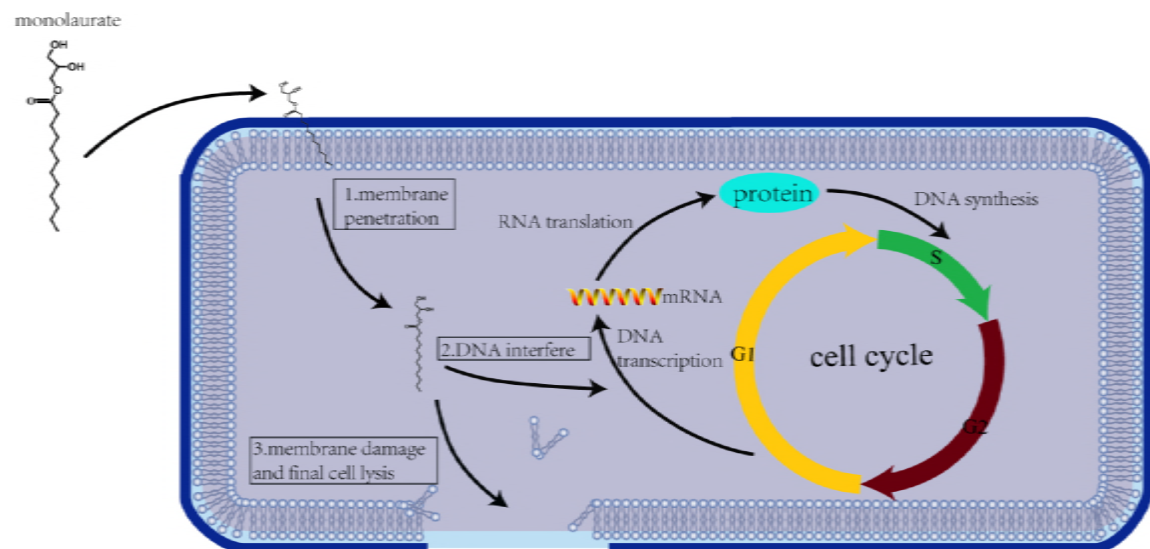

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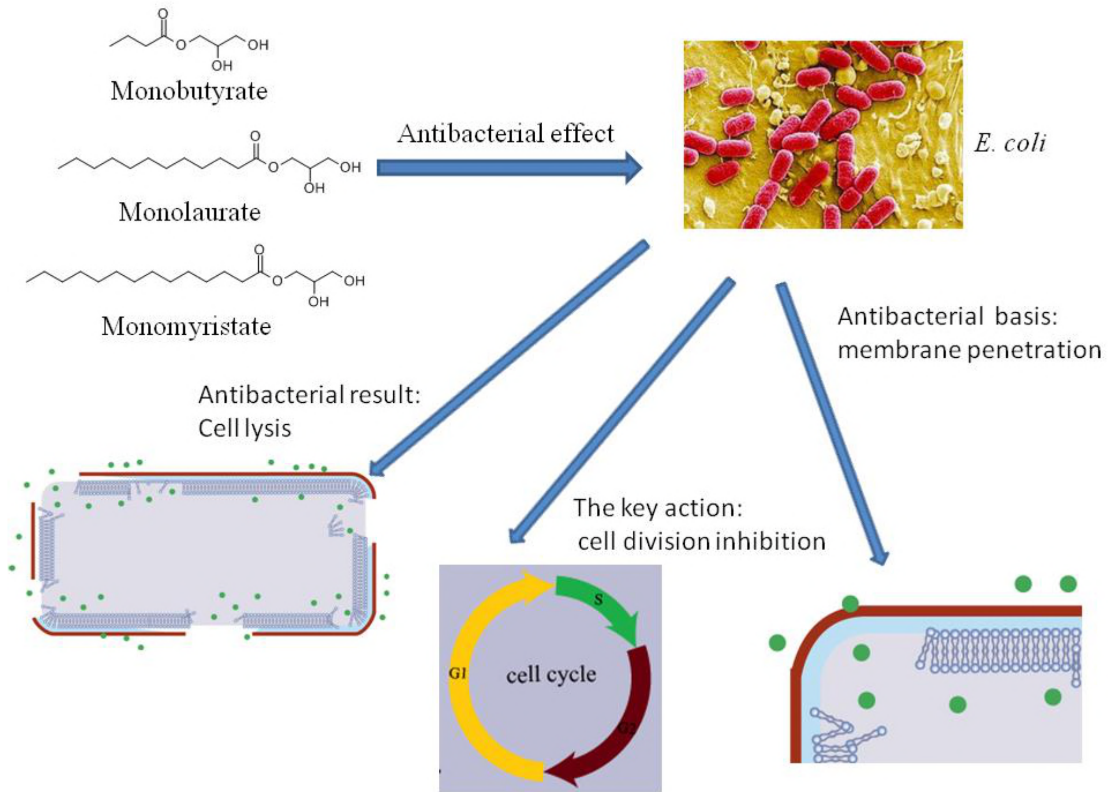

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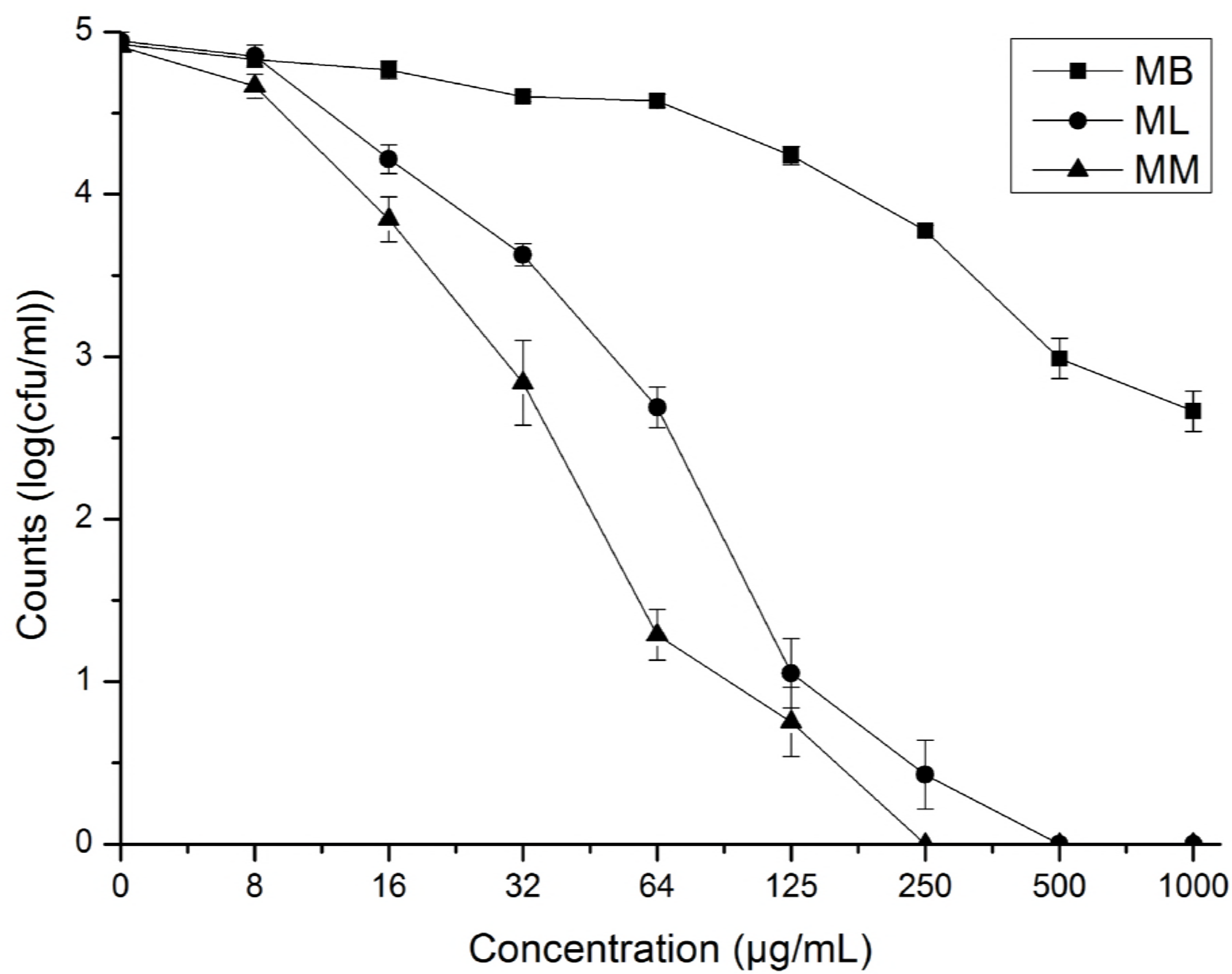

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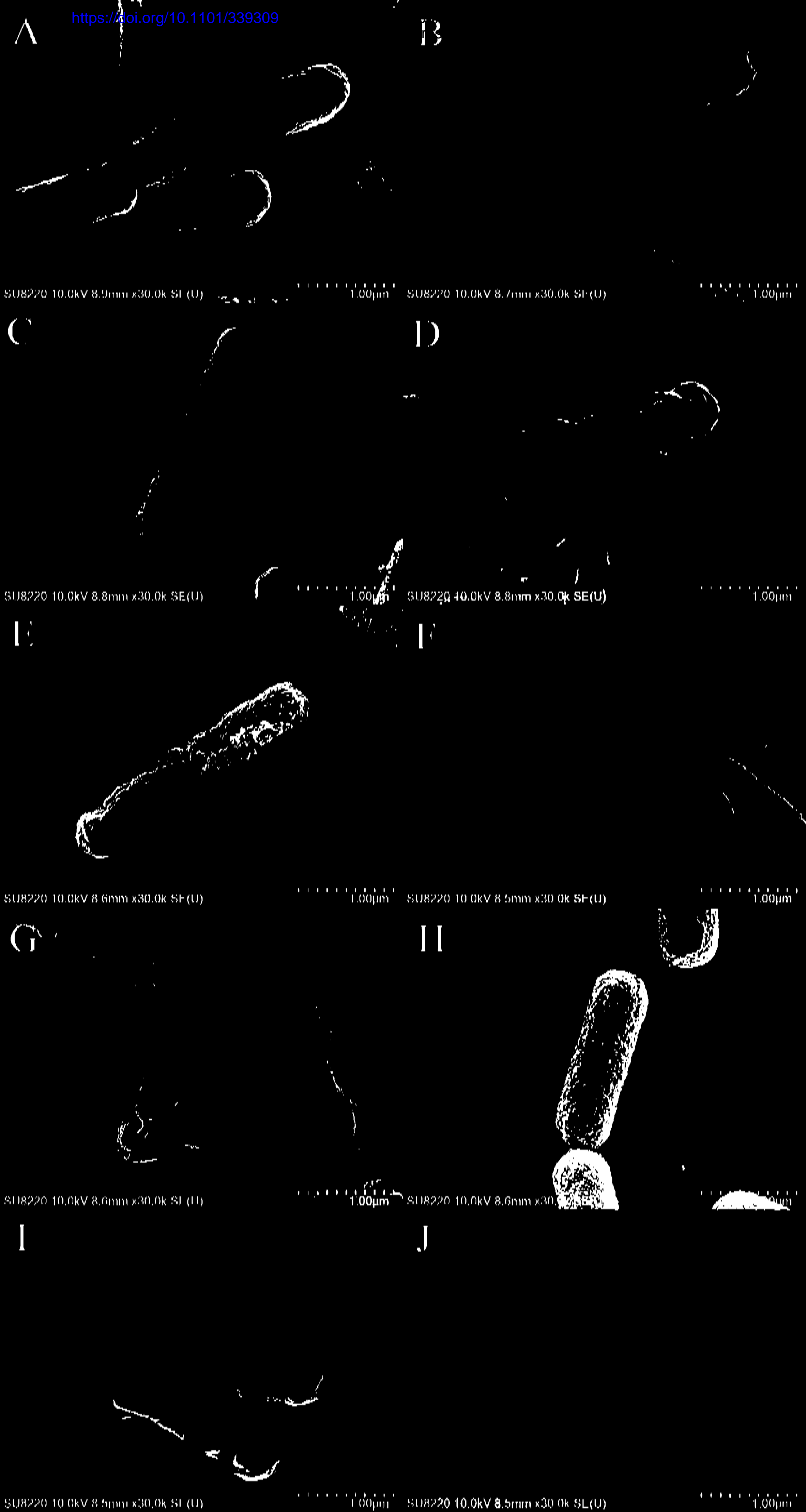

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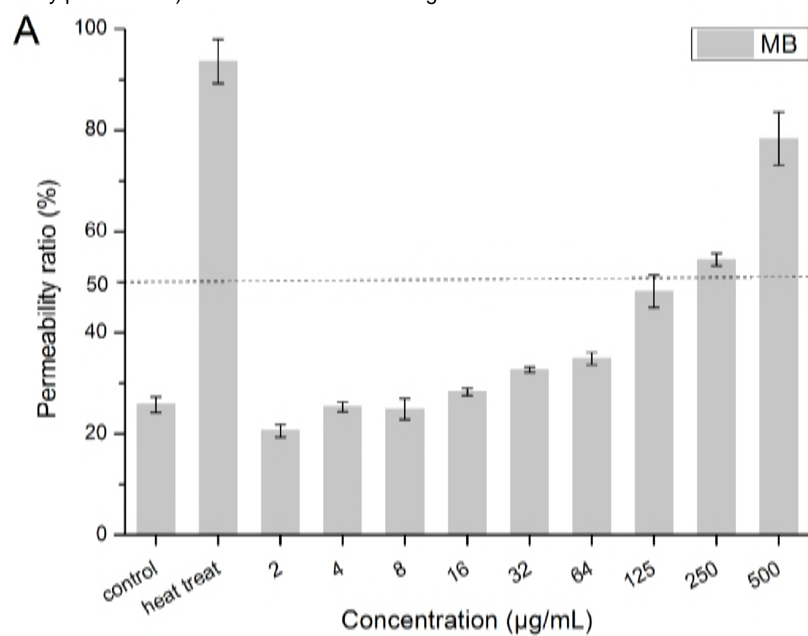

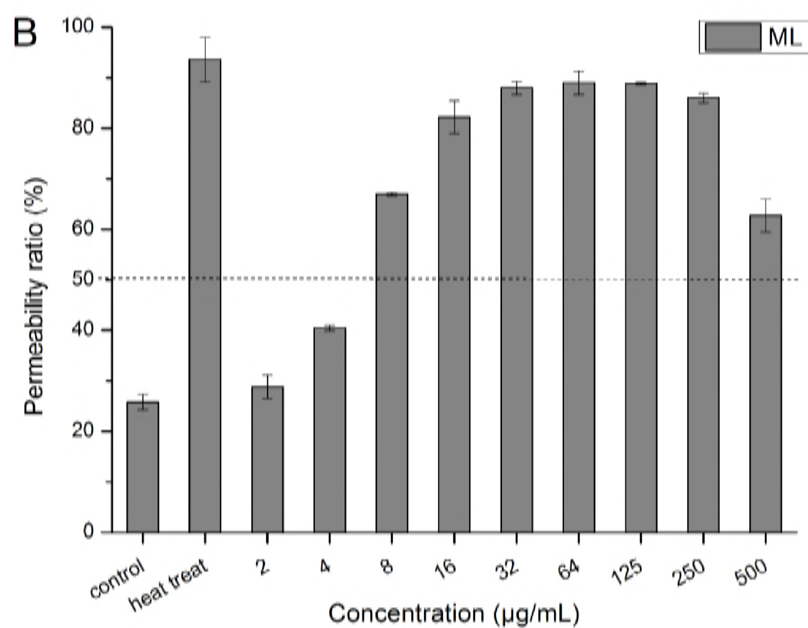

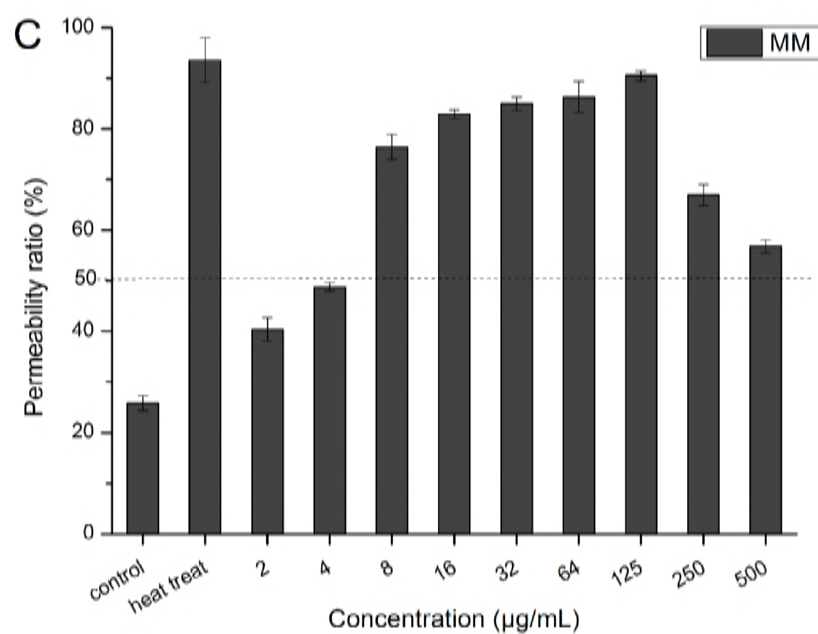

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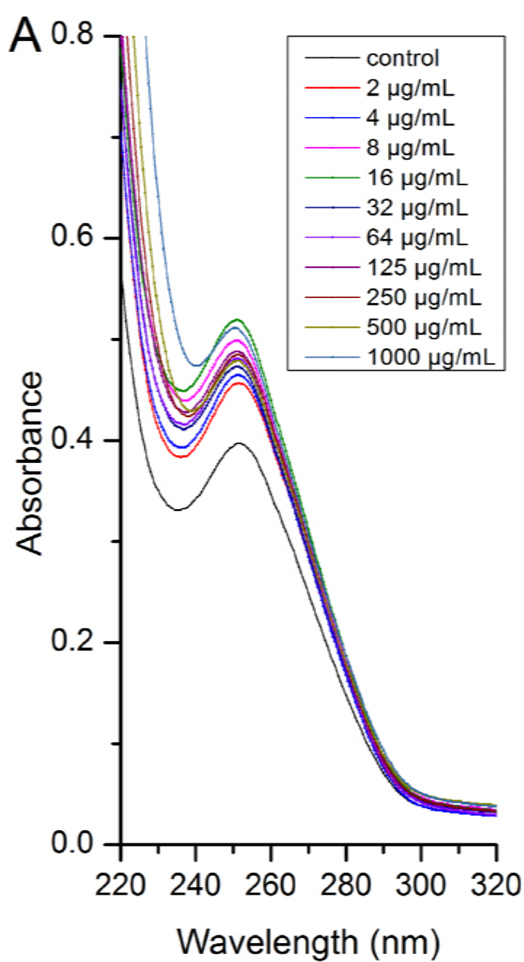

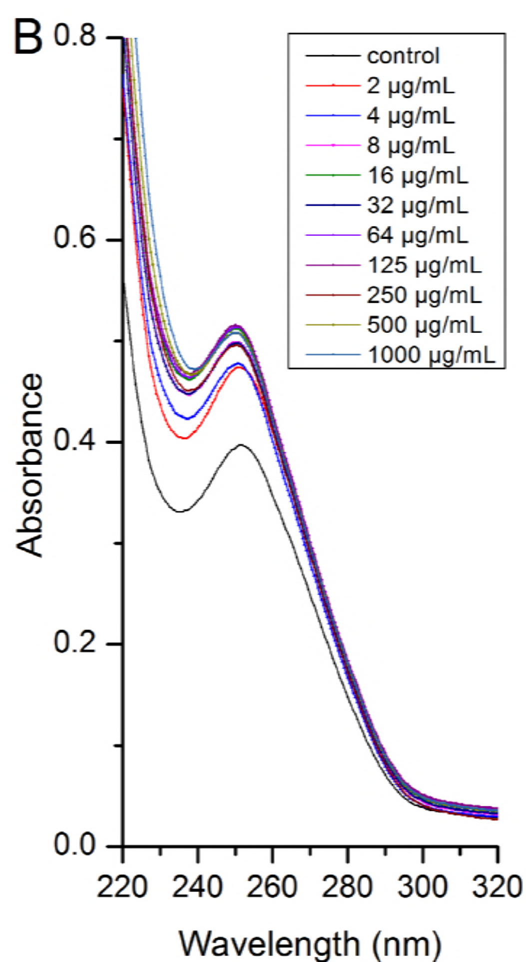

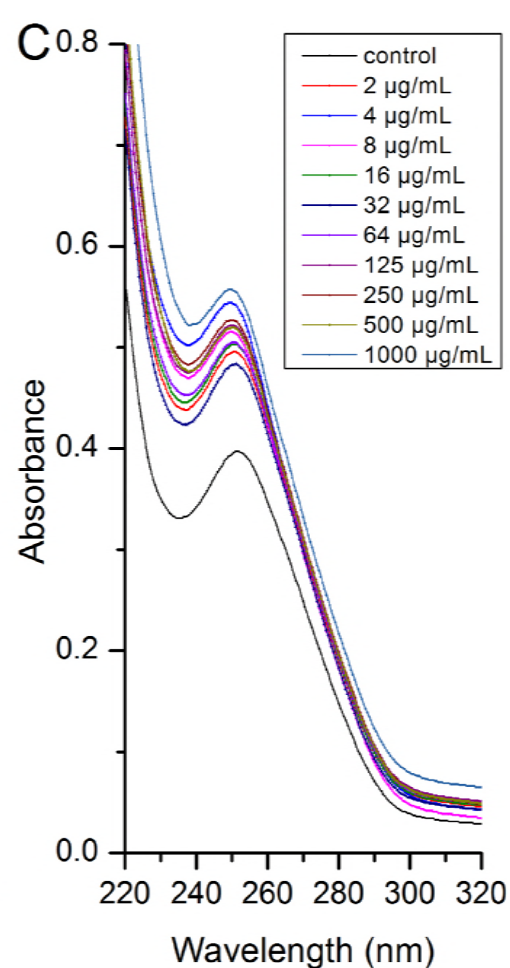

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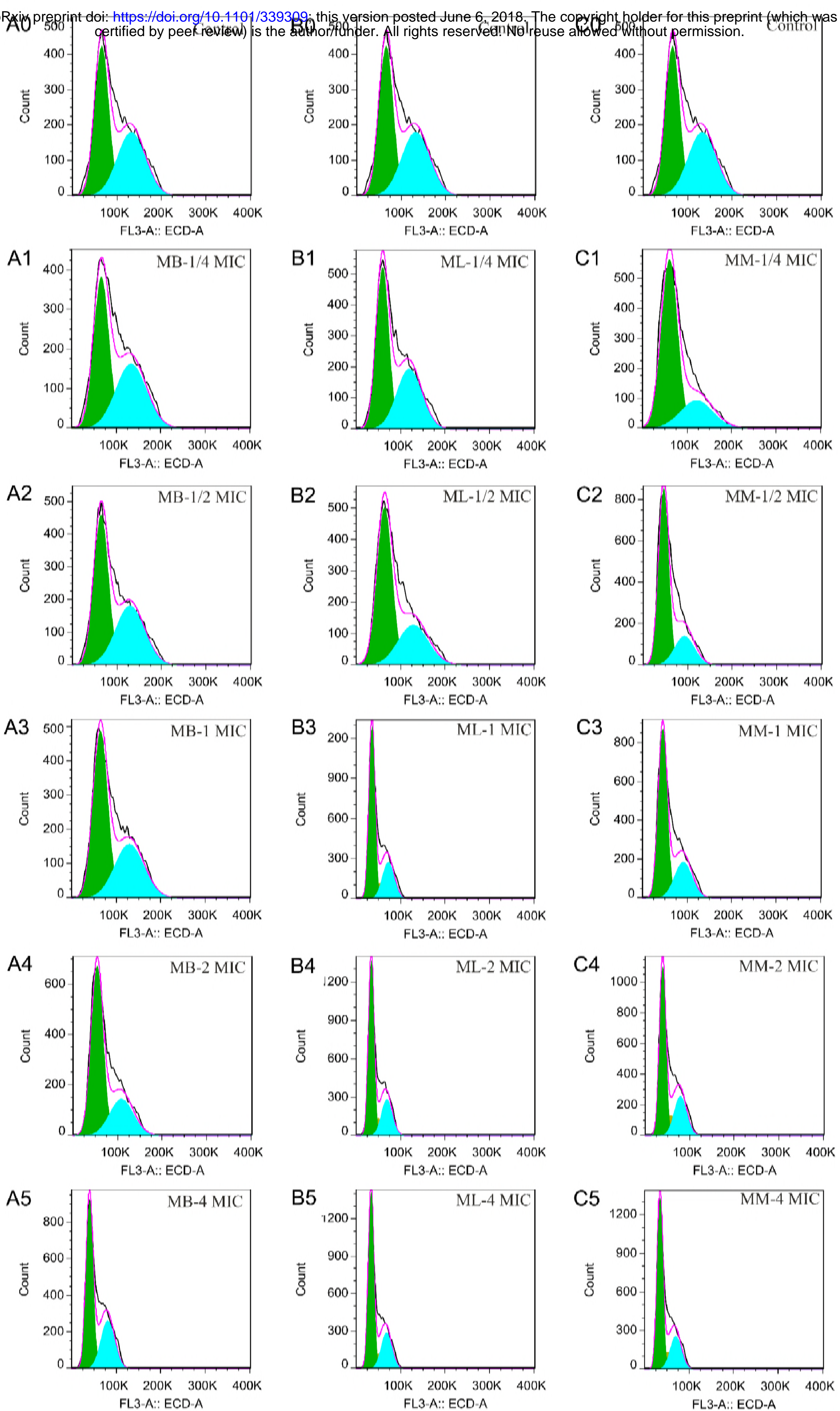

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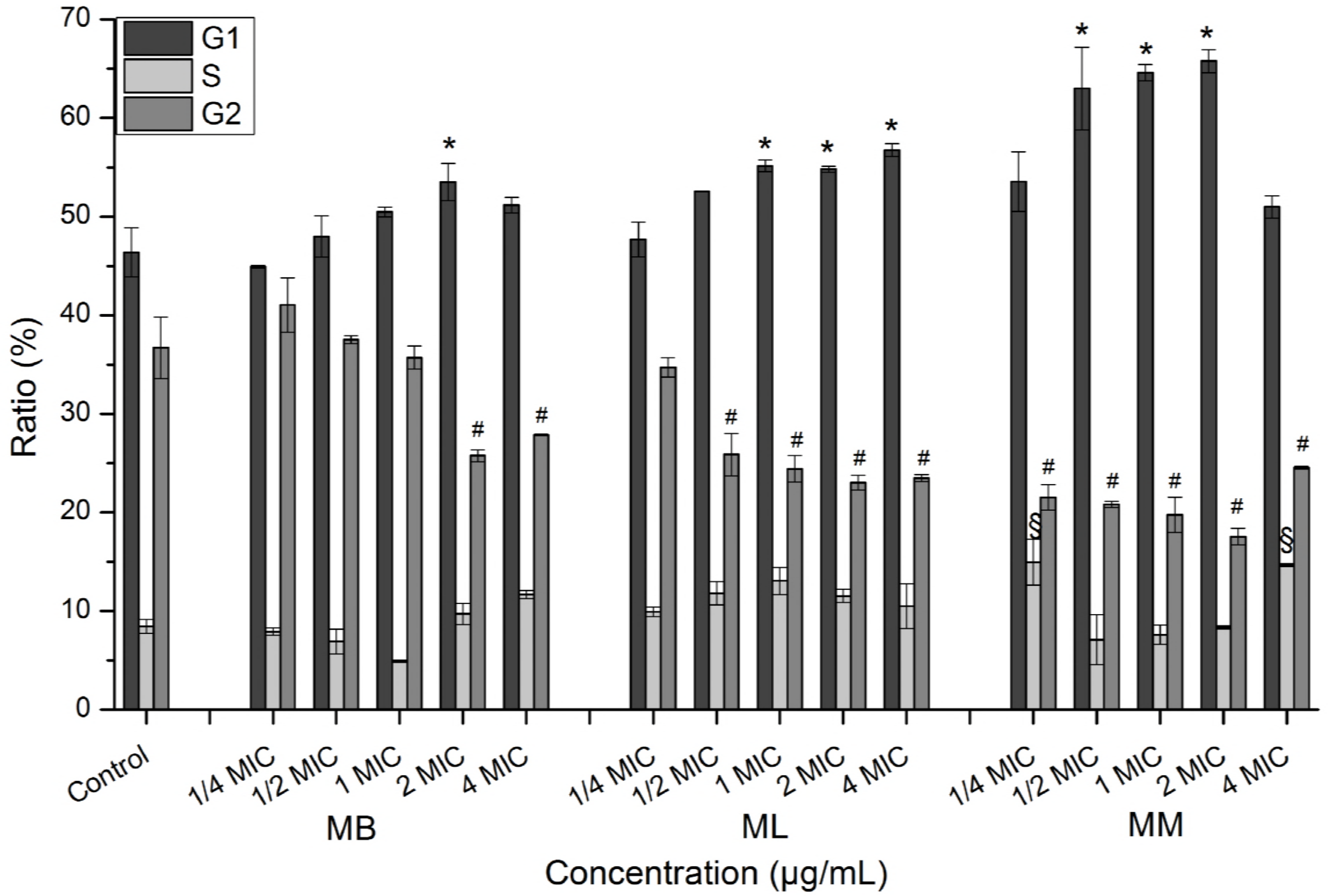

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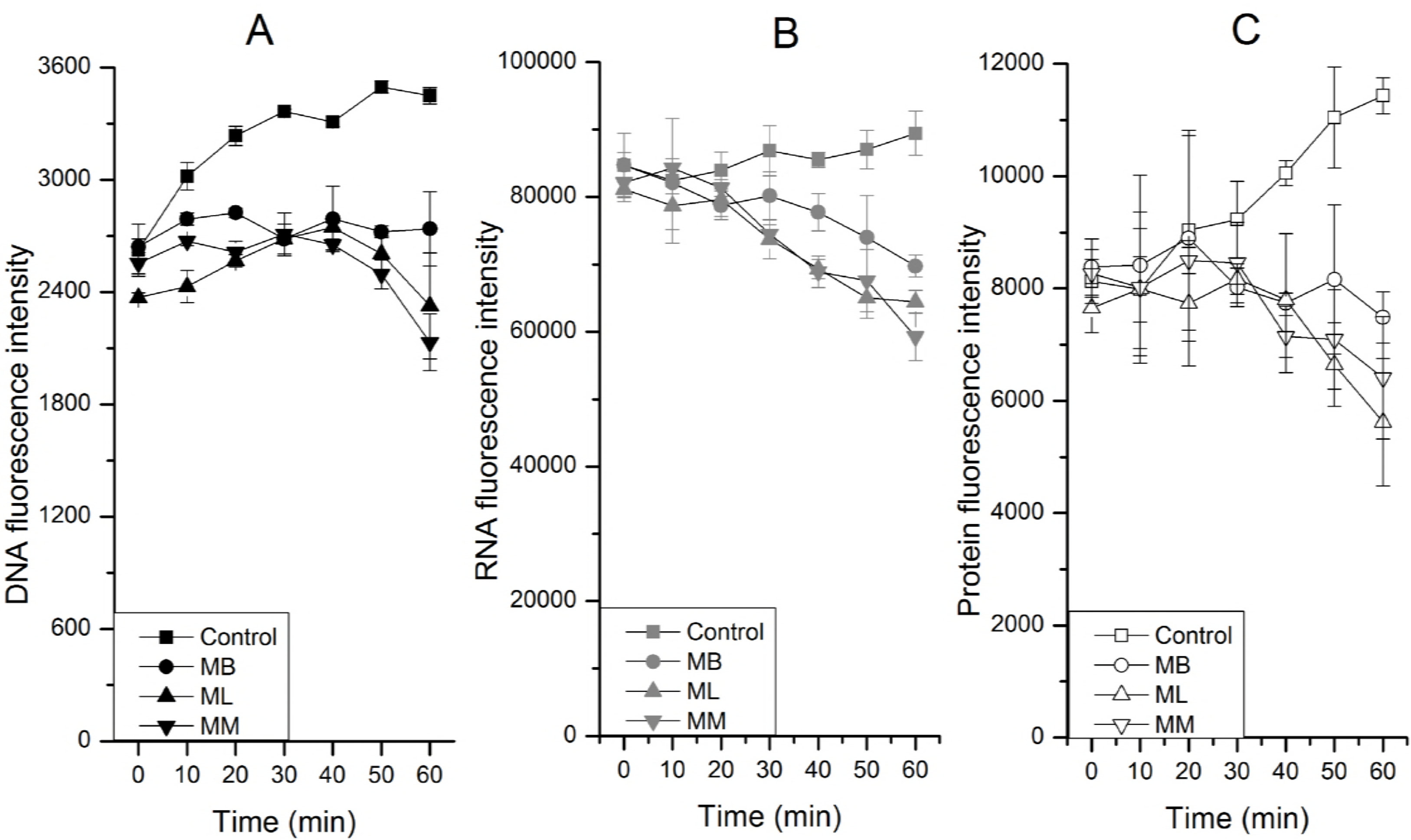

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