Arabica coffee extract shows antibacterial activity against *Staphylococcus epidermidis* and *Enterococcus faecalis* and low toxicity towards a human cell line

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Running title: Antibacterial activity and cytotoxicity of coffee extracts

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Abstract

The antimicrobial activity of a regular and decaffeinated Arabica coffee extract was evaluated against three different Gram-positive bacteria and two Gram-negatives, including pathogenic *Staphylococci* strains. The antimicrobial activity was shown to be independent from caffeine content and was more pronounced against the Gram-positive strains. The regular coffee extract exhibited a significant bacteriostatic effect against *Staphylococcus aureus* and *Staphylococcus epidermidis* at short exposure times and became bactericidal after prolonged exposure. The potential cytotoxicity of the regular coffee extract was also evaluated towards breast adenocarcinoma MCF7 cells, showing to become significant only after 24 h exposure and at a higher concentration than that producing the antibacterial effect. These results highlight the potential of a coffee extract as a naturally active antibacterial component for topical use such as for hand washing preparations to be used in health care units.

Keywords: Arabica coffee extract, antimicrobial activity, cytotoxicity

Abbreviations

MH, Mueller-Hinton broth; CQAs, caffeoylquinic acids; FQAs, ferruoylquinic acids; UHPLC, Ultra High Performance Liquid Chromatography; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; FIC, fractional inhibitory concentration; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; MTT, 1-(4, 5-Dimethylthiazol-2-yl)-3, 5-diphenylformazan; MRSA, methicillin-resistant *S. aureus*.

25 **1. Introduction**

26

27 The increase in the antimicrobial resistance in bacterial populations raises the question of an urgent
28 response in terms of new antimicrobial molecules (Zell & Goldmann, 2007). However the
29 development of new antibiotic molecules takes a long time scale and is expensive both in terms of
30 human and financial resources, therefore the preferred strategy is to optimize already existing
31 antimicrobial drugs or to combine multiple antibiotic compounds to improve their antimicrobial
32 potency. Furthermore, the overwhelming concern of the society over the safety of the synthetic
33 molecules has lead to an increased interest towards molecules of natural origin.
34 Some studies have reported antimicrobial property for coffee (Almeida, Farah, Silva, Nunan, &
35 Gloria, 2006; Daglia et al., 2007; Rurian-Henares & Morales, 2008; Tiwari et al., 2009), however
36 the antibacterial components responsible for the activity and the mechanisms of action have not
37 been fully elucidated yet (Mueller, Sauer, Weigel, Pichner, & Pischetsrieder, 2011). Previous
38 studies with *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 showed that
39 the antimicrobial potential of coffee is related to the roasting procedure and is dependent on the
40 degree of roasting, focusing the attention on the products of the Maillard reaction as potentially
41 responsible for the observed antimicrobial activity (Daglia, Cuzzoni, & Dacarro, 1994). In line with
42 this hypothesis, antimicrobial activities of melanoidins isolated from coffee have been reported
43 (Rurian-Henares & Morales, 2008). However, due to the extremely variable composition of these
44 molecules, derived from carbohydrates, proteins/amino acids and phenolic compounds formed
45 during the roasting procedure (Bekedam, Loots, Schols, Van Boekel, & Smit, 2008), the exact
46 mechanism by which the antimicrobial effect takes place remains difficult to elucidate.
47 Rufiàn-Henares and Morales showed that a coffee fraction, corresponding to high molecular weight
48 molecules such as melanoidins, was able to disrupt at the minimum inhibitory concentration both
49 the inner and outer membrane of an *E. coli* strain, leading to the release of intracellular molecules
50 (Rurian-Henares & Morales, 2008). Moreover, metal chelating properties were proposed as an

51 important feature to mediate the antibacterial activity of coffee (Rufian-Henares & de la Cueva,
52 2009).
53 To extend the use of coffee derivatives as antibacterial compounds for topical use, as active
54 components of hand washing preparations, it is essential to unravel the mechanisms mediating their
55 antimicrobial properties and to broaden the number of tested strains to define their spectrum of
56 activity. In this respect, *Staphylococcus epidermidis* and *Enterococcus faecalis* are two bacterial
57 species which have not been extensively tested in the past. The first is the dominant species among
58 the resident flora on hands (Rayan & Flournoy, 1987) and is one of the two most frequent cause of
59 nosocomial infection, together with *Staphylococcus aureus* (National Nosocomial Infections
60 Surveillance System, 2004). On the other hand *E. faecalis* is responsible of nosocomial infections
61 such as urinary tract and abdominal infections, bacteremia and endocarditis in patients with severe
62 concomitant diseases or with an impaired immune system (Mundy, Sahm, & Gilmore, 2000).
63 Contrary to what is observed for the antimicrobial effects of coffee extracts, less is known about its
64 potential cytotoxicity. Hegele et al. showed that the major cytotoxic component in Maillard reaction
65 mixtures and coffee (prepared as filter coffee and espresso) is represented by hydrogen peroxide
66 which is formed through an autooxidative process where polyphenolics reduce atmospheric oxygen
67 in the presence of transition metals (Hegele, Munch, & Pischetsrieder, 2009).
68 The aim of the present study was to investigate the antimicrobial activity of Arabica coffee extracts
69 against bacterial species such as *Staphylococcus epidermidis* and *Enterococcus faecalis*, which have
70 not been extensively studied in the past, and to assay possible cytotoxicity of the extracts towards
71 eukaryotic cells. Results are useful to evaluate the potentiality of the extracts as antimicrobials to be
72 used in topical preparations.
73

74 **2. Materials and methods**

75

76 **2.1. Bacterial strains, media and growth conditions**

77 The bacterial strains used were from the American Type Culture Collection (ATCC) and included
78 the three Gram-positive cocci *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis*
79 ATCC12228 and *Enterococcus faecalis* 29212, and the two Gram-negative bacilli *Escherichia coli*
80 ATCC25922 and *Salmonella enterica* ATCC14028. Bacterial cultures were grown in Mueller-
81 Hinton (MH) broth (Difco Laboratories, Becton Dickinson and Company, Sparks, MD) at 37°C
82 under aerobic conditions.

83

84 **2.2. Preparation of the regular and decaffeinated total coffee extracts**

85 Regular and decaffeinated coffee extracts were prepared starting from 6 g coffee powder (100%
86 Arabica, medium roasted), previously defatted by extraction with penthane, by solid-liquid
87 extraction under continuous stirring with 100 ml boiling water 10 min at 100°C. The aqueous
88 extracts were centrifuged 10 min at 1600 × g and the clear supernatant was then filtered through
89 0.45 μm filter and subsequently through 0.22 μm filter. Aliquots of the obtained coffee extracts
90 were freeze-dried and stored at -20°C until used. The average extraction yields were around 25%
91 (w/w).

92

93 **2.3. Chemicals**

94 Caffeine, trigonelline, formic acid, acetonitrile and methanol were purchased from Sigma-Aldrich
95 (Steinheim am Albuch, Germany); 5-, 4- and 3-caffeoylquinic acid (CQAs) and 3,4- 3,5- and 4,5-
96 dicaffeoylquinic acid were purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth,
97 Germany); ferruoylquinic acids (FQAs) were obtained from the division of organic chemistry and
98 biochemistry at Ruder Bošković Institute (Zagreb, Croatia) (Dokli, Navarini, & Hamersak, 2013).

99 Numbering of substituted position on CQAs and FQAs was designated according to the IUPAC
100 system. Water was purified on a Milli-Q system from Millipore (Bedford, MA).
101
102 **2.4. UHPLC analysis of the coffee extracts**
103 The analysis was performed using a 1290 UHPLC system (Agilent, Waldbronn, Germany),
104 consisting of a degasser, quaternary pump, thermostated column and diode array detector (DAD)
105 operating at 254 nm, 272 nm and 324 nm. Samples were prepared by dissolving the lyophilized
106 powder in a solution of 60% (v/v) methanol in Milli-Q water and then filtered through a 0.22 µm
107 filter. 2 µl of the sample were injected in the UHPLC system and the flow rate was 1.2 ml/min. For
108 caffeine, trigonelline and 3-, 4-, 5- chlorogenic acid, determinations were carried out using a 4.6
109 mm × 150 mm, 2.7 µm 120 SB-C18 Poroshell column (Agilent, Santa Clara, CA) and a gradient
110 elution (acetonitrile and 0.1% (v/v) formic acid). For minor compounds as ferruoylquinic acids, a
111 75 mm × 4.6 mm, 2.6 µm Kinetex Phenyl Hexyl column equipped with SecurityGuard™ Ultra
112 cartridges for Phenyl UHPLC (Phenomenex, Torrance, CA) and a similar elution gradient were
113 used. Identification and quantitation of compounds were performed by external calibration of
114 standard compounds on a 5-points calibration curve.
115 Confirmation of the caffeine content in the regular and decaffeinated extracts was performed via the
116 ISO 20481:2008 reference method, using a 1100 HPLC system (Agilent, Waldbronn, Germany)
117 equipped with a 4.6 mm × 150 mm, 5 µm MS-C18 XTerra column, isocratic elution of
118 water/methanol 76/24, 10 µl injection volume and 272 nm detection wavelength.
119
120 **2.5. Antimicrobial activity assays for caffeine and for the regular and decaffeinated coffee**
121 **extracts**
122 For the preparation of the stock solution of the regular and decaffeinated coffee extracts the
123 lyophilized powder was resuspended in sterile Milli-Q water at a final concentration ranging from
124 100 and 350 mg/ml for all the antimicrobial activity assays. For the caffeine stock a solution was

125 prepared dissolving 150 mg of caffeine powder (Sigma-Aldrich, St. Louis, MO) in 1 ml sterile
126 Milli-Q water at 80°C.
127 Minimum inhibitory concentration (MIC) values of the regular and decaffeinated coffee extracts
128 and of caffeine were determined using the broth microdilution susceptibility test following the
129 guidelines of the NCCLS with mid-log phase cultures. Serial two-fold dilutions of each extract or of
130 caffeine were prepared in a final volume of 50 µl in 96-well polystyrene plates (Sarstedt,
131 Nümbrecht, Germany) with MH broth. Each dilution series included control wells without the
132 extract or without caffeine. A volume of 50 µl of a bacterial suspension at a concentration of 5×10^5
133 cells/ml was then added to each well. The MIC was taken as the lowest concentration of regular and
134 decaffeinated coffee extract or of caffeine resulting in the complete inhibition of visible growth
135 after 20 h of incubation at 37°C. For the determination of the minimum bactericidal concentration
136 (MBC) 25 µl of broth from clear wells were spotted in triplicate on a MH agar plate which was then
137 incubated 20 h at 37°C. MBC was defined as the lowest concentration of coffee extract killing at
138 least 99.99% of the original inoculum.
139 To monitor bacterial growth inhibition, a bacterial suspension of 1×10^6 cells/ml was grown in a
140 96-well polystyrene plate (Sarstedt, Nümbrecht, Germany) with periodic shaking at 37°C in
141 presence of 5 mg/ml coffee extract for *E. coli*, *S. enterica* and *E. faecalis* or in presence of 0.5
142 mg/ml for *S. aureus* and *S. epidermidis*. The OD_{620} was measured on a microtiter plate reader
143 (Tecan Trading AD, Männedorf, Switzerland) every 10 min for *E. coli* and *S. enterica* or every 30
144 min for *E. faecalis*, *S. aureus* and *S. epidermidis*.
145 For the viable colony count a bacterial suspension of 1×10^6 cells/ml was grown with shaking at
146 37°C in presence of 1, 2, 4 mg/ml coffee extract for *S. aureus* and *S. epidermidis* or in presence of 5
147 mg/ml coffee extract for *E. faecalis*. At each time point (1, 2, 4 h) an aliquot of the sample was
148 serially diluted in fresh MH broth and 25 µl of the serial dilutions were spotted in triplicate on a
149 MH agar plate which was then incubated 20 h at 37°C to allow the viable colony count.
150

151 **2.5.1. Antimicrobial activity of the regular coffee extract in combination with vancomycin**

152 The activity of the regular coffee extract combined to vancomycin (Sigma-Aldrich, St. Louis, MO)
153 was evaluated by the checkerboard technique in a 96-well polystyrene plate (Euroclone, Milan,
154 Italy). Briefly, concentrations of vancomycin, ranging from 8 μM (12 μg/ml) to 0.125 μM (0.18
155 μg/ml), and regular coffee extract, ranging from 6 mg/ml to 0.09 mg/ml for *S. aureus* and *S.*
156 *epidermidis* or from 60 mg/ml to 0.93 mg/ml for *E. faecalis*, were combined in the standard MIC
157 format along with a bacterial suspension at a concentration of 5×10^5 cells/ml. The microplate was
158 incubated 20 h at 37°C and then the fractional inhibitory concentration (FIC) index was calculated
159 for each combination as follows: FIC index = Σ (FIC_A + FIC_B), where FIC_A is the MIC of
160 compound A in combination/MIC of compound A alone, and FIC_B is MIC of compound B in
161 combination/MIC of compound B alone. Synergy was defined for a FIC index of ≤ 0.5, indifference
162 for a FIC index of > 0.5 to ≤ 4, and antagonism for a FIC index of > 4 (Eliopoulos, 1996).

163

164 **2.6. Cytotoxicity towards eukaryotic cells**

165 The human breast carcinoma cell line was purchased from the ECACC N°86012803 (MCF7) and
166 cultured in Dulbecco's modified Eagle's (DMEM) High Glucose medium (Euroclone, Milan, Italy)
167 supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Euroclone, Milan, Italy), 2 mM L-
168 glutamine (Euroclone, Milan, Italy) and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Sigma-
169 Aldrich, St. Louis, MO).

170 For the cytotoxicity assay, the MCF7 cells were seeded at a density of 20,000 cells per well, in a
171 volume of 100 μl, in a 96-well polystyrene plate (Euroclone, Milan, Italy) and incubated at 37°C
172 with 5% CO_2. After 1 h, 2 h, 4 h, 24 h exposure to different extract concentrations (1, 2, 3, 4 mg/ml)
173 in supplemented DMEM High Glucose medium, the adherent cells were washed with Phosphate
174 Buffered Saline (PBS) (Sigma-Aldrich, St. Louis, MO) and 20 μl of 5 mg/ml 1-(4, 5-
175 Dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT) (Sigma-Aldrich, St. Louis, MO) were added
176 to 100 μl supplemented DMEM High Glucose medium in each well. After 4 h incubation at 37°C

177 with 5% CO_2, the culture medium supernatant was removed and the formazan was dissolved with
178 10% (w/v) tert-octylphenoxy poly(oxyethylene) ethanol (IGEPAL) (Sigma-Aldrich, St. Louis, MO)
179 in HCl 0.01 N overnight at 37°C. The absorbance at 620 nm was measured on a microtiter plate
180 reader (Tecan Trading AD, Männedorf, Switzerland).
181 For the 23 h recovery in fresh DMEM High Glucose medium after 1 h exposure to the extract, the
182 cells were washed with PBS and then 100 µl of fresh medium were added to each well. Afterwards
183 the microplate was incubated at 37°C with 5% CO_2 for the additional 23 h and then MTT was added
184 as described above.
185

186 **2.7. Statistical analysis**

187 The significance of differences among bacterial strains treated with increasing concentrations of
188 coffee extract for the viable colony count was assessed using GraphPad Prism (GraphPad Software,
189 La Jolla, CA) by the ANOVA multiple comparison test with Student-Newman-Keuls post test for
190 the *Staphylococci* strains and by the Unpaired t-test for the *E. faecalis* strain. For the cytotoxicity
191 assay towards breast adenocarcinoma MCF7 cells the significance of differences among the various
192 treatments was assessed using GraphPad Prism by the ANOVA multiple comparison test with
193 Student-Newman-Keuls post test.
194

195 **3. Results and Discussion**

196

197 **3.1. Analysis of the regular and decaffeinated coffee extracts**

198 The relative content of trigonelline, 3-, 4-, 5-CQAs, 3,4-, 3,5-, 4,5-diCQAs and 3-, 4-, 5-FQAs of
199 the regular and of the decaffeinated coffee extracts was determined by UHPLC-DAD. Results
200 indicate that the concentrations of all these components did not differ significantly between the two
201 coffee extracts and that the only difference was the relative content of caffeine (Table 1), which was
202 almost undetectable in the decaffeinated coffee extract. As expected, the specificity of the solvent

203 used in the decaffeination process resulted in a remarkable decrease in the caffeine content only
204 (Farah, de Paulis, Moreira, Trugo, & Martin, 2006) and this permits to put in evidence the role
205 played by caffeine which is not yet fully disclosed (Antonio et al., 2010; Almeida et al., 2012).
206

207 **3.2. Antimicrobial activity of the regular and decaffeinated coffee extracts**

208 The antimicrobial activity of a regular Arabica coffee extract was measured against three Gram-
209 positive and two Gram-negative bacterial strains. The results are expressed as the MIC values for
210 the tested strains (Table 2) and showed that the extract was active against all the strains. As a
211 general result Gram-positive bacteria showed a higher susceptibility to the extracts with respect to
212 the Gram-negative ones. The only exception was represented by *E. faecalis* which showed a MIC
213 value similar to that of the Gram-negative strains (Table 2). Results obtained with *S. aureus* and *E.*
214 *faecalis* are in agreement with previous data obtained using brewed coffee samples (Daglia, et al.,
215 1994; Rufian-Henares & de la Cueva, 2009). Excluding *E. faecalis*, the overall higher sensitivity of
216 the Gram-positive bacteria towards the extract could be due to the different composition of the
217 bacterial cell envelope in Gram-negative and positive bacteria, the latter lacking a low-permeability
218 barrier such as the outer membrane.

219 To evaluate the role of caffeine in the antimicrobial activity of the regular coffee extract, a
220 decaffeinated coffee extract (see Materials and methods) was also tested in parallel with pure
221 caffeine. The results showed that there are no differences between regular and decaffeinated
222 Arabica coffee extracts in the ability to inhibit the bacterial growth (Table 2). Furthermore the MIC
223 values for pure caffeine are much higher for all the tested bacterial strains than the caffeine
224 concentration determined in the regular coffee extract at the MIC value, which is < 0.1 mg/ml
225 (Table 1). These results are in agreement with those obtained by Manzhu Kang et al. who reported
226 that in presence of 4 mg/ml caffeine *E. coli* failed to grow (Kang et al., 2012).

227 These results highlight the predominant antibacterial effect of components other than caffeine in the
228 extract. Indeed Daglia et al. pointed to α-dicarbonyl compounds as the main agents responsible for

229 the antibacterial activity of roasted coffee against *Staphylococcus aureus* and *Streptococcus mutans*
230 (Daglia, et al., 2007). Moreover, other roasted coffee constituents including melanoidins (the final
231 products of the Maillard reaction), polyphenols, trigonelline and both caffeic and protocatechuic
232 acids have shown to exert antibacterial activity (Morales, Somoza, & Fogliano, 2012) Almeida et
233 al., 2012).
234 We then investigated the ability of the regular coffee extract to inhibit the bacterial growth even at
235 concentrations lower than the MIC values (Figure 1). In presence of 0.3-0.5 × MIC values of the
236 regular coffee extract all the tested bacterial strains showed a decreased growth rate. However the
237 highest inhibition of the bacterial growth was observed for *E. faecalis*: the effect of the extract on
238 the growth rate of this strain was significantly higher than that observed for *E. coli* or *S. enterica*,
239 even though the MIC value was comparable for the three strains.
240 To evaluate whether the extract has a bacteriostatic effect, we studied the short-term effect of the
241 extract against *S. aureus* and *S. epidermidis*, which showed the highest susceptibility to the extract
242 in terms of MIC values, and against *E. faecalis* to better understand the effect of the extract on the
243 growth rate of this strain.
244 Against both *Staphylococci* strains the extract showed a bacteriostatic effect which was appreciable
245 already after 1 h incubation and became more pronounced after prolonged incubation time at all the
246 tested concentrations, leading to a 2-log growth reduction after 4 h incubation at the maximum
247 concentration tested (4 mg/ml) for both *S. aureus* and *S. epidermidis* (Figure 2). These results
248 suggest that the antibacterial activity of the regular coffee extract is mainly bacteriostatic at short
249 exposure time and becomes bactericidal only after prolonged incubation. To confirm this
250 observation the MBC values were determined for both *S. aureus* and *S. epidermidis*. We found that
251 the MBC correspond to the MIC values of the extract for each strain (data not shown), indicating
252 that after prolonged incubation (20 h) the extract was able to kill bacteria. The observation that the
253 extract becomes bactericidal only after prolonged incubation time might suggest that it has a non-

254 lytic mechanism of action, which in contrast would have shown a strong and rapid bactericidal
255 effect. However further studies will be required to clarify this point.
256 On the opposite, the treatment of *E. faecalis* with 5 mg/ml extract produced a significant
257 bacteriostatic effect only after 4 h exposure to the extract (Figure 2B). This result, taken together
258 with the inhibition of the growth rate showed in Figure 1B, suggests that the extract exhibits a
259 delayed effect on this bacterial strain, beginning to significantly slow the bacterial growth and to
260 affect the bacterial viability only after 4 h treatment.
261 To test a potential synergic effect of the regular coffee extract in combination with an antibiotic
262 often used to treat nosocomial severe methicillin-resistant *S. aureus* (MRSA) infections(Rayner &
263 Munckhof, 2005), the antimicrobial activity of the extract was evaluated in combination with
264 vancomycin.
265 Given that vancomycin acts by inhibiting proper cell wall synthesis in Gram-positive bacteria, we
266 investigated whether the regular coffee extract enhanced its activity. The FIC indexes showed that
267 all combinations of regular coffee extract plus vancomycin did not exhibit any synergic effect
268 against *S. aureus* nor *S. epidermidis* ($0.5 < \text{FIC index} \leq 4$). Similar results were obtained also for the
269 combination of regular coffee extract plus vancomycin against *E. faecalis* ($0.5 < \text{FIC index} \leq 4$),
270 suggesting that this antibiotic and the extract act independently one from each other.
271

272 **3.3. Cytotoxicity effect of the regular coffee extract**

273 The eventual toxicity of the regular coffee extract was assayed towards breast adenocarcinoma
274 MCF7 cells (Figure 3), a cell line that is used as a model for cytotoxic studies of chemotherapeutic
275 agents. At a 3 mg/ml concentration, corresponding to 1.5- and 3-fold the MIC value for *S. aureus*
276 and *S. epidermidis* respectively, no significant toxicity was observed up to 4 h exposure to the
277 extract (Figure 3A). At this concentration, a significant decrease in cell viability was detected only
278 with a 24-h exposure. We further investigated the cytotoxicity of the extract after prolonged
279 exposure time showing that until 1 mg/ml the extract does not exhibit any effect on the viability of

280 the cells even after 24 h incubation (Figure 3B), while at 2 and 4 mg/ml the extract showed a
281 significant toxicity towards the MCF7 cells. However, when the cells were left for 23 h in fresh
282 medium, after the 1-h exposure, no toxicity was observed at any of the tested concentrations (Figure
283 3C), suggesting that, even if a damage might be produced by the extract after 1 h exposure, the cells
284 are able to repair it during the next 23 h.
285 Hydrogen peroxide, which in coffee is mainly formed by roasting products, was already shown to
286 play an important role in the cytotoxicity of coffee extracts towards bovine aorta endothelial cells
287 (Hegele, et al., 2009). This molecule might have a role also in cytotoxicity towards MCF7 cells and
288 further experiments will be needed to clarify this mechanism.
289 These data are encouraging considering that after 2 h exposure to the minimum concentration of
290 coffee extract (1 mg/ml) a clear antibacterial effect is already appreciable for both *S. aureus* and *S.*
291 *epidermidis*, while the toxicity towards the MCF7 cells begins to be significant only after 24 h
292 exposure to a concentration which is 4-fold (4 mg/ml) that producing a significant bacteriostatic
293 effect.
294

295 **4. Conclusions**

296 In conclusion, we showed that an Arabica regular coffee extract has a wide-spectrum antibacterial
297 activity. The extract was effective against *S. epidermidis*, which represent the dominant species
298 among the resident flora on hands, and has been shown to act as a reservoir for the transfer of
299 genetic elements to enhance the pathogenicity of *S. aureus* (Diep et al., 2006). The two
300 *Staphilococci* strains are often responsible of nosocomial infections resistant to the antibiotics
301 commonly used. In this respect we propose the use of the regular coffee extract as a component for
302 topical preparations to be used in healthcare units aimed at reducing the transmission of pathogens
303 by hand contact. Furthermore the observed antimicrobial activity of the extract against *E. faecalis*
304 and *S. aureus* might be promising also for its use as a food preservative. Indeed both bacterial
305 species may be present in many food products and can cause toxinfections (Giraffa, 2002;

306 Hennekinne, De Buyser, & Dragacci, 2012). Overall the kinetics of activity of the coffee extract
307 appear to be slower than that of other antimicrobials, with the extract showing mainly a
308 bacteriostatic effect at short exposure times, a feature that may be useful to arrest growth of
309 contaminant bacteria in food.
310 Finally, we showed that the Arabica coffee extract at the antibacterial concentrations does not cause
311 significant cytotoxic effects towards a model cell line. Therefore we believe that this kind of
312 compound deserves to be further studied with the aim to develop antibacterials for topical use, as
313 active components of hand washing preparations.
314

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316

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320

321 **References**

322 Almeida, A. A., Farah, A., Silva, D. A., Nunan, E. A., & Gloria, M. B. (2006). Antibacterial
323 activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *J*
324 *Agric Food Chem, 54*(23), 8738-8743.
325 Bekedam, E. K., Loots, M. J., Schols, H. A., Van Boekel, M. A., & Smit, G. (2008). Roasting
326 effects on formation mechanisms of coffee brew melanoidins. *J Agric Food Chem, 56*(16),
327 7138-7145.
328 Daglia, M., Cuzzoni, M.T., & Dacarro, C. (1994). Antibacterial activity of coffee. *J. Agric. Food*
329 *Chem. , 42*, 2270-2272.
330 Daglia, M., Papetti, A., Grisoli, P., Aceti, C., Spini, V., Dacarro, C.*, et al.* (2007). Isolation,
331 identification, and quantification of roasted coffee antibacterial compounds. *J Agric Food*
332 *Chem, 55*(25), 10208-10213.
333 Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G.*, et al.* (2006).
334 Complete genome sequence of USA300, an epidemic clone of community-acquired
335 meticillin-resistant Staphylococcus aureus. *Lancet, 367*(9512), 731-739.
336 Dokli, I., Navarini, L. , & Hamersak, Z. . (2013). Syntheses of 3-, 4-, and 5-O-feruloylquinic acids
337 *Tet. Asymm. , 24*, 785-790.
338 Eliopoulos, G. M. (1996). Antimicrobial combinations In Lorian V. (Ed.), Antibiotics in laboratory
339 medicine (pp. 330-393). Baltimore Williams & Wilkins
340 Farah, A., de Paulis, T., Moreira, D. P., Trugo, L. C., & Martin, P. R. (2006). Chlorogenic acids and
341 lactones in regular and water-decaffeinated arabica coffees. *J Agric Food Chem, 54*(2), 374-
342 381.
343 Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiol Rev, 26*(2), 163-171.
344 Hegele, J., Munch, G., & Pischetsrieder, M. (2009). Identification of hydrogen peroxide as a major
345 cytotoxic component in Maillard reaction mixtures and coffee. *Mol Nutr Food Res, 53*(6),
346 760-769.
347 Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012). Staphylococcus aureus and its food
348 poisoning toxins: characterization and outbreak investigation. *FEMS Microbiol Rev, 36*(4),
349 815-836.
350 Kang, T. M., Yuan, J., Nguyen, A., Becket, E., Yang, H., & Miller, J. H. (2012). The
351 aminoglycoside antibiotic kanamycin damages DNA bases in Escherichia coli: caffeine
352 potentiates the DNA-damaging effects of kanamycin while suppressing cell killing by
353 ciprofloxacin in Escherichia coli and Bacillus anthracis. *Antimicrob Agents Chemother,*
354 *56*(6), 3216-3223.
355 Morales, F. J., Somoza, V., & Fogliano, V. (2012). Physiological relevance of dietary melanoidins.
356 *Amino Acids, 42*(4), 1097-1109.
357 Mueller, U., Sauer, T., Weigel, I., Pichner, R., & Pischetsrieder, M. (2011). Identification of
358 H(2)O(2) as a major antimicrobial component in coffee. *Food Funct, 2*(5), 265-272.
359 Mundy, L. M., Sahm, D. F., & Gilmore, M. (2000). Relationships between enterococcal virulence
360 and antimicrobial resistance. *Clin Microbiol Rev, 13*(4), 513-522.
361 National Nosocomial Infections Surveillance System. (2004). National Nosocomial Infections
362 Surveillance (NNIS) System Report, data summary from January 1992 through June 2004,
363 issued October 2004. *Am J Infect Control, 32*(8), 470-485.
364 Rayan, G. M., & Flournoy, D. J. (1987). Microbiologic flora of human fingernails. *J Hand Surg*
365 *Am, 12*(4), 605-607.
366 Rayner, C., & Munckhof, W. J. (2005). Antibiotics currently used in the treatment of infections
367 caused by Staphylococcus aureus. *Intern Med J, 35 Suppl 2*, S3-16.
368 Rufian-Henares, J. A., & de la Cueva, S. P. (2009). Antimicrobial activity of coffee melanoidins-a
369 study of their metal-chelating properties. *J Agric Food Chem, 57*(2), 432-438.

370 Rurian-Henares, J. A., & Morales, F. J. (2008). Antimicrobial activity of melanoidins against
371 Escherichia coli is mediated by a membrane-damage mechanism. *J Agric Food Chem, 56*(7), 2357-2362.
372
373 Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen,
374 P. J. (2009). Application of natural antimicrobials for food preservation. *J Agric Food*
375 *Chem, 57*(14), 5987-6000.
376 Zell, B. L., & Goldmann, D. A. (2007). Healthcare-associated infection and antimicrobial
377 resistance: moving beyond description to prevention. *Infect Control Hosp Epidemiol, 28*(3),
378 261-264.
379
380

381 **Figure captions**

382

383 **Figure 1. Growth kinetics of different Gram-negative (A) and Gram-positive (B) bacterial**
384 **strains in presence of the regular coffee extract.** Bacterial suspensions of 10^6 cells ml^{-1} were
385 grown for 4 h (A) or 8 h (B) in absence (solid line) or in presence (dashed line) of the extract and
386 the OD_{620} was recorded every 10 min (A) or 30 min (B). Results are the mean of three independent
387 experiments ± SD.

388

389 **Figure 2. Viable colony counts of the Gram-positive strains exposed to the regular coffee**
390 **extract.** Bacteria were incubated for different hours in presence of different concentrations of the
391 extract, serially diluted with MH Broth, and plated to allow the viable colony counts. The results are
392 reported as CFU/ml (log scale) and represent the mean of three independent experiments ± SD. (A)
393 * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (ANOVA with Student-Newman-Keuls post test). (B) * $p<0.05$
394 (Unpaired t-test).

395

396 **Figure 3. Cytotoxicity towards breast adenocarcinoma MCF7 cells.** (A) Percentage of viable
397 cells compared to the control (no coffee extract, 100%) after increasing incubation times with 3
398 mg/ml extract (grey bars). (B) Percentage of viable cells compared to the control (no coffee extract,
399 100%) after 24 h exposure. (C) Percentage of viable cells compared to the control (no coffee
400 extract, 100%) after 1 h exposure and 23 h recovery in fresh medium. Results represent the mean ±
401 SEM of at least two independent experiments each performed in triplicate. ** $p<0.01$, *** $p<0.001$
402 vs untreated control (ANOVA with Student-Newman-Keuls post test).

Tables

Table 1. Relative content of trigonelline, caffeine, chlorogenic and dichlorogenic acids, and ferruoylquinic acids in coffee extracts.

	regular (mg/g)	**decaffeinated** (mg/g)
trigonelline	21	24
caffeine	38	< 2
caffeine[a]	36	1
3-CQA	11	11
4-CQA	12	11
5-CQA	21	22
3,4-diCQA	0.4	0.3
3,5-diCQA	0.1	0.1
4,5-diCQA	0.3	0.1
3-FQA	2.6	2.3
4-FQA	0.7	1.2
5-FQA	0.6	0.9

[a]Determined by the ISO 20481:2008 reference method.

Table 2. Sensitivity of different bacteria to total aqueous extracts obtained from Arabica coffee and to pure caffeine.

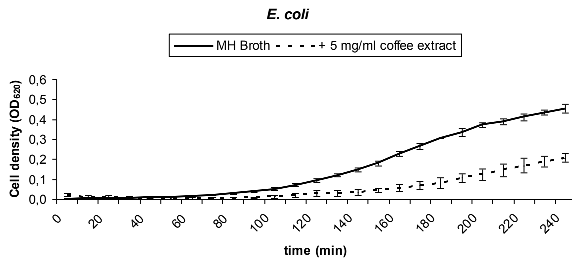
	MIC (mg/ml) [a]		
	regular	decaffeinated	caffeine
S. aureus ATCC25923	2	2	> 25
S. epidermidis ATCC12228	1	1	25
E. faecalis ATCC29212	15	15	12
E. coli ATCC25922	15	15	3
S. enterica ATCC14028	15	15	> 25

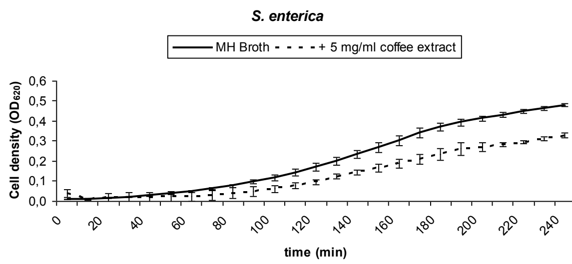
[a]Results are representative of three independent experiments.

Figure graphics

Figure 1

A





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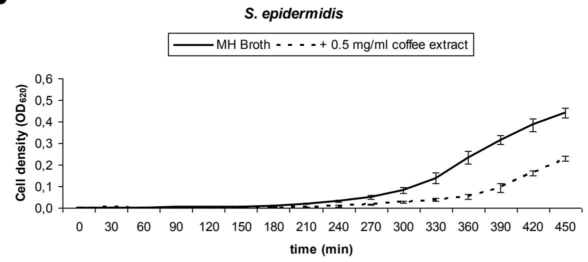
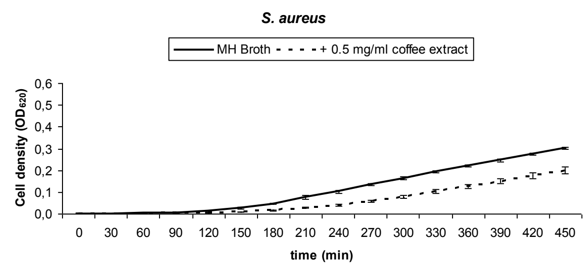




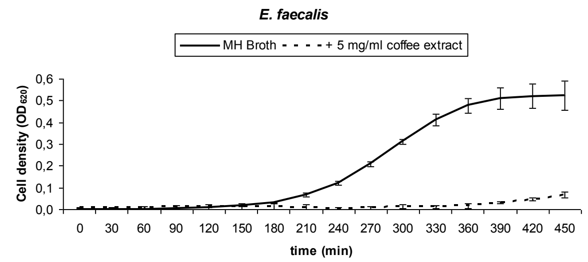
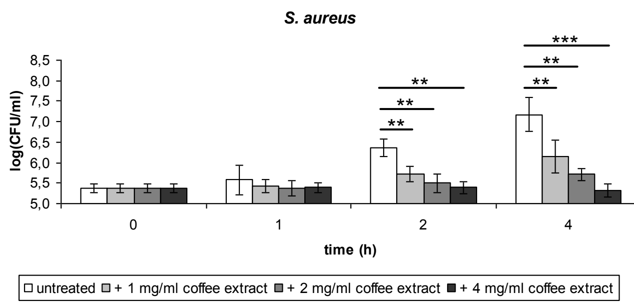


Figure 2

A



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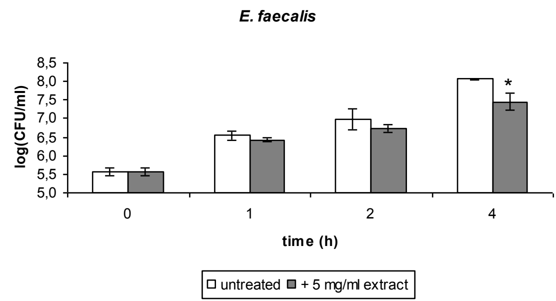

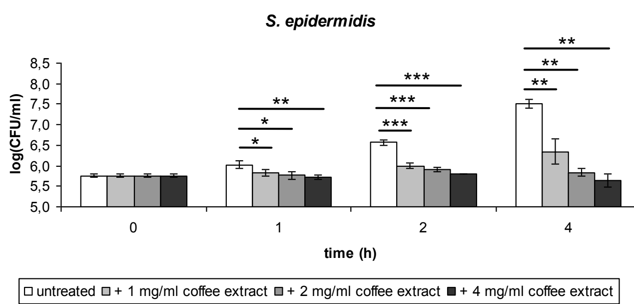

Figure 3