

1 **Coffee and spent coffee extracts protect against cell mutagens and inhibit growth**
2 **of food-borne pathogen microorganisms**

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21 Short title: Antimutagenic and antimicrobial activity of coffee and spent coffee

23 **ABSTRACT**

24 Coffee consumption decreases the risk of oxidative stress-related diseases. The by-
25 product obtained after brewing process (spent coffee) has also shown antioxidant
26 capacity. Spent coffee and coffee brews (filter and espresso) extracts were obtained
27 from Arabica and Robusta coffees, respectively. Spent coffee extracts showed slightly
28 high amounts in chlorogenic acids, but caffeine content was similar to their respective
29 coffee brew, with the Robusta samples being the richest. All coffee extracts exhibited
30 strong protection activity against indirect acting mutagen 2-AF (up to 92%), whereas
31 the protection against direct acting mutagen NPD was 12-35%, as measured by the
32 Ames Test. The growth inhibition of common food-borne pathogen and food spoilage
33 microorganisms by coffee extracts was also studied. Spent coffee extracts showed
34 antimicrobial activity, mainly against Gram-positive bacteria (*Staphylococcus aureus*,
35 *Listeria monocytogenes*) and yeast (*Candida albicans*). The role of phenolic acids,
36 caffeine and melanoidins in the antimutagenic and antimicrobial activities is discussed.
37 Thus, spent coffee extracts could be a potential source of bioactive compounds, thereby
38 becoming a promising new functional food ingredient.

39

40 **KEYWORDS:** coffee; by-products; antimicrobial; antimutagenicity; phenolics;
41 melanoidins.

42 **ABBREVIATIONS:** CGA: Chlorogenic acid; CQA: Caffeoylquinic acid; DiCQA:
43 Dicafeoylquinic acid; NPD: 4-Nitro-*O*-phenylenediamine; 2-AF: 2-Aminofluorene;
44 MIC: Minimum Inhibitory Concentration; CFU: Colony forming unit.

45 **1. Introduction**

46 Coffee is a worldwide food product with a total production of 8,700,000 kg in 2013
47 (ICO, 2013). Several studies have positively linked coffee consumption with a
48 decreased risk of oxidative stress-related diseases, such as cancer, cardiovascular
49 ailments, and diabetes, among others; thus, coffee has been proposed as a potential
50 functional food due to the presence of caffeine and phenolic compounds (Dorea & Da
51 Costa, 2005). Because different brewing processes extract different amounts of
52 bioactive compounds, the by-product generated after brewing processes, referred to as
53 spent coffee, could partially retain some of the bioactive compounds and consequently
54 their health-related properties, and it could possibly be considered as a novel and
55 sustainable functional ingredient. In a recent study, spent coffee was tested for its
56 antioxidant capacity measured by using chemical-based assays and in *in vitro* cell
57 cultures showing good capability for protecting against oxidation and DNA damage in
58 human cells (Bravo et al., 2012; Bravo, Arbillaga, De Peña, & Cid, 2013a).

59 Nowadays, consumers are requesting safe food products with beneficial health effects.
60 Therefore, the food industry is searching for new functional ingredients. The addition of
61 bioactive compounds capable of preventing pathological conditions caused by DNA
62 damages, such as cancer, might be a good strategy. Cancer is a leading cause of death
63 worldwide (8.2 million deaths in 2012) (WHO, 2012). Reactive oxygen species (ROS)
64 from endogenous and exogenous sources induce DNA changes, which can lead to cell
65 mutation (Klaunig & Kamendulis, 2004). Initial studies on coffee found potential
66 mutagenicity of coffee, although excessively heated brewed coffee samples (Kato,
67 Hiramoto, & Kikugawa, 1994) or extremely high coffee concentrations (Duarte et al.,
68 1999) were used for these analyses. In addition, non-physiological doses of coffee
69 compounds such as melanoidins or caffeine have been associated with a prooxidant

70 effect (Azam, Hadi, Khan, & Hadi, 2003; Caemmerer et al., 2012). On the contrary, a
71 small amount of coffee has a strong protective effect against oxidants (Stadler, Turesky,
72 Müller, Markovic, & Leong-Morgenthaler, 1994). Moreover, fruits, vegetables or herbs
73 with antioxidant properties and genoprotective effects have shown antimutagenic effects
74 (Edenharder, Sager, Glatt, Muckel, & Platt, 2002).

75 Another consideration that is essential in the development and production of foods,
76 including functional foods, is food safety. The European Food Safety Authority (EFSA)
77 reported high rates of outbreaks per population (1.2 per 100,000) in the EU in 2011
78 (EFSA, 2013), commonly caused by *Escherichia coli*, *Salmonella*, *Bacillus*, *Shigella*
79 and *Staphylococcus aureus*, among others. *Listeria monocytogenes* is a major risk
80 concern; in 2011, approximately 90% of the cases resulted in hospitalization and the
81 fatality rate was 10% (EFSA, 2013). In addition to health consequences,
82 microorganisms may cause food spoilage that can result in considerable economic loss
83 to producers and consumers. The food industry commonly uses preservatives,
84 preferably naturally occurring, to prevent microbial growth. Coffee has shown
85 antimicrobial activity against a broad range of microorganisms, including foodborne
86 pathogens (Almeida et al., 2012; Daglia, Cuzzoni, Dacarro, & Cesare, 1994; Martínez-
87 Tomé et al., 2011), but to the best of our knowledge, the antimicrobial activity of spent
88 coffee has not yet been evaluated.

89 The main aim of this study was to assess the potential antimutagenic and antimicrobial
90 activity of spent coffee extracts due to the presence of high amounts of bioactive
91 compounds in order to suggest their use as natural functional food ingredients.
92 Therefore, the protection of spent coffees and their respective coffee brews against
93 acting mutagens (Ames Test), as well as the capability to act as a food preservative

94 inhibiting the growth of a broad range of food-borne pathogens and food spoilage
95 microorganisms, has been evaluated.

96 **2. Material and methods**

97 **2.1 Preparation of coffee brews and spent coffee extracts.**

98 Roasted coffee from Guatemala (*Coffea arabica*, referred to as Arabica, 3.03% water
99 content, $L^* = 25.40 \pm 0.69$, roasted at 219 °C for 905 s) and Vietnam (*Coffea canephora*
100 var. robusta, referred to as Robusta, 1.59% water content, $L^* = 24.92 \pm 0.01$, roasted at
101 228 °C for 859 s) was provided by a local factory. Coffee beans were ground for 20 s
102 using a grinder (Moulinex super junior “s”, Paris, France). The L^* value was analyzed
103 by means of a tristimulus colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan),
104 using the D65 illuminant and CIE 1931 standard observer.

105 Filter coffee brew was prepared with an Ufesa Avantis coffeemaker (24 g coffee/400
106 mL water, 6 min at 90 °C). Espresso coffee brew was prepared with a Saeco Aroma
107 coffeemaker (7 g coffee/40 mL, 24 s at 90 °C). Coffee residues, called spent coffee,
108 were dried for 2 h at 102 ± 3 °C in a JP Selecta oven (Barcelona, Spain) and defatted
109 with petroleum ether (1:11, w/v) for 3 h at 60 °C in a Soxhlet extraction system (Büchi,
110 Flawil, Switzerland). Next, 24 g of spent coffee was extracted with 400 mL of water
111 using a filter coffeemaker (6 min at 90 °C). Both coffee brews and spent coffee extracts
112 were lyophilized using a Cryodos Telstar (Terrassa, Spain).

113 **2.2 Chlorogenic acids (CGA) and caffeine HPLC analysis.**

114 Extract preparation and cleanup were carried out on a C₁₈ Sep-Pak cartridge (Millipore
115 Waters, Milford, MA, USA) according to Bicchi et al. (1995). Briefly, an aliquot of the
116 sample (6 mL) was loaded onto the cartridge, previously conditioned with MeOH (5
117 mL) and Milli-Q water (3 mL). The cartridge was then eluted with 20 mL of
118 MeOH/Milli-Q water (40/60). The compounds were analyzed by HPLC following the

119 method described by Farah et al. (2005), with some modifications (Bravo et al., 2012).
120 Briefly, 100 μ L of sample were injected into an analytical HPLC unit model 1100
121 (Agilent Technologies, Palo Alto, CA, USA) equipped with a reversed-phase Poroshell
122 120 C-18 (2.7 μ m particle size, 250 x 4.6 mm) column at 25°C. The chromatographic
123 separation was performed using a gradient of methanol (Panreac, Barcelona, Spain)
124 (solvent A) and Milli-Q water acidulated with phosphoric acid (pH 3.0, solvent B) at a
125 constant flow of 0.8 mL/min as described by Bravo et al. (2012). Chromatograms were
126 recorded at 325 nm for chlorogenic acids (CGA) and 276 nm for caffeine.
127 Individualized identification of chlorogenic acid (3-, 4- and 5-caffeoylquinic acids and
128 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids) and caffeine was carried out by comparing the
129 retention time and the photodiode array spectra with those of their reference standard
130 compounds. Pure reference standards of 5-caffeoylquinic acid (5-CQA) and caffeine
131 were obtained from Sigma-Aldrich (St. Louis, MO, USA), and pure reference standards
132 of 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids were purchased from Phytolab
133 (Vestenbergsgreuth, Germany). Calibration curves of 5-caffeoylquinic acid (5-CQA)
134 and caffeine standard were used for quantifying. Coefficients of linearity for the
135 calibration curves were typically $R^2 > 0.99$. Quantification of the other chlorogenic acids
136 was carried out using the area of 5-CQA standard combined with molar extinction
137 coefficients of the respective chlorogenic acid as reported by Trugo and Macrae (1984)
138 and Farah et al. (2005).

139 **2.3 Melanoidins (Abs 420 nm)**

140 Fifty microlit of each sample were diluted to 2 mL with demineralized water.
141 Absorbance was measured at 420 nm, after exactly 2 min in a 3 mL cuvette (1 cm
142 length) with a Lambda 25 UV-vis spectrophotometer (Perkin-Elmer Instruments,
143 Madrid, Spain) connected to a temperature controlled chamber (25 °C).

144 **2.4 Antimutagenic activity**

145 The antimutagenic activity was evaluated using the *Salmonella* (*S. Typhimurium* His⁻,
146 TA98 strain, Moltax, NC, USA) mutagenicity test (Ames Test) described by Maron and
147 Ames (1983). First, toxicity and mutagenicity assays were performed using the same
148 conditions as those of the Ames test, with and without metabolic activation of rat liver
149 homogenate (S9 mix). No toxic or mutagenic effects were shown at the tested
150 concentrations of 2.4, 4.8 and 9.6 mg of lyophilized spent coffee extracts per plate; 2.4,
151 4.8 and 7.2 mg of lyophilized coffee brew extracts per plate; standard solutions of
152 caffeoylquinic acid (190, 320 and 750 µg/ plate); caffeine (120, 250 and 700 µg/ plate);
153 caffeic acid (120, 230 and 480 µg/ plate); ferulic acid (20, 70 and 180 µg/ plate); or
154 CQA/Caffeine standards mixtures (190/120, 320/350, 750/350 and 750/750). Next, a
155 bacterial suspension of 2.0×10^8 cfu/mL was prepared in order to determine the
156 antimutagenic activity. Assays without metabolic activation (-S9) were performed
157 mixing 50 µL of each sample or standard solution with 450 µL of phosphate buffer
158 (0.1M, pH 7.4), 100 µL of bacteria suspension and 50 µL of mutagen solution (NPD 20
159 µg/plate). After 60 min of incubation, 2 mL of molten top agar supplemented with
160 traces of histidine and biotin (Sigma-Aldrich St. Louis, MO, USA) (50 µM each, final
161 concentration) were added, rapidly vortexed and poured onto agar plates. Assays with
162 metabolic activation (+S9) were performed similarly, replacing phosphate buffer by an
163 equal volume of S9 mix and the mutagen solution by 2-AF 10 µg/plate. Negative and
164 positive controls were also included in each assay. Each experimental condition was
165 tested in triplicate. After 48 h of incubation at 37 °C, the revertant colonies were
166 counted. Three independent assays were carried out for each experimental condition.
167 The data was expressed as the number of revertants (cfu/plate) and as the percentage of

168 inhibition: (Sample plate revertants - spontaneous revertants) / (positive control
169 revertants- spontaneous revertants) x 100.

170

171 **2.5 Antimicrobial susceptibility tests**

172 *2.5.1 Microbial strains*

173 Eight bacterial and two fungal strains coming from the Spanish Collection of Culture
174 Strains (CECT) were selected as representatives of pathogen and spoilage
175 microorganisms: *Staphylococcus aureus* CECT 240 (ATCC 6538P), *Listeria*
176 *monocytogenes* CECT 911 (ATCC 19112), *Bacillus subtilis* CECT 356 (ATCC 6633),
177 *Escherichia coli* CECT 434 (ATCC 25922), *Pseudomonas aeruginosa* CECT 108
178 (ATCC 27853), *Salmonella Choleraesuis* CECT 443 (ATCC 13311), *Candida albicans*
179 CECT 1002 (ATCC 18804) and *Aspergillus niger* CECT 2574 (ATCC 16404).

180 *2.5.2 Agar-well diffusion method*

181 Lyophilized samples were dissolved in distilled water at a concentration of 160 mg
182 lyophilized/mL for spent coffee (equivalent to 20 mL of original sample), and 122 and
183 260 mg lyophilized/mL for Arabica and Robusta coffee brew (equivalent to 10 mL of
184 original sample), respectively. A millilit of each bacterial suspension (10^6 cfu/mL) was
185 added to sterilized 90-mm petri plates. Next, 50 mL of sterilized Mueller-Hinton agar
186 (BD, Franklin Lakes, NJ, USA) were added to each plate and, after homogenization, the
187 plates were dried for 20 min at room temperature. Wells measuring 6-mm in diameter
188 were cut from the agar and 50 μ L of the coffee samples were added. Ampicillin (10
189 μ g/well), gentamicin (10 μ g/well) (BD, Franklin Lakes, NJ, USA) and amphotericin-B
190 (100 μ g/well) (Marnes-La-Coquette, France) were used as positive controls. The plates
191 were incubated at $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 24 h. Three independent assays were carried out for

192 each experimental condition. The diameter (mm) of the inhibition zone formed around
193 the well was measured (CLSI, 2006).

194 *2.5.3 Minimum inhibitory concentration (MIC) determination*

195 The MICs of coffee samples were determined by a broth dilution method in microtiter
196 plates (CLSI, 2006). Twofold serial dilutions of the products under study were carried
197 out using Mueller-Hinton broth (200 μ L/well after addition of the inoculum). The tested
198 concentration ranges (mg lyophilized/mL) for each coffee sample were: Arabica and
199 Robusta spent coffee (0.08-160), Arabica coffee brew (0.06-122) and Robusta coffee
200 brew (0.12-260). Following incubation of microtiter plates at 37 $^{\circ}$ C \pm 1 $^{\circ}$ C for 24 h
201 (bacterial strains) or 30 $^{\circ}$ C \pm 1 $^{\circ}$ C for 72 h (fungal strains), turbidity was measured in a
202 spectrophotometer reader at 595 nm (SPECTRA MR Dynex technology, Chantilly, VA,
203 USA). Positive (Mueller-Hinton broth) and negative wells (Mueller-Hinton +
204 Ampicillin 0.2 μ g/mL or Amphotericin-B 0.2 μ g/mL) were used as controls. Each
205 experimental condition was tested in triplicate. MIC was considered as the lowest
206 concentration of coffee sample that inhibited the growth of microorganism.

207 *2.5.4 Growth curves in the presence of coffee extracts*

208 Overnight cultures were diluted using Mueller-Hinton broth to yield an inoculum of
209 10^6 cfu/mL. Product concentrations of 0 (control) and 12 mg lyophilized/mL for Arabica
210 and Robusta spent coffee were added to the inoculum, and incubation was initiated at 37
211 $^{\circ}$ C \pm 1 $^{\circ}$ C. Samples were taken immediately and at 1, 24, 48 and 72 hours of exposure to
212 then be serially diluted and plated on Tryptone soy agar (Biomerieux, Marcy l'Etoile,
213 France). Following incubation at 37 $^{\circ}$ C \pm 1 $^{\circ}$ C for 24 h, colonies were counted and the
214 number of viable bacteria was expressed in cfu/mL.

215 **2.6 Statistical Analysis**

216 Each parameter was analyzed in triplicate. Results are shown as the mean \pm standard
217 deviation (SD). Comparisons were performed by the non-parametric Mann–Whitney U-
218 test for the antimutagenic parameters. A probability of $p \leq 0.05$ was accepted as the
219 level of significance. One-way analysis of variance (ANOVA) was applied for the
220 antimicrobial parameters. A Tukey test was applied as a test *a posteriori* with a level of
221 significance of 95%. All statistical analyses were performed using STATA v.12.0.

222

223 **3. Results and discussion**

224 **3.1 Bioactive compounds**

225 Two coffees from the most consumed varieties (Arabica and Robusta) were selected due
226 to their phytochemical differences. Filter and espresso coffee brewing processes are the
227 most universal methods used for preparing a cup of coffee, not only at domestic levels
228 but also in workplaces and coffee shops. These two coffee extraction methodologies
229 yield approximately 16.6% (w/w) in terms of final lyophilized powder of coffee brew
230 extract. Residues (spent coffee) after preparation of coffee brews with filter (Arabica)
231 and espresso (Robusta) coffeemakers were obtained and desiccated for subsequent
232 preparation of aqueous extracts using a previously optimized methodology (Bravo,
233 Monente, Juárez, De Peña, & Cid, 2013b). Spent coffee extraction yielded
234 approximately 10% (w/w) in terms of final lyophilized powder of spent coffee extract.
235 These two spent coffee extracts were selected because they showed the highest
236 antioxidant activity in chemical-based assays and in cell cultures (Bravo et al., 2012;
237 2013a).

238 The main bioactive coffee compounds (chlorogenic acids, caffeine and melanoidins)
239 were measured to characterize both spent coffee and coffee brew lyophilized extracts.
240 The results are shown in Table 1. The most abundant chlorogenic acids (CGA) in

241 coffee, which are caffeoylquinic acids formed by esterification between quinic acid and
242 one or two moieties of caffeic acid, were identified and quantified (3-CQA, 4-CQA, 5-
243 CQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA). Both spent coffee extracts showed
244 slightly greater amounts of all the CQAs and diCQAs than their respective coffee
245 brews, with the difference ($p < 0.05$) in Robusta samples being quite significant. Arabica
246 spent coffee extract had a final concentration of total chlorogenic acids (CQA and
247 diCQA) of 84.22 mg/g, whereas Robusta spent coffee extract had 66.09 mg/g.
248 Monocaffeoylquinic acids (CQA), and mainly 5-CQA, followed by their isomers 4-
249 CQA and 3-CQA were the most abundant chlorogenic acids in both spent and coffee
250 brew extracts. Among dicaffeoylquinic acids, 4,5-diCQA was the most abundant closely
251 followed by 3,4-diCQA and 3,5-diCQA. Furthermore, the caffeine content was similar
252 in the extracts of spent coffee and their respective coffee brews, with the Robusta
253 samples being the richest. All these results concur with those reported by Bravo et al.
254 (2012), whose publication discussed the extractability of all these hydrophilic bioactive
255 compounds and compared the results obtained with results reported for industrial
256 soluble and espresso spent coffee grounds (Mussatto, Ballesteros, Martins, & Teixeira,
257 2011; Cruz et al., 2012). Finally, espresso coffee brew had the most significantly
258 highest absorbance at 420 nm which measures brown Maillard Reaction Products, such
259 as melanoidins (0.418 vs. 0.133-0.165), concurring with previous studies which showed
260 that espresso coffeemakers extract the highest amount of Maillard Reaction Products in
261 comparison with other brewing methods

262 **3.2 Antimutagenic activity**

263 After characterization of the extracts, the potential antimutagenic effect of spent coffee
264 extracts and coffee brews was evaluated using the *S. Typhimurium* test strain TA98
265 (Ames test). Coffee samples were tested against the mutagenic activity of 4-nitro-o-

266 phenylene-diamine (NPD) and 2-aminofluorene (2-AF) (positive controls). In addition,
267 commercial standards were analyzed in order to estimate the contribution of certain
268 coffee components to the antimutagenic activity. The revertants number of each sample
269 was compared with the positive control, and the inhibition percentage was calculated.
270 Results showed that all coffee samples are active against both direct (NPD) and indirect
271 (2-AF) acting mutagens (Table 2).

272 Spent coffee extracts were more antimutagenic than their respective coffee brews
273 against the action of the direct acting mutagen NPD. Robusta spent coffee was the most
274 protective sample (up to 35%), while the Arabica spent coffee inhibition percentage
275 ranged from 12 to 26%. With regard to coffee brews, Robusta had a protective effect
276 between 20 and 23%, whereas Arabica coffee brew had the least antimutagenic effect,
277 showing no significant differences. These results suggest that the components in spent
278 coffee extracts and coffee brews are active against the mutagenic action of NPD. First,
279 phenolic compounds might have a positive contribution; in fact, previous findings have
280 reported that these compounds might play an important role in the antimutagenic
281 activity of fruit, vegetables or herbs (Edenharder et al., 2002). Similar concentrations of
282 the main bioactive compounds in spent coffee extracts and coffee brews were tested to
283 determine their role in antimutagenic activity (Table 3). The results indicated that 5-
284 CQA standard was highly effective in the inhibition of NPD mutagen (30-56%), mainly
285 due to caffeic acid, which has similar antimutagenic activity (38-41%). Furthermore,
286 feruloylquinic acids are common phenolic acids found in coffee samples; they are an
287 ester linkage between a quinic acid and a ferulic acid. Due to the fact that there are no
288 commercial standards of this chlorogenic acid, three concentrations of ferulic acid were
289 tested. The results showed that this compound might also have an important
290 contribution to the antimutagenic activity of spent coffee (37-51% protection). Shushi

291 and Kaur (2008) reported that the methoxy group on the phenyl ring is responsible for
292 the antimutagenic activity of ferulic acid.

293 Apart from the activity of phenolic compounds, another coffee bioactive compound
294 such as caffeine could contribute to the mutagen inhibition. Caffeine standard solutions
295 had high inhibition percentages (33-56%) against the mutagenic agent, showing a dose-
296 dependent pattern. In order to better simulate different spent coffee and coffee brew
297 matrices, mixtures of low, medium and high concentrations of 5-caffeoylquinic acid and
298 caffeine were also tested. The results from 5-CQA and caffeine mixtures did not show
299 any additional or synergistic effects, because similar inhibition percentages have been
300 found when comparing with individual standards (40-53%). The mixtures of similar
301 amounts of both compounds (750/700 and 320/350 µg/plate) were more efficient.
302 Therefore, these last results could partially explain the highest inhibition percentage
303 observed in Robusta spent coffee extract with similar concentrations of naturally-
304 occurring CQAs and caffeine. Nevertheless, coffee samples were less effective than
305 standard solutions, probably due to the presence of many other compounds, which may
306 act as antagonists.

307

308 Substances may become mutagenic agents after metabolic process, which it is the case
309 of the aromatic amine 2-aminofluorene (2-AF). S9 mix is rat liver microsomal fraction
310 containing phase I and II metabolic enzymes. Moreover, the metabolic activation
311 involves the cytochrome-based P450 metabolic oxidation system, where the arylamino
312 group of 2-AF is oxidized to the N-hydroxy-derivatives (Wang & Guengerich, 2013).
313 These electrophilic products are highly mutagenic due to their capability to form DNA
314 adducts (Heflich & Neft 1994). The results showed that spent coffee extracts and coffee
315 brews had a stronger response against the mutagenic activity of 2-AF than NPD. The

316 highest tested concentrations of spent coffee extracts and coffee brews yielded almost
317 complete protection against the mutagen action (89-92%). A clear dose-dependent
318 pattern was observed; for example, Arabica and Robusta spent coffee extract protection
319 percentages were in the range of 5–92% (2.4–9.6 mg/plate). Similar to the
320 aforementioned results (-S9), phenolic compounds and caffeine standard solutions were
321 effective reducing the mutagenicity caused by the mutagen 2-AF. In this case, CQA and
322 caffeic acid showed a higher antimutagenic effect than ferulic acid. All phenolic
323 compounds showed dose-dependent pattern. In contrast, no differences were observed
324 among high and low concentrations of caffeine. Furthermore, the inhibition percentages
325 of the standard mixtures were lower than the ones found for coffee samples, indicating
326 that other compounds, such as Maillard Reaction Products like melanoidins, might
327 participate actively against indirect acting mutagens.

328 These data demonstrate that both spent coffee extracts were able to reduce the activity
329 of direct and indirect acting mutagens. Moreover, the assays with standard solutions
330 confirmed that naturally-occurring coffee compounds (phenolic acids and caffeine) are
331 active contributors to the antimutagenic activity. In addition, the results highlight that
332 4.8 mg of spent coffee extract is an effective dose for reducing (up to 18-45%) the
333 activity of both direct (20 µg of NPD) or indirect (10 µg 2-AF) acting mutagens. This
334 quantity of spent coffee extract contains approximately 300 µg of CQAs and caffeine.
335 The protection mechanism is not completely understood, but results suggest a possible
336 direct action against free radicals and an indirect mechanism for DNA protection. The
337 antioxidant capacity of phenolic acids, caffeine or melanoidins could be associated with
338 the inhibition of mutagens, through scavenging activity against free radicals (Azam,
339 Hadi, Khan, & Hadi, 2003, Farombi & Kamendulis, 2005, Rufian-Henares & Morales
340 2007). The high level of antioxidant activity of the tested spent coffee extracts (Bravo et

341 al., 2012) and their capability to reduce ROS level and DNA strand breaks induced by
342 H₂O₂ in a human cell line (Bravo et al., 2013a) may support the radical scavenging
343 theory. In addition, the samples might also protect-DNA through other ways. Abraham
344 et al. (1991) found genotoxicity inhibition of several carcinogens by coffee in *in-vivo*
345 studies. Indirect acting mutagen could also be blocked by interfering with the enzymatic
346 process or competing for the metabolic paths. Some authors have reported that phenolic
347 acids had selective inhibitory effects on cytochrome P450 (Teel & Huynh 1998).
348 Furthermore, caffeine is metabolized through cytochrome P450 enzymatic path which
349 might cause a decrease in the revertants number, due to a competitive inhibition
350 (Weisburger, Dolan & Pittman, 1998).

351 Thus, spent coffee extract compounds were capable of inhibiting, of destroying or of
352 avoiding DNA damage caused by direct and indirect acting mutagens, and
353 consequently, might be linked with the prevention of earlier stages of carcinogenesis.

354 **3.3 Antimicrobial activity**

355 A requirement for a new food ingredient, whether it be functional or not, is that it be
356 safe; this ingredient would become even more valuable if it also contributed to food
357 safety. Due to the fact that coffee has antimicrobial activity, and spent coffee extracts
358 contain the same bioactive compounds, discussed earlier in this paper, these extracts
359 would probably be good candidates as suitable food ingredients for preventing or
360 delaying microbial growth that may cause food borne-diseases or food spoilage. Thus,
361 three assays were used to evaluate the antimicrobial activity of coffee samples against
362 some of the most common food-borne pathogens (*Salmonella*, *Listeria monocytogenes*,
363 *Staphylococcus aureus*, *Escherichia coli*) and food spoilage microorganisms (*Bacillus*
364 *subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*). First, a
365 screening test was carried out to determine the sensibility of each microorganism to

366 spent coffee and coffee brew samples. Next, the lowest amount of extract capable of
367 inhibiting microorganism growth was estimated. Finally, the antimicrobial effect of
368 spent coffee extracts was measured at 24, 48 and 72 hours of exposure.

369 The inhibition diameters of the agar-well diffusion method (Table 4) showed that all the
370 tested samples were more active against Gram-positive bacteria (*S. aureus*, *L.*
371 *monocytogenes*, *B. subtilis*) than against Gram-negative ones (*E. coli*, *S. Choleraesuis*,
372 *Ps. aeruginosa*). *B. subtilis* was the least sensitive Gram-positive bacterium to coffee,
373 coinciding with results reported by Murthy and Manonmani (2009). This higher
374 resistance could be related to the capability to produce endospores, a mechanism linked
375 to the increase of resistance to environmental conditions (Russell, 1991). Furthermore,
376 no antimicrobial activity was observed against *A. niger*, whereas *Candida albicans*
377 demonstrated more sensitivity to the samples. The data indicated that spent coffee
378 extracts were more efficient than Robusta coffee brew due to the fact that smaller
379 amounts of Arabica and Robusta spent coffee extract (160 mg/mL) yielded similar
380 results ($p > 0.05$) to those obtained for Robusta coffee brew (260 mg/mL).

381 The minimum inhibitory concentration (MIC) results (Table 5) partially coincided with
382 those obtained in the agar diffusion method, because Gram-positive bacteria (*S. aureus*
383 and *L. monocytogenes*) required smaller quantities of coffee extracts to induce growth
384 inhibition. *S. aureus* showed the lowest MIC, needing only 5 mg/mL of Arabica spent
385 coffee extract to inhibit the pathogen growth. However, it must be pointed out that
386 coffee extracts with concentrations less than 160 mg had antibacterial activity against
387 Gram-negative strains. A possible explanation could be that high molecular weight
388 compounds which are partially responsible for antibacterial activity interfere with the
389 diffusion through the agar (Cagri, Ustunol, & Ryser, 2001). Moreover, *Ps. aeruginosa*
390 and *S. Choleraesuis* showed similar or lower MICs than *B. subtilis* and *C. albicans*. The

391 data showed that amounts ranging from 5 to 80 mg/mL of spent coffee were capable of
392 inhibiting the growth of a broad range of microorganisms with concentrations of 10^6
393 cfu/mL; however, the most resistant bacteria (*E. coli*) required larger quantities.

394 Finally, three of the most susceptible microorganisms were chosen for studying their
395 growth in the presence of low concentrations of Arabica and Robusta spent coffee (12
396 mg/mL) throughout time. The number of viable cells (log cfu/mL) at 1, 24, 48 and 72 h
397 of exposure are shown in Figure 1. As expected, *S. aureus* was the most sensitive
398 microorganism to the tested extracts, with a maximum reduction of 2 to 4 logarithms
399 compared to the control after 24 h of exposure, and still remains approximately 2.5 to 3
400 log after 72 hours (Figure 1a). It must be pointed out that Robusta spent coffee showed
401 bacteriostatic activity during the first 24 h (no increased concentration from the initial
402 one). On the other hand, a slight growth inhibition (1 log cfu/mL after 72 hours) was
403 observed in the cases of *B. subtilis* and *C. albicans* cultures (Figure 1b, 1c). However,
404 this reduction was higher than expected, since the amount of spent coffee was fourfold
405 lower than *B. subtilis* and *C. albicans* MICs. Therefore, our data suggest that small
406 amounts of spent coffee extracts may cause a bacteriostatic effect on microorganisms
407 during long exposures.

408 The response of each microorganism to coffee samples might be influenced by a
409 number of factors. Firstly, structural differences between Gram-negative and Gram-
410 positive bacteria, specifically in the outer membrane, have been associated with
411 resistance patterns. Secondly, the differences in the antimicrobial activity could also be
412 affected by the variation of the concentrations of the phytochemicals among coffee
413 samples (Table 1). Our data suggest that Gram-positive bacteria appear to be more
414 susceptible to phenolic acids, coinciding with that which was reported by Del Castillo et
415 al. (2007). Several authors have proposed that the hydroxycinnamic acids, and

416 specifically the hydroxyl groups on chlorogenic acids, are responsible for the
417 antimicrobial activity, probably due to their capability of disrupting the cell membrane
418 permeability (Lou, Wang, Zhu, Ma, & Wang, 2011). In our study, Gram positive
419 bacteria (*S. aureus*, *L. monocytogenes* and *B. subtilis*) were inhibited by small amounts
420 of spent coffee extracts (CQAs ranged from 0.4 to 4.4 mg/mL), while higher
421 concentrations were needed to inhibit *S. Choleraesuis*, *Ps. aeruginosa*, *E. coli* and
422 *Candida albicans* (CQAs ranged from 1.6 to 10 mg/mL). Therefore, these results
423 suggest that the antimicrobial activity against more resistant microorganisms was due to
424 other coffee components. Although the data did not show a strong connection between a
425 high content of caffeine and lower inhibition diameters or MICs, some authors have
426 reported that caffeine has antimicrobial activity against Gram-negative bacteria
427 (Almeida et al., 2012; Almeida et al., 2006). Melanoidins have been proposed as highly
428 active against resistant microorganisms (Einarsson et al., 1983; Rufián-Henares &
429 Morales, 2007; Stauder et al., 2010). Our results strongly suggest that a high content of
430 melanoidins (Robusta coffee brew) is responsible for the growth inhibition of Gram-
431 negative bacteria. Rufián-Henares and De la Cueva (2009) propose that antimicrobial
432 activity of melanoidins could be mediated by metal chelating mechanisms. Therefore,
433 the complex mixture of compounds found in spent coffee (phenolic compounds,
434 caffeine and melanoidins) is able to act as a suitable antimicrobial agent for extent food
435 shelf-life, mainly against Gram-positive bacteria and yeast.

436 In conclusion, the antimutagenic and antimicrobial activity of spent coffee found in this
437 study suggests that this by-product could be considered as a potential food ingredient
438 for enhancing functional properties and extending the shelf-life of foods, or also for
439 pharmaceutical and cosmetic purposes. Although further research is needed to study the
440 stability of compounds in a food matrix and while undergoing industrial processes, the

441 results support the idea that spent coffee is an accessible, sustainable, and major source
442 of bioactive compounds with potential health benefits.

443

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450

451 The authors declare no conflict of interest.

452

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583 in 2012. http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

584 **Table 1.** Characterization of bioactive compounds of spent coffee and coffee brew
 585 extracts

	Concentration (mg/g lyophilized)			
	Arabica filter		Robusta espresso	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
3-caffeoylquinic acid (3-CQA)	20.66±0.70 ^a	18.10±0.82 ^a	18.14±0.48 ^a	12.86±0.73 ^b
4-caffeoylquinic acid (4-CQA)	24.75±0.54 ^a	21.22±1.09 ^a	19.40±2.18 ^{ab}	15.43±0.92 ^b
5-caffeoylquinic acid (5-CQA)	36.78±1.01 ^b	33.30±0.66 ^b	25.74±0.63 ^a	20.94±0.84 ^a
3,4-dicaffeoylquinic acid (3,4-diCQA)	0.71±0.10 ^{ab}	0.50±0.03 ^a	0.93±0.02 ^b	0.48±0.00 ^a
3,5-dicaffeoylquinic acid (3,5-diCQA)	0.54±0.14 ^a	0.41±0.10 ^a	0.66±0.01 ^a	0.41±0.05 ^a
4,5-dicaffeoylquinic acid (4,5-diCQA)	0.77±0.12 ^a	0.56±0.03 ^a	1.21±0.02 ^b	0.69±0.09 ^a
Total CQA+diCQA (CGA)	84.22	74.10	66.09	50.80
Caffeine	49.35±6.55 ^a	48.04±2.37 ^a	72.92±0.68 ^b	81.37±0.50 ^b

586 Values are expressed as means ± standard deviation from three experiments

587 **Table 2.** Effects of coffee samples against the mutagenic effects of NPD and 2-AF on
 588 *S. Typhimurium* TA98

	Concentration mg/plate	- S9		+ S9	
		Revertants (CFU/plate)	% Inhibition	Revertants (CFU/plate)	% Inhibition
Arabica Filter					
Spent coffee	2.4	1093 ± 32*	26	1576 ± 112	5
	4.8	1203 ± 102*	18	944 ± 199*	45
	9.6	1288 ± 88	12	195 ± 18.96*	92
Coffee Brew	2.4	1271 ± 116	14	1391 ± 57*	16
	4.8	1450 ± 110	1	475 ± 162*	74
	7.2	1358 ± 148	11	203 ± 94*	92
Robusta Espresso					
Spent coffee	2.4	1000 ± 155*	33	1536 ± 164	7
	4.8	968 ± 47*	35	1171 ± 97*	30
	9.6	1068 ± 84*	28	241 ± 50*	89
Coffee Brew	2.4	1136 ± 131*	23	1372 ± 113*	12
	4.8	1149 ± 53*	22	454 ± 172*	66
	7.2	1141 ± 120*	20	186 ± 81*	92
NPD		1461 ± 105			
2-AF				1651 ± 50	
Spontaneous revertants		47 ± 22		68 ± 70	

589 NPD: 4-nitro-o-phenylene-diamine (20 µg /plate) and 2-AF: 2-aminofluorene (10 µg /plate) were used as
 590 positive controls for -S9 and +S9, respectively. *Significantly different from positive control (95%
 591 significance level).

592 Values are expressed as means ± standard deviation from three independent experiments.

593 **Table 3.** Effects of standard solutions against the mutagenic effects of NPD and
 594 2-AF on *S. Typhimurium* TA98

Standard Solution	Concentration µg/plate	- S9		+ S9	
		Revertants (CFU/plate)	% Inhibition	Revertants (CFU/plate)	% Inhibition
5- Caffeoylquinic acid	190	927 ± 174*	56	1219 ± 105	4
	320	1144 ± 49*	45	892 ± 135*	30
	750	1439 ± 197*	30	859 ± 102*	33
Caffeic acid	120	1284 ± 166*	38	1182 ± 145	7
	230	1203 ± 57*	42	1019 ± 86*	20
	480	1215 ± 103*	41	820 ± 62*	36
Ferulic acid	20	905 ± 68*	57	1165 ± 92	8
	70	1207 ± 138*	42	1045 ± 181	18
	180	1304 ± 171*	37	1011 ± 78*	21
Caffeine	120	1388 ± 48*	33	1020 ± 143*	20
	350	1071 ± 34*	48	1045 ± 137	18
	700	908 ± 116*	56	1048 ± 127*	18
Standard mixture	5-CQA/Caffeine				
	190 / 120	1243 ± 137*	40	839 ± 176*	35
	320 / 350	1040 ± 62*	50	761 ± 126*	41
	750 / 350	1189 ± 157*	43	703 ± 155*	46
	750 / 700	971 ± 112*	53	851 ± 55*	34
NPD		2051 ± 101			
2-AF				1269 ± 41	
Spontaneous revertants		28 ± 7		28 ± 3	

595 NPD: 4-nitro-o-phenylene-diamine (20 µg /plate) and 2-AF: 2-aminofluorene (10 µg /plate) were used as
 596 positive controls for -S9 and +S9, respectively. *Significantly different from positive control (95%
 597 significance level).

598 Values are expressed as means ± standard deviation from three independent experiments.

599

600 **Table 4.** Inhibition zones obtained with the agar-well diffusion method (diameter
 601 in mm).

Organisms	Inhibition zones (mm)			
	Arabica Filter		Robusta espresso	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
<i>S. aureus</i>	17.3 ± 0.6 ^{ab}	15.3 ± 0.6 ^a	15.3 ± 1.2 ^a	19.7 ± 1.2 ^b
<i>L. monocytogenes</i>	20.0 ± 3.6 ^a	19.0 ± 2.7 ^a	18.0 ± 2.0 ^a	22.3 ± 3.1 ^a
<i>B. subtilis</i>	8.8 ± 0.3 ^a	9.7 ± 0.6 ^a	ND	11.9 ± 0.2 ^a
<i>E. coli</i>	ND	ND	ND	10.3 ± 1.2
<i>S. Choleraesuis</i>	ND	ND	ND	10.7 ± 0.6
<i>Ps. aeruginosa</i>	ND	ND	ND	10.3 ± 0.6
<i>C. albicans</i>	15.3 ± 1.2 ^{ab}	13.7 ± 0.6 ^a	15.7 ± 1.5 ^{ab}	17.7 ± 0.6 ^b
<i>A. niger</i>	ND	ND	ND	ND

602 Values are expressed as means ± standard deviation of three experiments. In each row, different
 603 superscripts indicate significant differences ($p < 0.05$) among samples. Positive control inhibition
 604 zones (mm): *S. aureus* 34.0 ± 0, *L. monocytogenes* 30.3 ± 1.5, *B. subtilis* 16.0 ± 1, *E. coli* 12.7 ± 0.6,
 605 *S. Choleraesuis* 18.3 ± 1.5, *Ps. aeruginosa* 19.7 ± 0.6, *C. albicans* 20.3 ± 1.5.

606 ND (not detected)

607 **Table 5.** Minimum inhibitory concentration values for coffee extracts and coffee brews
 608 against different microorganisms.

Organisms	MIC (mg/mL)			
	Arabica filter		Robusta espresso	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
<i>S. aureus</i>	5	7.5	10	8.1
<i>L. monocytogenes</i>	20	30	20	16.3
<i>B. subtilis</i>	40	60	40	32.5
<i>E. coli</i>	80	60	160	32.5
<i>S. choleraesuis</i>	40	30	40	16.3
<i>Ps. aeruginosa</i>	40	60	80	16.3
<i>C. albicans</i>	40	60	40	32.5

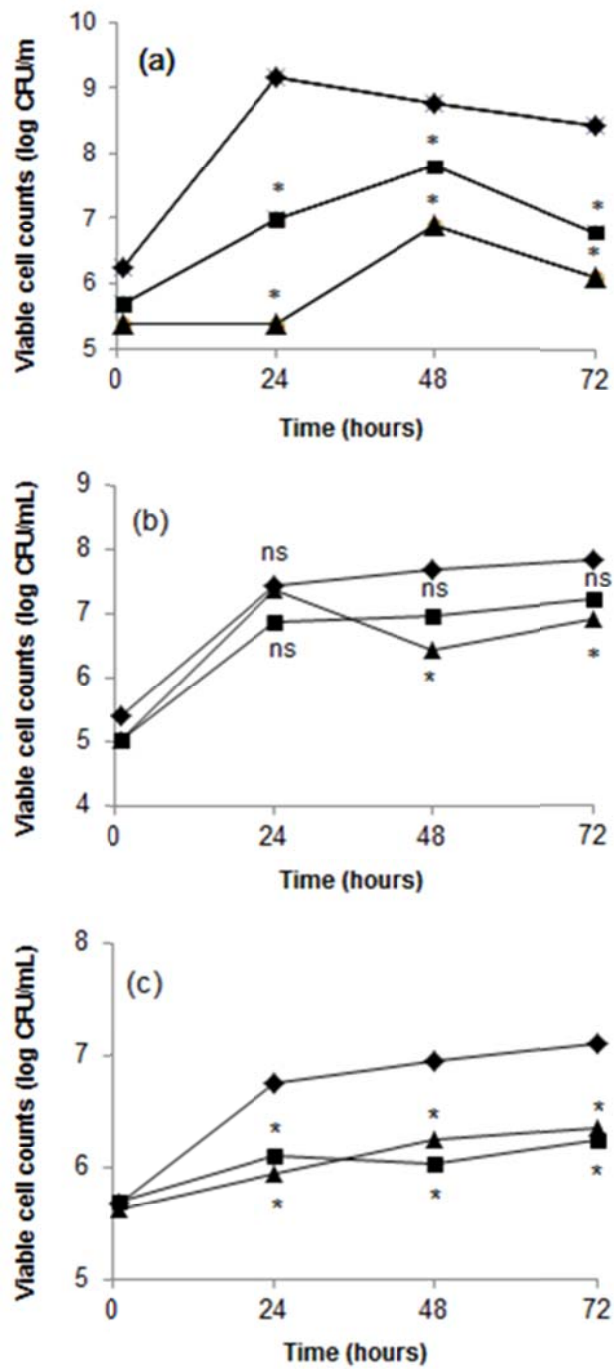
609 Three independent experiments showed identical values.

610 **Figure caption**

611 **Figure 1.** Growth curves of spent coffee extracts on (a) *S. aureus*, (b) *B. subtilis*, (c) *C.*
612 *albicans*. (◆) Control, (■) Arabica spent coffee, (▲) Robusta spent coffee.

613

616 **Figure 1.** Growth curves of spent coffee extracts on (a) *S. aureus*, (b) *B. subtilis*, (c) *C.*
 617 *albicans*. (◆) Control, (■) Arabica spent coffee, (▲) Robusta spent coffee.



617