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

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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 Dicaffeoylquinic acid; NPD: 4-Nitro-*O*-phenylenediamine; 2-AF: 2-Aminofluorene;
44 MIC: Minimum Inhibitory Concentration; CFU: Colony forming unit.

1. Introduction

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

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

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

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

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

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

2. Material and methods

2.1 Preparation of coffee brews and spent coffee extracts.

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

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

2.2 Chlorogenic acids (CGA) and caffeine HPLC analysis.

Extract preparation and cleanup were carried out on a C_{18} Sep-Pak cartridge (Millipore Waters, Milford, MA, USA) according to Bicchi et al. (1995). Briefly, an aliquot of the sample (6 mL) was loaded onto the cartridge, previously conditioned with MeOH (5 mL) and Milli-Q water (3 mL). The cartridge was then eluted with 20 mL of 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 µL 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 µm 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).

2.4 Antimutagenic activity

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

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

2.5 Antimicrobial susceptibility tests

2.5.1Microbial strains

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

2.5.2 Agar-well diffusion method

Lyophilized samples were dissolved in distilled water at a concentration of 160 mg lyophilized/mL for spent coffee (equivalent to 20 mL of original sample), and 122 and 260 mg lyophilized/mL for Arabica and Robusta coffee brew (equivalent to 10 mL of original sample), respectively. A millilit of each bacterial suspension (10^6 cfu/mL) was added to sterilized 90-mm petri plates. Next, 50 mL of sterilized Mueller-Hinton agar (BD, Franklin Lakes, NJ, USA) were added to each plate and, after homogenization, the plates were dried for 20 min at room temperature. Wells measuring 6-mm in diameter were cut from the agar and 50 µL of the coffee samples were added. Ampicillin (10 µg/well), gentamicin (10 µg/well) (BD, Franklin Lakes, NJ, USA) and amphotericin-B (100 µg/well) (Marnes-La-Coquette, France) were used as positive controls. The plates were incubated at 37 ºC ± 1 ºC for 24 h. Three independent assays were carried out for

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

2.5.3 Minimum inhibitory concentration (MIC) determination

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

2.5.4 Growth curves in the presence of coffee extracts

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

2.6 Statistical Analysis

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

3. Results and discussion

3.1 Bioactive compounds

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

The main bioactive coffee compounds (chlorogenic acids, caffeine and melanoidins) were measured to characterize both spent coffee and coffee brew lyophilized extracts. 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-

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

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

al., 2012) and their capability to reduce ROS level and DNA strand breaks induced by H_2O_2 in a human cell line (Bravo et al., 2013a) may support the radical scavenging theory. In addition, the samples might also protect-DNA through other ways. Abraham et al. (1991) found genotoxicity inhibition of several carcinogens by coffee in *in-vivo* studies. Indirect acting mutagen could also be blocked by interfering with the enzymatic process or competing for the metabolic paths. Some authors have reported that phenolic acids had selective inhibitory effects on cytochrome P450 (Teel & Huynh 1998). Furthermore, caffeine is metabolized through cytochrome P450 enzymatic path which might cause a decrease in the revertants number, due to a competitive inhibition (Weisburger, Dolan & Pittman, 1998).

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

3.3 Antimicrobial activity

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

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

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

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

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

In conclusion, the antimutagenic and antimicrobial activity of spent coffee found in this study suggests that this by-product could be considered as a potential food ingredient for enhancing functional properties and extending the shelf-life of foods, or also for pharmaceutical and cosmetic purposes. Although further research is needed to study the 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
453 **REFERENCES**
454 Abraham, S. K. (1991). Inhibitory effects of coffee on the genotoxicity of carcinogens
455 in mice. *Mutation Research Letters, 262*(2), 109-114.

456 Almeida, A. A. P., Naghetini, C. C., Santos, V. R., Antonio, A. G., Farah, A., & Glória,
457 M. B. A. (2012). Influence of natural coffee compounds, coffee extracts and increased
458 levels of caffeine on the inhibition of *Streptococcus mutans*. *Food Research*
459 *International, 49*(1), 459-461.

460 Almeida, A. A. P., Farah, A., Silva, D. A. M., Nunan, E. A., & Glória, M. B. A. (2006).
461 Antibacterial activity of coffee extracts and selected coffee chemical compounds against
462 enterobacteria. *Journal of Agricultural and Food Chemistry, 54*(23), 8738-43.

463 Azam S., Hadi, N., Khan, N., & Hadi, S. (2003). Antioxidant and prooxidant properties
464 of caffeine, theobromine and xanthine. Medical Science Monitor, 9, 325-330.

465 Bicchi, C., Binello, A., Pellegrino, G., & Vanni, A. (1995). Characterization of green
466 and roasted coffees through the chlorogenic acid fraction by HPLC-UV and principal
467 component analysis. *Journal of Agricultural and Food Chemistry, 43*(6), 1549-1555.

468 Bravo, J., Juaniz, I., Monente, C., Caemmerer, B., Kroh, L., De Peña, M. P., & Cid, C.
469 (2012). Evaluation of spent coffee obtained from the most common coffeemakers as a
470 source of hydrophilic bioactive compounds. *Journal of Agricultural and Food*
471 *Chemistry, 60*(51), 12565-73.

Bravo, J., Arbillaga, L., De Peña, M. P., & Cid, C. (2013a). Antioxidant and genoprotective effects of spent coffee extracts in human cells. *Food and Chemical Toxicology, 60*(0), 397-403.

Bravo, J., Monente, C., Juániz, I., De Peña, M. P., & Cid, C. (2013b). Influence of extraction process on antioxidant capacity of spent coffee. *Food Research International, 50*(2), 610-616.

Caemmerer, B., Chodakowski, K., Gienapp, C., Wohak, L., Hartwig, A., & Kroh, L. (2012). Pro-oxidative effects of melanoidin–copper complexes on isolated and cellular DNA. *European food research and technology, 234*(4), 663-670.

Cagri, A., Ustunol, Z., & Ryser, E. T. (2001). Antimicrobial, Mechanical, and Moisture Barrier Properties of Low pH Whey Protein-based Edible Films Containing p-Aminobenzoic or Sorbic Acids. *Journal of Food Science, 66*(6), 865-870.

CLSI 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard 7th ed. M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.

Cruz, R., Cardoso, M. M., Fernandes, L., Oliveira, M., Mendes, E., Baptista, P., Morais, S., & Casal, S. (2012). Espresso coffee residues: A valuable source of unextracted compounds. *Journal of Agricultural and Food Chemistry,* 60(32), 7777-7784.

Daglia, M., Cuzzoni, M., & Dacarro, C. (1994). Antibacterial activity of coffee. *Journal of Agricultural and Food Chemistry,* 42 (10), 2270-2272.

Del Castillo, M. D., Ferrigno, A., Acampa, I., Borrelli, R. C., Olano, A., Martínez-Rodríguez, A., & Fogliano, V. (2007). In vitro release of angiotensin-converting enzyme inhibitors, peroxyl-radical scavengers and antibacterial compounds by enzymatic hydrolysis of glycated gluten. *Journal of cereal science*, 45(3), 327-334.

Dorea, J., & Da Costa, T. (2005). Is coffee a functional food? *The British journal of nutrition, 93*(6), 773-782.

Duarte, M. P., Laires, A., Gaspar, J., Leão, D., Oliveira, J. S., & Rueff, J. (1999). Genotoxicity of instant coffee: possible involvement of phenolic compounds. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 442*(1), 43-51.

Edenharder, R., Sager, J. W., Glatt, H., Muckel, E., & Platt, K. L. (2002). Protection by beverages, fruits, vegetables, herbs, and flavonoids against genotoxicity of 2-acetylaminofluorene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in metabolically competent V79 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 521*(1–2), 57-72.

EFSA, (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control), (2013). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011; EFSA Journal 2013, 11(4):3129.

Einarsson, H., Snygg, B. G., & Eriksson, C. (1983). Inhibition of bacterial growth by maillard reaction products. *Journal of Agricultural and Food Chemistry*, 31(5), 1043-1047.

Farah, A., De Paulis, T., Trugo, L., & Martin, P. (2005). Effect of roasting on the formation of chlorogenic acid lactones in coffee. *Journal of Agricultural and Food Chemistry, 53*(5), 1505-1513.

Farombi, E. F., & Fakoya, A. (2005). Free radical scavenging and antigenotoxic activities of natural phenolic compounds in dried flowers of *Hibiscus sabdariffa L*. *Molecular nutrition & food research, 49*(12), 1120-1128.

Heflich, R. H., & Neft, R. E. (1994). Genetic toxicity of 2-acetylaminofluorene, 2-aminofluorene and some of their metabolites and model metabolites. *Mutation Research/Reviews in Genetic Toxicology*, 318(2), 73-174.

International Coffee Organization ICO. (2013). Exporting countries: total production http://www.ico.org/prices/po.htm

Kato, T., Hiramoto, K., & Kikugawa, K. (1994). Possible occurrence of new mutagens with the DNA breaking activity in coffee. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 306*(1), 9-17.

Klaunig, J. E., & Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annual Review of Pharmacology and Toxicology, 44*(1), 239-267.

López Galilea, I., De Peña, M. P., & Cid, C (2007). Correlation of selected constituents with the total antioxidant capacity of coffee beverages: influence of the brewing procedure. *Journal of Agricultural and Food Chemistry, 55*(15), 6110-6107.

Lou, Z., Wang, H., Zhu, S., Ma, C., & Wang, Z. (2011). Antibacterial Activity and Mechanism of Action of Chlorogenic Acid. *Journal of Food Science, 76*(6), M398-M403.

Maron, D. M., & Ames, B. N. (1983). Revised methods for the *Salmonella* mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects, 113*(3–4), 173-215.

Martínez-Tomé, M., Jiménez-Monreal, A., Garcia-Jiménez, L., Almela, L., García-Diz, L., Mariscal-Arcas, M., & Murcia, M. A. (2011). Assessment of antimicrobial activity of coffee brewed in three different ways from different origins. *European food research and technology, 233*(3), 497-505.

Murthy, P., & Manonmani, H. K. (2009). Physico-chemical, antioxidant and antimicrobial properties of Indian monsooned coffee. *European food research and technology, 229*(4), 645-650.

Mussatto, S. I., Ballesteros, L. F., Martins, S., & Teixeira, J. A. (2011). Extraction of antioxidant phenolic compounds from spent coffee grounds. *Separation and Purification Technology, 83*(0), 173-179.

548 Rufián-Henares, J. M., & Morales, F. (2007). Functional properties of melanoidins: In
549 vitro antioxidant, antimicrobial and antihypertensive activities. *Food Research*
550 *International*, 40(8), 995-1002.

551 Rufián-Henares, J. M., & Morales, F. (2008). Microtiter plate-based assay for screening
552 antimicrobial activity of melanoidins against E. coli and S. aureus. *Food Chemistry*,
553 111(4), 1069-1074.

554 Rufián-Henares, J., & De la Cueva, S. P. (2009). Antimicrobial activity of coffee
555 melanoidins-a study of their metal-chelating properties. *Journal of Agricultural and*
556 *Food Chemistry*, 57(2), 432-8.

557 Russell, A. D. (1991). Mechanisms of bacterial resistance to non-antibiotics: food
558 additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology*,
559 *71*(3), 191-201.

560 Shishu, & Kaur, I. P. (2008). Antimutagenicity of curcumin and related compounds
561 against genotoxic heterocyclic amines from cooked food: The structural requirement.
562 *Food Chemistry,* 111(3), 573-579.

563 Stadler, R. H., Turesky, R. J., Müller, O., Markovic, J., & Leong-Morgenthaler, P.
564 (1994). The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity.
565 *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 308*(2),
566 177-190.

567 Stauder, M., Papetti, A., Mascherpa, D., Schito, A.M., Gazzani, G., Pruzzo, C., &
568 Daglia, M. (2010). Antiadhesion and antibiofilm activities of high molecular weight
569 coffee components against Streptococcus mutans. *Journal of Agricultural and Food*
570 *Chemistry*, 58(22), 11662-6.

571 Teel, R. W., & Huynh, H. (1998). Effect of phytochemicals on cytochrome P450-linked
572 alkoxyresorufin O-dealkylase activity. *Phytotherapy Research*, 12(2), 89-93.

573 Trugo, L.C., & Macrae, R. (1984). A study of the effect of roasting on the chlorogenic
574 acid composition of coffee using HPLC. *Food Chemistry*, 15(3), 219-227.

575 Wang, K., & Guengerich, F. P. (2013). Reduction of Aromatic and Heterocyclic
576 Aromatic N-Hydroxylamines by Human Cytochrome P450 2S1. *Chemical research in*
577 *toxicology*, 26(6), 993-1004.

578 Weisburger, J. H., Dolan, L., & Pittman, B. (1998). Inhibition of PhIP mutagenicity by
579 caffeine, lycopene, daidzein, and genistein. *Mutation Research/Genetic Toxicology and*
580 *Environmental Mutagenesis*, 416(1–2), 125-128.

581 WHO, International Agency for Research on Cancer. (2012). All Cancers (excluding
582 non-melanoma skin cancer) Estimated Incidence, Mortality and Prevalence Worldwide
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).

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

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

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