1   **TITLE:**

2   **Bioactive properties of a propolis-based dietary supplement and its use in**

3   **combination with mild heat for apple juice preservation**

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Abstract

This study characterizes the antioxidant and antibacterial properties of a propolis-based dietary supplement (PDS) and investigates its incorporation into apple juice to decrease the intensity of the heat treatment required to inactivate 5 $\log_{10}$ cycles of *Escherichia coli* O157:H7. As the source of propolis, we used a PDS containing 0.2 mg/µL of propylene glycol-extracted propolis (propolis). The total phenolic content and antioxidant activity (IC_{50}) of the PDS were 82.15 ± 3.53 mg/g and 0.055 ± 0.003 mg/mL, respectively. Regarding antimicrobial activity, propolis (0.2 mg/mL) was very effective under acidic pH against *Listeria monocytogenes* EGD-e, inactivating more than 5 $\log_{10}$ cell cycles in 1 h, but hardly inactivated or sub-lethally injured *E. coli* O157:H7 Sakai. However, incorporating propolis (0.2 mg/mL) into acidic buffer decreased the time needed to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 Sakai at 51 °C by 40 times. Moreover, when combined with heat in apple juice, propolis (0.1 mg/mL) reduced the thermal treatment time and temperature needed to inactivate 5 $\log_{10}$ cycles of *E. coli* by 75% and 3 °C, respectively. The corresponding PDS concentration did not decrease the organoleptic properties of the apple juice, which implies the possibility of obtaining a sensorially appealing, low-pasteurized apple juice with the functional properties provided by propolis.

Keywords: Propolis, Bioactive properties, *Escherichia coli* O157:H7, Heat, Apple juice, Sensory analysis

1. Introduction

Propolis is a bee product collected by honeybees (*Apis mellifera* L.) from tree buds; it is used in beehives as a protective barrier against pathogenic microorganisms (Silva et al., 2012). In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils (EOs), 5% pollen, and 5% various other substances, including organic debris (Marcucci, 1995).

Considering its role as bees' chemical weapon, it is no surprise that propolis has been subjected to intensive studies describing its antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, immunostimulating, and anticancer properties (Silva et al., 2012). Due to these properties, alongside consumers' growing demand for "green products," and the fact that its main constituents are generally recognized as safe (GRAS) substances (Burdock, 1998), propolis is gaining popularity as a natural preservative for new food applications and is being added to foods and drinks as a source of bioactive compounds to improve health (Mishima et al., 2005; Moreira et al., 2008).

On the other hand, extensive research is being carried out to develop new preservation methods, in an attempt to achieve food safety goals while maintaining high sensorial and nutritional food quality. According to the "hurdle theory" proposed by Leistner and Gorris (1995), the combination of a low-intensity thermal treatment and antimicrobial compounds could provide an enhanced antimicrobial effect, resulting in fewer undesirable effects. Previously, our research group explored the application of combined preservation processes based on the simultaneous action of mild heat and natural substances of vegetable origin, such as essential oils (EOs) and their constituents, observing synergistic effects (Espina et al., 2011; Espina et al., 2010; Espina et al., 2012; Somolinos et al., 2010). On the other hand, propolis has been

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suggested to act synergistically with heat in meat products (Kim et al., 2014), which might be related to presence of EO constituents (such as terpenoids and phenolic compounds) (Burt, 2004; Kumazawa et al., 2004).

This potential synergism between propolis and heat could be exploited to design preservation treatments for heat-sensitive and sweet-flavored foods, such as apple juice, so that thermal treatments could be applied at lower intensity. Moreover, the preservation of apple juice needs to be improved, as it has been involved in recent outbreaks of *E. coli* O157:H7 (Parish, 2009). Because this microorganism has been pointed out as the most heat- and acid-resistant pathogen in acidic juices (Mazzotta, 2001), the Food and Drug Administration's Guidance for Industry implemented a rule requiring that all apple juice producers achieve a 5-$\log_{10}$ reduction of *E. coli* O157:H7 to ensure the safety of their product (FDA, 2001).

Therefore, this study was conducted to characterize a propolis-based dietary supplement (PDS) containing 0.2 mg/µL of propylene glycol–extracted propolis (propolis) and evaluate its possible use as a natural additive in apple juice. More specifically, the aims of this work were (i) to characterize the bioactive properties of PDS (in terms of phenolic content and antioxidant activity), (ii) evaluate the effectiveness of propolis against Gram-positive and Gram-negative representative bacteria (*L. monocytogenes* and *E. coli*, respectively), (iii) evaluate its use in combination with mild heat to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 cells in apple juice, and (iv) determine the hedonic acceptability of apple juice with propolis.

2. Material and Methods

2.1. Propolis sample

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The sample used in this investigation was a dietary food supplement containing raw propolis provided by Miel El Albar (Lechón, Zaragoza, Spain). According to the producer, the main plant species that contributed to the propolis were poplar (*Populus* spp.), oak (*Quercus* spp.), and pine (*Pinus* spp.). The raw propolis was collected and macerated for 48 h under agitation at 36 ºC in propylene glycol. Wax and debris were removed by double filtration, obtaining a tincture (the propolis-based dietary supplement, PDS). The final concentration of propolis in PDS was 0.2 mg/µL.

2.2. Analysis of bioactive compounds and determination of antioxidant activity of the propolis-based dietary supplement

The total polyphenol content (TPC), flavonoid content (FC), and 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical-scavenging activity were determined by analyzing 6 replicates of the same sample. Spectrophotometric lectures were carried out in a Helios Gamma Thermo Electron Corporation Spectrophotometer (United Kingdom).

The TPC was determined by the Folin–Ciocalteu method (Millena Popova, Silici, Kaftanoglu, & Bankova, 2005). One mL of a test solution of PDS was transferred to a 50-mL volumetric flask containing 15 mL distilled water and 4 mL of the Folin–Ciocalteu reagent (Panreac, Spain); 6 mL of a 20% sodium carbonate solution (w/v) (sodium carbonate anhydrous, Panreac, Spain) were then added. The rest of the volume was made up with distilled water to 50 mL. After 2 h, the absorbance was measured at 760 nm. A blank solution was included in each assay, with 1 mL of methanol (Labscan, Poland) instead of the test solution. A calibration curve of standard caffeic acid (Sigma-Aldrich, USA) was employed (10–50 mg/mL; $y = 0.0055x - 0.0283$; $R^2 = 0.9984$). The results were expressed as mg caffeic acid equivalents (CAEs)/g PDS.

The aluminum chloride method (Silva et al., 2012) was used to determine the FC. Briefly, 250 µL of a test solution of PDS were mixed with 1.25 mL of distilled H_2O and 75 µL of a 5%-$NaNO_2$ solution (sodium nitrite, Panreac, Spain). After 5 min, 150 µL of a 10%–$AlCl_3$ H_2O solution (aluminum chloride hexahydrate, Panreac, Spain) was added. After 6 min, 500 µL of 1-M NaOH (Panreac, Spain) and 275 µL of distilled H_2O were added to the mixture. The solution was well mixed, and the intensity of pink color was measured at 510 nm. In an analogous procedure, 250 µL of a blank solution was used instead of the test solution. Catechin (Sigma-Aldrich, USA) standard solutions (0.01–0.09 mg/mL) were used to construct the calibration curve (y = 3.7300 x − 0.0098; $R^2 = 0.9998$). The results were expressed as mg catechin equivalents (CEs)/g PDS.

The DPPH free radical method is an antioxidant assay based on electron transfer that produces a violet solution in ethanol. This free radical, which is stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to a colorless ethanol solution. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry. This test was performed as described by Miguel et al. (2010). 50 µL of different concentrations of PDS were added to 2 mL of 60-µM methanolic solution of DPPH (Sigma-Aldrich, USA). The absorbance measurements were read at 517 nm, after 20 min of incubation time at room temperature (A1). The absorption of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control (A0). The percentage inhibition [(A0 – A1 / A0) * 100] was plotted against the different concentrations of the commercial sample. IC_{50} was determined (mg/mL) as the concentration of the commercial sample able to scavenge 50% of DPPH free radicals. The results were expressed as IC_{50} (mg/mL) of PDS.

2.3. Micro-organisms and growth conditions

E. coli O157:H7 Sakai *stx* 1A⁻/*stx* 2A⁻ was kindly provided by Kyu-Tae Chang (The National Primate Research Center, KRIBB, Ochang, South Korea). This strain was isolated from an outbreak involving white radish sprout (Michino et al., 1999). *L. monocytogenes* EGD-e was kindly provided by Prof. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). During this investigation, the cultures were maintained and kept frozen at −80 °C in cryovials. Broth subcultures were prepared by inoculating one single colony from a plate into a test tube containing 5 mL of sterile tryptic soy broth (Biolife, Milan, Italy) with 0.6% yeast extract added (Biolife) (TSBYE). After inoculation, the tubes were incubated overnight at 37 °C (*E. coli* O157:H7 Sakai) or 30 ℃ (*L. monocytogenes* EGD-e). Along with these subcultures, 250-mL Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final concentration of 10^4 cells/mL. These flasks were incubated under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at the appropriate temperature until the stationary growth phase was reached (24 ± 2 h). The stationary phase was chosen because microorganisms show higher resistance to heat at this stage than at the exponential phase (Hansen and Rieman, 1963), as well as to match previously published data (Espina et al., 2011; Espina et al., 2010; Espina et al., 2012; Somolinos et al., 2010).

2.4. Evaluation of the antimicrobial properties of propolis

Propolis was added, in the form of PDS, to determine its antimicrobial properties. As the solvent in PDS, propylene glycol did not affect microbial growth or inactivation under the conditions tested (data not shown); for comparison purposes with

previous works, we expressed the concentrations of propolis added in mg of propolis per mL of treatment medium.

The propolis was screened to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *L. monocytogenes* EGD-e and *E. coli* O157:H7 Sakai. Tubes containing 5 mL of TSBYE and different concentrations of propolis (0.008–2 mg/mL) were inoculated to a final concentration of 10^5 cells/mL. A negative control (without microorganisms), positive control (without propolis), and diluent control (the amount of propylenglicol corresponding to the maximum propolis concentration assayed (2 mg/mL)) were also prepared. After 24 h of incubation at the appropriate temperature in a shaking thermostatic bath (Bunsen, mod. BTG, Madrid, Spain), 100 μL of each tube were spread-plated in tryptic soy agar (Biolife) with 0.6% yeast extract added (TSAYE). The plates were incubated at corresponding temperatures for 24 h. The MIC was the lowest concentration of propolis at which bacteria failed to grow, showing counts equals to the initial concentration. The MBC was defined as the lowest concentration of propolis that inactivates 99.9% of an inoculated sample, showing counts below 10^2 colony-forming units (CFU)/mL. The evaluations of MIC and MBC were carried out in triplicate.

Moreover, the antimicrobial properties of propolis were evaluated by determining the bacterial inactivation, as a function of the treatment medium pH. A vigorous shaking method was used to prepare propolis suspensions in citrate–phosphate buffers (McIlvaine buffer) at pH 7.0 and 4.0.

Cells from stationary-phase cultures were added at final concentrations of 3 x 10^7 CFU/mL to buffers, both with and without propolis (0.2 mg/mL). The buffer pH was not modified as a consequence of adding antimicrobial compounds. Antimicrobial compound treatments were carried out at 20 ºC for 24 h. Samples were taken at 1, 6, and

191 24 h, and the survivors and sub-lethally injured cells were enumerated, as described

192 below. Previous experiments showed that untreated cells of *E. coli* O157:H7 Sakai and

193 *L. monocytogenes* EGD-e at concentrations of 10^7 CFU/mL were insensitive to

194 incubation in citrate–phosphate buffers at pH 7.0 or 4.0 for 24 h at 20 ºC (data not

195 shown).

196

197 *2.5. Measurement of cell inactivation by heat treatments and propolis*

198 Heat and combined treatments were carried out in a specially designed thermo-

199 resistometer, as previously described (Condón et al., 1993). Briefly, this device uses a

200 thermocouple (Pt 100) to monitor the temperature during the heat treatment and for the

201 injection of the inoculum. Once the temperature stabilized, 0.2 mL of an adequately

202 diluted cell suspension was injected with a disposable syringe into the 400-mL

203 treatment chamber containing the treatment medium under constant agitation. The

204 initial bacterial concentration was approximately 3 x 10^7 CFU/mL, in order to match

205 previously published data (Espina et al., 2010) and to allow for the detection of 5 $\log_{10}$

206 cycles of inactivation. The treatment media included a sterile McIlvaine buffer of pH

207 4.0 and commercial apple juice (Don Simón, Murcia, Spain) of pH 3.7, as well as these

208 media with propolis added (0.1 and 0.2 mg/mL). Samples were taken, and the survivors

209 were enumerated.

210

211 *2.6. Counts of viable cells*

212 After treatment, the samples were adequately diluted in 0.1% w/v peptone water

213 (Oxoid, Hampshire, England). Next, 0.1-mL samples were pour-plated onto TSAYE,

214 which was used as a recovery medium. The plates were incubated for 24 h at 37 °C.

215 After incubation, the CFUs were counted with an improved image analyzer automatic

216   counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom), as

217   previously described (Condón et al., 1996). Inactivation was expressed in terms of the

218   extent of reduction in $\log_{10}$ counts (CFU) after any treatment.

219

220   *2.7. Determination of sublethally injured cells*

221   After treatment, the samples were also plated onto TSAYE with 4% sodium

222   chloride (Fisher Scientific, Loughborough, United Kingdom) added (TSAYE-SC), as

223   well as onto TSAYE with 0.25% bile salts (Oxoid, Hampshire, England) added

224   (TSAYE-BS), to evaluate the cytoplasmic membrane damage and outer membrane

225   damage, respectively (Mackey, 2000). These concentrations of sodium chloride and bile

226   salts were previously determined as the maximum non-inhibitory concentrations (data

227   not shown) for untreated cells. The samples recovered in the selective media were

228   incubated for 48 h at 37 °C. After incubation, the CFUs were also counted. The extent

229   of sub-lethal injury was expressed as the difference between the $\log_{10}$ counts on a non-

230   selective medium (TSAYE) and the $\log_{10}$ counts on selective media (TSAYE-SC and

231   TSAYE-BS).

232

233   *2.8. Resistance parameters to heat and combined processes*

234   Survival curves were obtained by plotting the $\log_{10}$ fractions of survivors versus

235   the treatment times at constant temperature and propolis concentrations. As linear,

236   concave upward and concave downward survival curves were observed, a mathematical

237   model based on a Weibull-like distribution was used. The model is described by the

238   Mafart equation (Mafart, Couvert, Gaillard, & Leguerinel, 2002):

239

240    $$\log_{10} S(t) = -(t / \delta)^{\rho} \tag{1}$$

241

242   where $S(t)$ is the survival fraction, t is the holding time (min), δ is the scale parameter

243   (min), and ρ is the shape parameter (dimensionless). The δ value represents the

244   treatment time needed to reduce the first 1 $\log_{10}$ cycle of the population. Depending on

245   the survival curve, the ρ value will be: $\rho < 1$ (concave upward), $\rho = 1$ (linear), or $\rho > 1$

246   (concave downward). The GraphPad Prism® software (GraphPad Software, Inc., San

247   Diego, CA, USA) was used to fit the model to the experimental data and to calculate the

248   δ and ρ parameters.

249   Thermal death time (TDT) curves were obtained by plotting the $\log_{10}$ of the

250   times to inactivate 5 $\log_{10}$ cell cycles versus their corresponding heating temperature.

251   The R^2 coefficients and statistical significant differences (*t*-test and ANOVA) ($p = 0.05$)

252   were calculated with GraphPad Prism® software.

253

254   *2.9. Sensory test*

255   A sensory test was performed by a panel consisting of 77 untrained judges who

256   tasted 2 sets of 4 samples each. Each set of 4 samples consisted of commercial apple

257   juice (Don Simón, Murcia, Spain) with increasing concentrations of propolis added. For

258   each sample, 20 mL of juice was offered in a cup. The samples were presented in

259   counter-balanced order, and yogurt was offered as a palate cleanser. For the first set, the

260   panelists were asked to determine the hedonic acceptance of the 4 coded samples by

261   ranking them on a 1–9 scale (from "dislike extremely" to "like extremely"); they were

262   also asked their purchase intention (yes/no). For the second set, the panelists were

263   shown the added concentrations of propolis in each sample; then, a short summary on

264   the published health benefits of propolis and their purchase intentions (yes/no) were

obtained. The results were analyzed statistically with IBP SPSS Statistics 19 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Bioactive compounds of propolis

Propolis usually contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, and amino acids (Kumazawa et al., 2004). Among them, the bioactive properties of propolis have mainly been associated with its content in polyphenols (Burdock, 1998; Kumazawa et al., 2004). For this reason, total polyphenol content (TPC) and its main group flavonoids (flavonoid content, FC) were measured in our propolis-based dietary supplement (PDS) before testing its antioxidant and antibacterial activities. As in other studies, the quantification of these compounds into groups with the same or similar chemical structure was preferred to the quantification of individual components because the former correlates better with biological activity (Jug et al., 2014; Popova et al., 2004).

The chemical composition of propolis depends on the phytogeographic characteristics of the site of collection, but is also influenced by the use of different extraction methods and solvents (Burdock, 1998; Cunha et al., 2004; Kumazawa et al., 2004). Furthermore, due to the lack of a standardized extraction process, TPC and FC can be expressed in different terms, like as a fraction of the dry or concentrated propolis in the solution (Cunha et al., 2004; Moreira et al., 2008). In our PDS, we determined a TPC of 82.15 ± 3.53 mg/g CAEs and a FC of 0.096 ± 0.003 mg/g CEs. This TPC is within the medium-high range, similar to that described for propolis from Greece, Cyprus, and Spain (Kalogeropoulos et al., 2009; Kumazawa et al., 2013), while the FC

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290 is low, like that observed in other Mediterranean propolis from Malta, whose bioactive

291 properties were ascribed to its high concentration of diterpenes (Popova et al., 2011).

292 Further investigations on the vegetation in the collection site and on the chemical profile

293 should be performed in order to fully define the chemotype of our propolis.

294

295 *3.2. Antioxidant activity of propolis*

296   As a measure of the antioxidant activity, DPPH free radical–scavenging activity

297 was determined in our PDS, obtaining an IC_{50} value of 0.055 ± 0.003 mg/mL, which

298 was within the common range of other analyzed propolis samples (0.030–0.115 mg/mL)

299 (Jug et al., 2014; Moreira et al., 2008). This result indicated a similar DPPH free

300 radical–scavenging activity to that of other extensively studied natural antioxidants,

301 such as origanum and thyme essential oils (Prakash et al., 2015), and resveratrol

302 derivatives (He and Yan, 2013).

303   The naturally high antioxidant potential of propolis has been ascribed to the

304 capacity of polyphenols to reduce the oxidative damage caused by free radicals in

305 cellular biomolecules (Burdock, 1998). Like the antimicrobial properties of propolis, its

306 antioxidant activity can also be promoted as being of pharmaceutical interest and as a

307 potential application in food preservation to help prevent the undesirable effect of

308 oxidation reactions in foods.

309

310 *3.3. Evaluation of the antimicrobial properties of propolis*

311   The antimicrobial activities of propolis were evaluated by determining the MIC

312 and MBC, as well as by testing microbial inactivation as a function of the treatment

313 medium pH. As shown in Table 1, *L. monocytogenes* EGD-e was more sensitive to

314 propolis than *E. coli* O157:H7 Sakai, which confirms previous studies pointing to

315   Gram-positive bacteria as the most susceptible to propolis (Burdock, 1998). Propolis

316   showed bacteriostatic and bactericidal activity against *L. monocytogenes* EGD-e but

317   only bacteriostatic activity against *E. coli* O157:H7 Sakai under the concentrations

318   tested (up to 2 mg/mL of propolis). The comparison of these results with those of other

319   natural antimicrobials tested under the same experimental conditions shows that

320   propolis was more effective than citrus, juniper, cypriol, eucalyptus, and rosemary EOs,

321   but less effective than pennyroyal mint and thyme EOs, as well as individual EO

322   constituents such as thymol, carvacrol, borneol, and linalool (Ait-Ouazzou et al., 2011;

323   Ait-Ouazzou et al., 2012; Espina et al., 2011). The strong antimicrobial and antioxidant

324   activities in our propolis could be partly due to its non-flavonoid TPC, but the

325   antimicrobial activity of the terpenoids, as well as the possible synergistic interactions

326   among its major and minor components, are not discarded.

327   The pH of the treatment medium is one of the major environmental factors

328   affecting microbial resistance to physical or chemical inactivating agents (Burt, 2004;

329   Hansen and Rieman, 1963). However, to the best of our knowledge, the influence of

330   acid pH on the bactericidal effect of propolis has not been previously tested. The action

331   of propolis (0.2 mg/mL) on the survival of both microorganisms was tested in buffer at

332   pH 7.0 and 4.0 for 1, 6, and 24 h at 20 ºC (Table 2). The inactivation at pH 7.0 was

333   coincident with the bactericidal activity that was previously pointed out in Table 1:

334   While more than 5 $\log_{10}$ cycles of *L. monocytogenes* EGD-e cells were inactivated after

335   24 h, less than 1 $\log_{10}$ cycles of *E. coli* O157:H7 Sakai cells were affected. The

336   reduction of the treatment medium pH to 4.0 significantly increased the sensitivity of *L.*

337   *monocytogenes* EGD-e to propolis, achieving more than 5 $\log_{10}$ cycles of inactivation in

338   1 h; however, it hardly affected the resistance of *E. coli* O157:H7 Sakai, with less than 2

339     $\log_{10}$ cycles inactivated in 24 h.

340   Evaluating the survivors using selective recovery media showed that propolis

341   caused sub-lethal injuries on the cytoplasmic membranes of most *L. monocytogenes*

342   EGD-e after 1 h of incubation at pH 7.0 (Table 2): While 1.3 $\log_{10}$ cycles of cells were

343   inactivated, 3.7 extra $\log_{10}$ cell cycles were sub-lethally injured. Injured cells were

344   finally inactivated by propolis after 24 h of incubation at 20 ºC. In contrast, none of the

345   *E. coli* O157:H7 Sakai cells were sub-lethally injured at the cytoplasmic or outer

346   membranes after 24 h of incubation. The higher resistance among Gram-negative

347   bacteria to certain antimicrobial compounds has been attributed to the presence of an

348   outer membrane, which acts as a barrier to lipophilic compounds (Burt, 2004). This

349   outer membrane avoids the action of propolis against the sensitive cytoplasmic

350   membrane. To the best of our knowledge, no attempts to evaluate sub-lethal injury in

351   cell membranes of propolis-treated cells using the selective medium plating technique

352   have been carried out before.

353   As shown, propolis might perform as an effective antimicrobial against *L.*

354   *monocytogenes* EGD-e at very low doses (0.2 mg/mL) and at both treatment medium's

355   pH; however, *E. coli* O157:H7 Sakai was hardly affected under the same treatment

356   conditions. Since higher doses of propolis might not be suitable for application by the

357   food industry, from a sensorial and economical point of view, our goal was to

358   investigate the application of low doses of propolis in combination with mild heat, in

359   order to design a new combined process to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7

360   Sakai. In previous studies, combinations of mild heat and low doses (0.2 µL/mL) of

361   EOs and EO constituents were investigated, and synergistic effects against *E. coli*

362   O157:H7 were described in both laboratory media and fruit juices (Ait-Ouazzou et al.,

363   2012; Ait-Ouazzou et al., 2013; Espina et al., 2014; Espina et al., 2012). For instance,

364   the combination of mild heat and carvacrol, which is also a constituent of some propolis

365 extracts (Segueni et al., 2010), was effective against *E. coli* O157:H7 suspended in

366 mango, orange, apple, and tomato juices (Ait-Ouazzou et al., 2013). This combined

367 process allowed for a significant reduction in the heat treatment intensity, which avoids

368 undesirable effects on food quality. Therefore, the first step was to demonstrate the

369 synergism between heat and propolis in laboratory media, and later its persistence in

370 apple juice as a food matrix.

371

372 *3.4. Study of the synergistic effect of heat and propolis in laboratory media*

373 To evaluate the synergistic lethal effect of heat and propolis on *E. coli* O157:H7

374 Sakai, the action of each hurdle acting alone was firstly investigated. The experiments

375 were performed using a McIlvaine buffer of pH 4.0, close to that of fruit juices, in order

376 to more deeply investigate the mechanisms and kinetics of inactivation.

377 Fig. 1 shows the survival curves for a heat treatment of 51 ºC by recovering the

378 survivors on the non-selective TSAYE medium and the selective TSAYE-SC and

379 TSAYE-BS media. As linear and concave downward survival curves were observed, a

380 mathematical model based on the Weibull distribution (Mafart et al., 2002) was used to

381 fit the curves obtained in TSAYE ($R^2 \geq 0.92$) and to calculate the time needed to

382 inactivate up to 5 $\log_{10}$ cell cycles (Table 3). The thermal treatment at 51 ºC inactivated

383 5 $\log_{10}$ cycles of microorganisms in approximately 44 min. Nevertheless, the

384 inactivation kinetics in the non-selective medium were not linear because a 20 min

385 "shoulder" was observed, adjusting the ρ value to 2.34. On the other hand, at 20 ºC, the

386 population of *E. coli* O157:H7 suspended in a buffer of pH 4.0 with propolis (0.2

387 mg/mL) added hardly decreased 0.3 $\log_{10}$ cycles after 45 min (data not shown). A dose

388 of 0.2 mg/mL was chosen for comparing the efficacy of this antimicrobial with others,

389 such as EOs and their constituents, which were previously tested at the same

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concentrations, alone or in combination with heat treatments, by our research group (Ait-Ouazzou et al., 2012; Ait-Ouazzou et al., 2013; Espina et al., 2010; Espina et al., 2014; Espina et al., 2012).

Alternatively to the 44 min required for the thermal treatment, the combined treatment at 51 ºC with 0.2 mg/mL of propolis added inactivated 5 $\log_{10}$ cycles of the initial population in approximately 1 min (Table 3; Fig. 1). These results demonstrated that the addition of propolis to the treatment medium before heating achieved more than 4 extra $\log_{10}$ cycles of inactivation after only 1 min of treatment, which means that propolis and heat acted synergistically, reducing the time needed to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 by more than 40 times, in comparison with thermal treatment at the same temperature. From a kinetic of inactivation point of view, and in contrast to the curves observed for thermal treatments, the survival curves of *E. coli* O157:H7 after the combined processes with propolis did not show any shoulder.

The synergism observed when combining mild heat and EOs, or their constituents, is related to the inactivation of heat-injured cells, especially of those with damaged outer membranes. Heat damaged the outer membrane, facilitating the access and/or action of the lipophilic compounds. However, in the presence of propolis, this hypothesis does not seem to be suitable, since the degree of inactivation reached by the combined treatment was much greater than that predicted by the survival curves obtained in TSAYE-BS (Fig. 1). It seems more likely that heat might facilitate the diffusion of propolis constituents into the lipid phase of the membrane, allowing them to penetrate the cell and act in the cytoplasm. This was the greatest synergistic effect observed by our research group when combining mild heat and natural antimicrobials under the same experimental conditions (Ouazzou et al., 2012; Ait-Ouazzou et al., 2013; Espina et al., 2010; Espina et al., 2014; Espina et al., 2012). Thus, the combination of

415 mild heat and propolis might be proposed for alternative food preservation treatments or

416 even as a cleaning and disinfection method.

417

418 *3.5. Study of the synergistic effect of heat and propolis in commercial apple juice*

419 Microbial heat resistance usually is higher in food than in buffers of the same

420 pH (Manas and Pagán, 2005). Food components such as salts, sugars, proteins, and fats

421 might help to protect cells against heat damage. Synergism might also be influenced by

422 the interaction of food constituents with barriers or microorganisms.

423 Our results demonstrated that the time needed to inactivate 5 $\log_{10}$ cycles of *E.*

424 *coli* O157:H7 by heat at 51 ºC, when suspended in apple juice, increased from 44 (in

425 buffer of pH 4.0) to 61 min (in apple juice) (Fig. 2; Table 3). The kinetics of the

426 inactivation also showed a pronounced shoulder in apple juice; similarly, the survival

427 curves obtained in TSAYE media required 30% extra time to reach the 5 $\log_{10}$ cycles of

428 inactivation. The synergism was also reduced, since the time required to achieve the 5

429 $\log_{10}$ cycles of inactivation increased from 1 (in buffer of pH 4.0) to 9.8 min (in apple

430 juice) (Table 3). Despite the partial loss of synergism between heat and propolis, the

431 time to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 using the combined treatment was

432 6.25 times shorter than that required when applying heat alone. Thus, the synergism's

433 effectiveness between heat and propolis extract was similar to that observed when

434 applying mild heat and citral to apple juice (Espina et al., 2010) or mild heat and lemon

435 EO or limonene to orange juice (Espina et al., 2014).

436

437 *3.6. Study of the hedonic acceptability of commercial apple juice in the presence of*

438 *propolis*

18

439   Because sensory evaluation is the key to ensuring compliance with the quality

440   and marketability requirements of food products, this study aimed to determine an

441   acceptable threshold concentration of propolis extract in apple juice through a sensory

442   test. This experiment was carried out before exploring the influence of the treatment

443   temperature in the efficacy of the combined treatment, in order to evaluate this relevant

444   aspect with a tolerable propolis dose.

445   Fig. 3 shows the box-and-whiskers plots corresponding to the hedonic data

446   collected from the sensory tests when the panelists were not revealed the concentrations

447   of propolis in each sample. Since not all of the hedonic data could be fitted to a normal

448   distribution, Kruskal–Wallis tests were performed to compare the results from the

449   sensory tests. No statistically significant differences were found between the control and

450   the samples with 0.05 and 0.1 mg/mL of propolis added. On the contrary, the sample

451   with 0.2 mg/mL of propolis added was significantly less appreciated than the rest of the

452   samples ($p < 0.05$). As a conclusion, the apple juice treated with heat and up to 0.1

453   mg/mL of propolis would be, in terms of hedonic evaluation, as acceptable as a sample

454   with no propolis extract added ($p > 0.05$). Moreover, the purchase intention of panelists

455   did not decrease after knowing the content of propolis in each sample ($p > 0.05$); on the

456   contrary, the buying intention for apple juice with 0.05 mg/mL of propolis increased by

457   22%. This could indicate that the health and pharmaceutical benefits of propolis could

458   account for the commercialization of propolis-enriched apple juice as a functional food

459   with good sensory properties, meeting consumers' demands for healthy, nutritious, and

460   tasty food.

461

462   *3.7. Study of the influence of treatment temperature on the synergistic effect*

463   Once the synergism of the combined process had been characterized at 51 ℃

464   and the maximum acceptable hedonic concentration was chosen, the final step was to

465   elucidate whether the effectiveness of the synergistic effect would be maintained when

466   reducing the concentration of propolis extract and approaching pasteurization

467   temperatures. For this purpose, thermal death time (TDT) curves were obtained in apple

468   juice that was heat treated at 51–63 ℃ and with 0.2 mg/mL (reference concentration)

469   and 0.1 mg/mL (maximum acceptable hedonic concentration) of propolis extract added

470   (Fig. 4).

471   Fig. 4 shows the TDT curves obtained from plotting the $\log_{10}$ values of the times

472   to inactivate 5 $\log_{10}$ cycles for each temperature. This inactivation level matches FDA's

473   recommendation for the hygienization of acidic fruit juices (FDA, 2001). The TDT

474   curves were described by the following equations:

475 $$\log_{10} t = -0.1493 \cdot T + 9.42 \qquad (R^2 = 0.98) \qquad (2)$$

476 $$\log_{10} t = -0.1433 \cdot T + 8.63 \qquad (R^2 = 0.95) \qquad (3)$$

477 $$\log_{10} t = -0.1905 \cdot T + 10.67 \qquad (R^2 = 0.94), \qquad (4)$$

478

479   where t is the time to inactivate 5 $\log_{10}$ of the initial population of *E. coli* O157:H7 and

480   T is the temperature of the control thermal treatment (Eq. 2) and the thermal treatments

481   in the presence of 0.1 mg/mL (Eq. 3) or 0.2 mg/mL propolis (Eq. 4).

482   As shown in Fig. 4, the synergism observed at 51° C in the presence of the

483   reference concentration (0.2 mg/mL) of propolis was maintained or even slightly

484   increased when increasing the thermal treatment up to 57 °C ($p \leq 0.05$). The reduction

485   of propolis concentration from 0.2 to 0.1 mg/mL (maximum acceptable hedonic

486   concentration) caused a significant reduction in the effectiveness of the combined

487   process. The synergistic effect observed at 51 °C in the presence of 0.1 mg/mL of

488　propolis caused a reduction of 75% in the treatment time, compared to the thermal

489　treatment acting alone. The synergism was constant in the presence of 0.1 mg/mL of

490　propolis, when increasing the thermal treatment up to 60 °C, because no statistically

491　significant differences were observed between the slopes of the TDT curves defined by

492　Eq. 3 ($z = 7.0 \pm 0.7$ °C) and Eq. 2 ($z = 6.7 \pm 0.2$ °C) ($p > 0.05$). From these equations, it

493　can be observed that, at 60 °C, the combined process also caused a reduction in the

494　treatment time by 4 times, as observed at 51 °C. As a consequence, the application of

495　these combined processes at higher temperatures, such as those used during the current

496　LTLT (low temperatures–long time) (60 – 65 °C) pasteurization processes, would likely

497　result in a similar synergistic effect. The same conclusion was drawn from the

498　investigation of the effect of temperature on the synergistic inactivation of *E. coli*

499　O157:H7 by heat and lemon EO in apple juice (Espina et al., 2012), as well as on

500　orange EO and limonene in orange juice (Espina et al., 2014). As in those natural

501　products, the antimicrobial constituents of propolis seem to be resistant to heat

502　denaturation under the treatment conditions assayed.

503　　　　Fig. 4 suggests two possibilities for reducing the intensity of thermal treatments.

504　As the treatment time required to inactivate 5 $\log_{10}$ cycles was reduced by 4 times in the

505　presence of propolis, the amount of processed apple juice could be increased by more

506　than four time with the same equipment, with regards to heat treatments without

507　propolis. On the other hand, the same microbial inactivation levels achieved after 2.8

508　min of treatment at 60 °C with no antimicrobials (Eq. 2) were reached in combination

509　with 0.1 mg/mL of propolis at 57 °C (Eq. 3)—3 °C lower. Thus, this decrease in the

510　treatment temperature to achieve the same inactivation levels is expected to have a

511　positive impact on the nutritional and organoleptic properties of apple juice (Vikram et

512　al., 2005), as well as provide advantages for the food industry, such as energy cost

513   reductions in achieving the desired safety level. Other demonstrated properties of

514   propolis, such as its antifungal activity in juices (Koc et al., 2007), could also become

515   additional advantages to be considered. These results show the potential of propolis in

516   combination with heat to improve preservation of apple juice. Further research on the

517   influence of environmental factors, such as pH, food matrix or concentration of propolis

518   on these synergistic effects, is needed in order to develop secondary and tertiary models

519   to adequately predict microbial inactivation and to optimize combined processes of heat

520   treatments in presence of propolis.

521

522   **4. Conclusions**

523   This study has characterized the bioactive compounds and analyzed the

524   antioxidant activity of a propolis-based dietary supplement (PDS) from Spain. The total

525   phenolic content of the PDS was 82.15 ± 3.53 mg CAEs/g, with an antioxidant activity

526   (IC_{50}) of 0.055 ± 0.003 mg/mL. Regarding its antimicrobial activity, this study has

527   revealed the greater inactivation and occurrence of sub-lethal injury by propolis

528   treatments at acidic pH and on a Gram-positive bacterium than at neutral pH and on a

529   Gram-negative bacterium. From these results, propolis might perform as an effective

530   antimicrobial against *L. monocytogenes* EGD-e at very low doses (0.2 mg/mL),

531   although it hardly affected *E. coli* O157:H7 Sakai.

532   Strong synergistic, lethal effects against *E. coli* O157:H7 Sakai were shown

533   using mild heat and propolis, since the addition of 0.2 mg/mL of propolis to a pH 4.0

534   buffer reduced the heating time needed to inactivate 5 $\log_{10}$ cell cycles by more than 40

535   times. In apple juice, the controlled incorporation of 0.1 mg/mL of propolis reduced the

536   thermal treatment required to reach the goal inactivation level by at least 4 times or 3

537   ºC. As this propolis concentration was sensorially acceptable, a less intense

pasteurization process would be expected to improve the organoleptic and nutritional properties of apple juice, besides increasing its industrial performance. Furthermore, given the phenolic content and high antioxidant properties of propolis (besides other possible health benefits), the present study contemplates its incorporation into apple juice, not only to improve the preservation methodology, but also as a means of creating a new "green-labeled" functional food.

Acknowledgements

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Figure Legends

Fig. 1. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU/mL) to a heat treatment at 51 °C in citrate-phosphate buffer of pH 4.0, and recovered in TSAYE (○), TSAYE-SC (□), TSAYE-BS (◊), or recovered in TSAYE after a combined treatment of heat and propolis (0.2 mg/mL) (●). Data represent the means ± standard error of the mean (error bars) of at least three independent experiments.

Fig. 2. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU/mL) to a heat treatment at 51 °C in apple juice, and recovered in TSAYE (○), TSAYE-SC (□), TSAYE-BS (◊), or recovered in TSAYE after a combined treatment of heat and of propolis (0.2 mg/mL) (●). Data represent the means ± standard error of the mean (error bars) of at least three independent experiments.

Fig. 3. Box-and-whisker plots showing the hedonic data values for apple juices with increasing concentrations of propolis (0, 0.05, 0.1 and 0.2 mg/mL). In each plot, horizontal lines correspond to the minimum value, the percentiles 25, 50 and 75 (ranging from 1 to 9 in the scale), and the maximum value. The asterisk represents statistically significant differences with the 0 % juice.

Fig. 4. Log_{10} times (min) for inactivation of 5 log_{10} cycles of *Escherichia coli* O157:H7 (initial concentration, 3×10^7 CFU/mL) at different treatment temperatures in

710   apple juice, with no propolis added (●), with 0.1 mg/mL (○) or 0.2 mg/mL (□) of
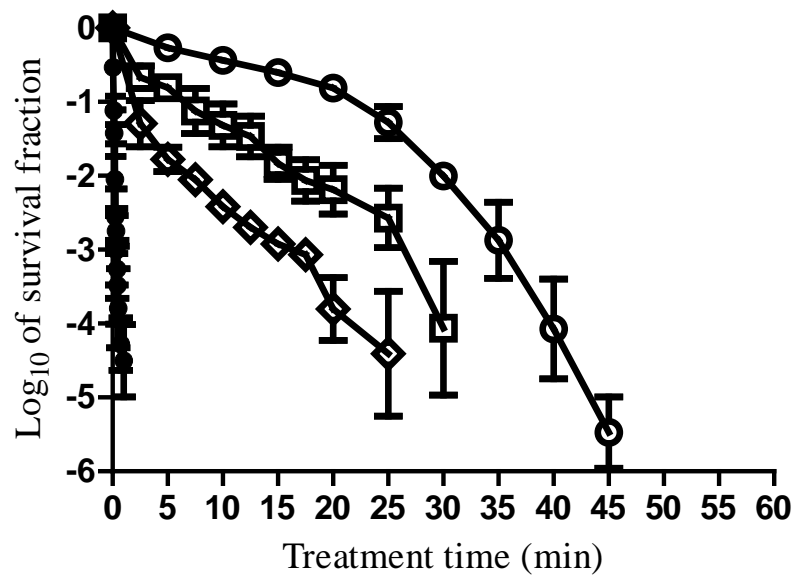
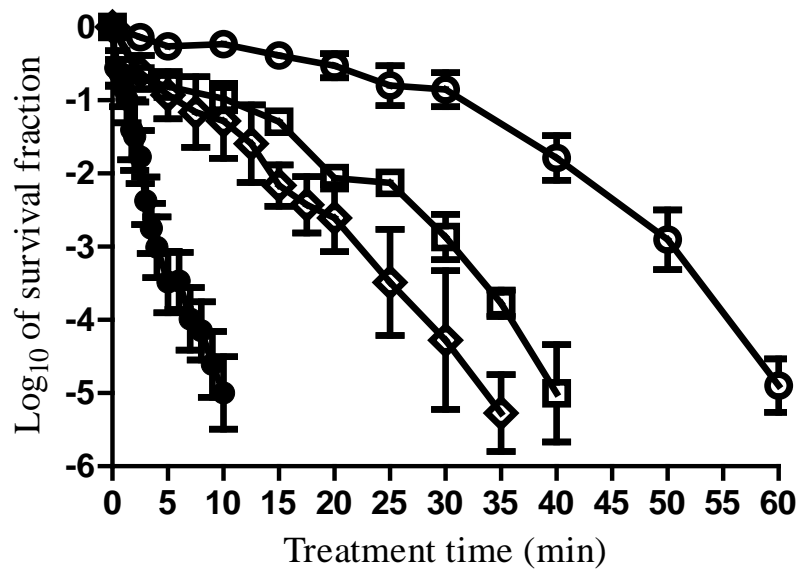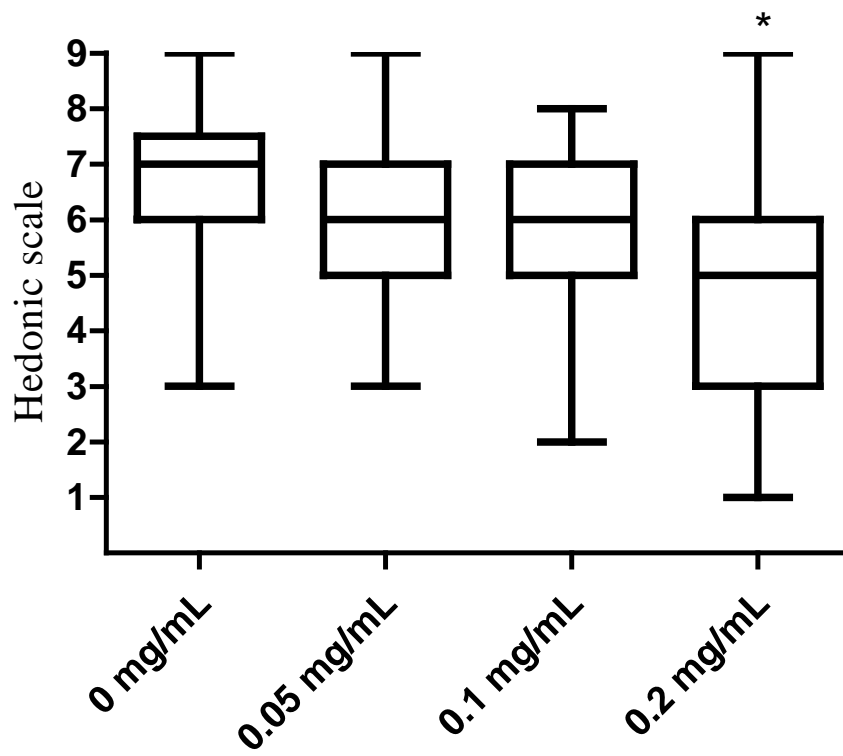711   propolis added. Cells were recovered in TSAYE.

Fig.1.
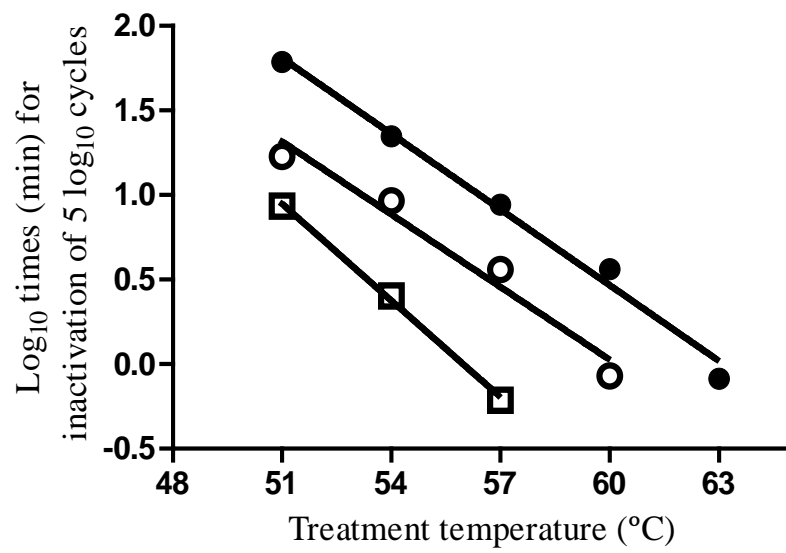
Fig.2.

Fig.3.

Fig.4.

1   **Table 1.** Minimum inhibitory concentration (MIC) and minimum

2   bactericidal concentration (MBC) of propolis (mg/mL).

Strains Tested	MIC	MBC
Listeria monocytogenes EGD-e	0.05	0.11
Escherichia coli O157:H7 Sakai	0.2	>0.2

3

4

Table 2. Log_{10} cycles of inactivation (mean ± standard deviation) of *Escherichia coli* O157:H7 Sakai and *Listeria monocytogenes* EGD-e

after a treatment with propolis (0.2 mg/mL) at 20 ℃. Cells were treated in citrate-phosphate buffer of pH 4.0 or pH 7.0 and recovered in TSAYE,

TSAYE-SC and TSAYE-BS.

Treatment Medium	Recovery Medium	Strains tested					
		E. coli O157:H7 Sakai			*L. monocytogenes* EGD-e		
		1 h	6 h	24h	1 h	6 h	24 h
pH 4	TSAYE	0.29 ± 0.27	0.91 ± 0.72	1.58 ± 0.27	> 5.0	> 5.0	> 5.0
	TSAYE-SC	0.39 ± 0.30	0.99 ± 0.64	2.01 ± 0.88	> 5.0	> 5.0	> 5.0
	TSAYE-BS	0.54 ± 0.45	1.13 ±0.62	1.71 ± 0.94			
pH 7	TSAYE	0.08 ± 0.11	0.16 ± 0.12	0.53 ± 0.16	0.38 ± 0.13	1.27 ± 0.31	> 5.0
	TSAYE-SC	0.32 ± 0.17	0.33 ± 0.18	0.89 ± 0.13	0.71 ± 0.27	5.07 ± 1.33	> 5.0
	TSAYE-BS	0.30 ± 0.20	0.57 ± 0.30	0.86 ± 0.07			

8
9

10 **Table 3.** Regression parameters (δ, time to inactivate 5 $\log_{10}$ cell cycles, and ρ) and the

11 goodness of fit (R^2 and Root Mean Square Error (RMSE)) estimated from the fit of

12 equation 1 to experimental data of *Escherichia coli* O157:H7 Sakai (initial

13 concentration: 3 x 10^7 CFU/mL) heat-treated at 51 ºC and recovered in TSAYE. CI:

14 Confidence Interval.

Treatment Medium	δ (min) (95% CI)	Time for 5-$\log_{10}$ red. (min) (95% CI)	ρ (95% CI)	R^2	RMSE
McIlvaine buffer pH 4	21.96 (19.96-23.96)	43.70 (42.09-45.31)	2.34 (1.98-2.70)	0.93	0.85
McIlvaine buffer pH 4 + propolis (0.2 mg/mL)	0.052 (0.026-0.078)	0.97 (0.77-1.17)	0.55 (0.43-0.67)	0.84	1.08
Apple juice	30.86 (27.85-33.88)	61.22(58.88-63.55)	2.35 (1.96-2.75)	0.92	0.85
Apple juice + propolis (0.2 mg/mL)	1.02 (0.75-1.23)	9.80 (8.80-10.81)	0.71 (0.61-0.81)	0.87	0.99

15