TITLE:

Bioactive properties of a propolis-based dietary supplement and its use in combination with mild heat for apple juice preservation

Author names: Adrián Luis-Villaroya, Laura Espina, Diego García-Gonzalo, Susana Bayarri, Consuelo Pérez and Rafael Pagán*.

Affiliation: Departamento de Producción Animal y Ciencia de los Alimentos. Facultad de Veterinaria. Universidad de Zaragoza.

C/ Miguel Servet, 177, 50013, Zaragoza, Spain.

Corresponding author: Dr. Rafael Pagán Tomás

Address: Dpto. PACA. Facultad de Veterinaria. Universidad de Zaragoza.

C/ Miguel Servet, 177, 50013, Zaragoza, Spain.

Phone number: 34-976-761581

Fax. number: 34-976-761590

E-mail: pagan@unizar.es

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

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

92 The sample used in this investigation was a dietary food supplement containing
93 raw propolis provided by Miel El Albar (Lechón, Zaragoza, Spain). According to the
94 producer, the main plant species that contributed to the propolis were poplar (*Populus*
95 spp.), oak (*Quercus* spp.), and pine (*Pinus* spp.). The raw propolis was collected and
96 macerated for 48 h under agitation at 36 ºC in propylene glycol. Wax and debris were
97 removed by double filtration, obtaining a tincture (the propolis-based dietary
98 supplement, PDS). The final concentration of propolis in PDS was 0.2 mg/µL.
99
100 *2.2. Analysis of bioactive compounds and determination of antioxidant activity of the*
101 *propolis-based dietary supplement*
102 The total polyphenol content (TPC), flavonoid content (FC), and 2, 2-diphenyl-
103 1-picryl-hydrazyl-hydrate (DPPH) free radical-scavenging activity were determined by
104 analyzing 6 replicates of the same sample. Spectrophotometric lectures were carried out
105 in a Helios Gamma Thermo Electron Corporation Spectrophotometer (United
106 Kingdom).
107 The TPC was determined by the Folin–Ciocalteu method (Millena Popova,
108 Silici, Kaftanoglu, & Bankova, 2005). One mL of a test solution of PDS was transferred
109 to a 50-mL volumetric flask containing 15 mL distilled water and 4 mL of the Folin–
110 Ciocalteu reagent (Panreac, Spain); 6 mL of a 20% sodium carbonate solution (w/v)
111 (sodium carbonate anhydrous, Panreac, Spain) were then added. The rest of the volume
112 was made up with distilled water to 50 mL. After 2 h, the absorbance was measured at
113 760 nm. A blank solution was included in each assay, with 1 mL of methanol (Labscan,
114 Poland) instead of the test solution. A calibration curve of standard caffeic acid (Sigma-
115 Aldrich, USA) was employed (10–50 mg/mL; $y = 0.0055x - 0.0283$; $R^2 = 0.9984$). The
116 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.

142 *2.3. Micro-organisms and growth conditions*

143 *E. coli* O157:H7 Sakai *stx* $1A^-$/*stx* $2A^-$ was kindly provided by Kyu-Tae Chang
144 (The National Primate Research Center, KRIBB, Ochang, South Korea). This strain was
145 isolated from an outbreak involving white radish sprout (Michino et al., 1999). *L.*
146 *monocytogenes* EGD-e was kindly provided by Prof. Chakraborty (Institute for Medical
147 Microbiology, Giessen, Germany). During this investigation, the cultures were
148 maintained and kept frozen at −80 °C in cryovials. Broth subcultures were prepared by
149 inoculating one single colony from a plate into a test tube containing 5 mL of sterile
150 tryptic soy broth (Biolife, Milan, Italy) with 0.6% yeast extract added (Biolife)
151 (TSBYE). After inoculation, the tubes were incubated overnight at 37 °C (*E. coli*
152 O157:H7 Sakai) or 30 ºC (*L. monocytogenes* EGD-e). Along with these subcultures,
153 250-mL Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final
154 concentration of 10^4 cells/mL. These flasks were incubated under agitation (130 rpm;
155 Selecta, mod. Rotabit, Barcelona, Spain) at the appropriate temperature until the
156 stationary growth phase was reached (24 ± 2 h). The stationary phase was chosen
157 because microorganisms show higher resistance to heat at this stage than at the
158 exponential phase (Hansen and Rieman, 1963), as well as to match previously published
159 data (Espina et al., 2011; Espina et al., 2010; Espina et al., 2012; Somolinos et al.,
160 2010).

161

162 *2.4. Evaluation of the antimicrobial properties of propolis*

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

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

168 The propolis was screened to determine the minimum inhibitory concentration
169 (MIC) and minimum bactericidal concentration (MBC) against *L. monocytogenes* EGD-
170 e and *E. coli* O157:H7 Sakai. Tubes containing 5 mL of TSBYE and different
171 concentrations of propolis (0.008–2 mg/mL) were inoculated to a final concentration of
172 10^5 cells/mL. A negative control (without microorganisms), positive control (without
173 propolis), and diluent control (the amount of propylenglicol corresponding to the
174 maximum propolis concentration assayed (2 mg/mL)) were also prepared. After 24 h of
175 incubation at the appropriate temperature in a shaking thermostatic bath (Bunsen, mod.
176 BTG, Madrid, Spain), 100 μL of each tube were spread-plated in tryptic soy agar
177 (Biolife) with 0.6% yeast extract added (TSAYE). The plates were incubated at
178 corresponding temperatures for 24 h. The MIC was the lowest concentration of propolis
179 at which bacteria failed to grow, showing counts equals to the initial concentration. The
180 MBC was defined as the lowest concentration of propolis that inactivates 99.9% of an
181 inoculated sample, showing counts below 10^2 colony-forming units (CFU)/mL. The
182 evaluations of MIC and MBC were carried out in triplicate.

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

187 Cells from stationary-phase cultures were added at final concentrations of 3 x
188 10^7 CFU/mL to buffers, both with and without propolis (0.2 mg/mL). The buffer pH
189 was not modified as a consequence of adding antimicrobial compounds. Antimicrobial
190 compound treatments were carried out at 20 ºC for 24 h. Samples were taken at 1, 6, and

24 h, and the survivors and sub-lethally injured cells were enumerated, as described below. Previous experiments showed that untreated cells of *E. coli* O157:H7 Sakai and *L. monocytogenes* EGD-e at concentrations of 10^7 CFU/mL were insensitive to incubation in citrate–phosphate buffers at pH 7.0 or 4.0 for 24 h at 20 ºC (data not shown).

2.5. Measurement of cell inactivation by heat treatments and propolis

Heat and combined treatments were carried out in a specially designed thermo-resistometer, as previously described (Condón et al., 1993). Briefly, this device uses a thermocouple (Pt 100) to monitor the temperature during the heat treatment and for the injection of the inoculum. Once the temperature stabilized, 0.2 mL of an adequately diluted cell suspension was injected with a disposable syringe into the 400-mL treatment chamber containing the treatment medium under constant agitation. The initial bacterial concentration was approximately 3 x 10^7 CFU/mL, in order to match previously published data (Espina et al., 2010) and to allow for the detection of 5 $\log_{10}$ cycles of inactivation. The treatment media included a sterile McIlvaine buffer of pH 4.0 and commercial apple juice (Don Simón, Murcia, Spain) of pH 3.7, as well as these media with propolis added (0.1 and 0.2 mg/mL). Samples were taken, and the survivors were enumerated.

2.6. Counts of viable cells

After treatment, the samples were adequately diluted in 0.1% w/v peptone water (Oxoid, Hampshire, England). Next, 0.1-mL samples were pour-plated onto TSAYE, which was used as a recovery medium. The plates were incubated for 24 h at 37 °C. After incubation, the CFUs were counted with an improved image analyzer automatic

counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom), as previously described (Condón et al., 1996). Inactivation was expressed in terms of the extent of reduction in $\log_{10}$ counts (CFU) after any treatment.

2.7. Determination of sublethally injured cells

After treatment, the samples were also plated onto TSAYE with 4% sodium chloride (Fisher Scientific, Loughborough, United Kingdom) added (TSAYE-SC), as well as onto TSAYE with 0.25% bile salts (Oxoid, Hampshire, England) added (TSAYE-BS), to evaluate the cytoplasmic membrane damage and outer membrane damage, respectively (Mackey, 2000). These concentrations of sodium chloride and bile salts were previously determined as the maximum non-inhibitory concentrations (data not shown) for untreated cells. The samples recovered in the selective media were incubated for 48 h at 37 °C. After incubation, the CFUs were also counted. The extent of sub-lethal injury was expressed as the difference between the $\log_{10}$ counts on a non-selective medium (TSAYE) and the $\log_{10}$ counts on selective media (TSAYE-SC and TSAYE-BS).

2.8. Resistance parameters to heat and combined processes

Survival curves were obtained by plotting the $\log_{10}$ fractions of survivors versus the treatment times at constant temperature and propolis concentrations. As linear, concave upward and concave downward survival curves were observed, a mathematical model based on a Weibull-like distribution was used. The model is described by the Mafart equation (Mafart, Couvert, Gaillard, & Leguerinel, 2002):

$$\mathrm{Log}_{10}\, S(t) = -(t / \delta)^{\rho} \qquad (1)$$

where $S(t)$ is the survival fraction, t is the holding time (min), δ is the scale parameter (min), and ρ is the shape parameter (dimensionless). The δ value represents the treatment time needed to reduce the first 1 $\log_{10}$ cycle of the population. Depending on the survival curve, the ρ value will be: $\rho < 1$ (concave upward), $\rho = 1$ (linear), or $\rho > 1$ (concave downward). The GraphPad Prism® software (GraphPad Software, Inc., San Diego, CA, USA) was used to fit the model to the experimental data and to calculate the δ and ρ parameters.

Thermal death time (TDT) curves were obtained by plotting the $\log_{10}$ of the times to inactivate 5 $\log_{10}$ cell cycles versus their corresponding heating temperature. The R^2 coefficients and statistical significant differences (t-test and ANOVA) ($p = 0.05$) were calculated with GraphPad Prism® software.

2.9. Sensory test

A sensory test was performed by a panel consisting of 77 untrained judges who tasted 2 sets of 4 samples each. Each set of 4 samples consisted of commercial apple juice (Don Simón, Murcia, Spain) with increasing concentrations of propolis added. For each sample, 20 mL of juice was offered in a cup. The samples were presented in counter-balanced order, and yogurt was offered as a palate cleanser. For the first set, the panelists were asked to determine the hedonic acceptance of the 4 coded samples by ranking them on a 1–9 scale (from “dislike extremely” to “like extremely”); they were also asked their purchase intention (yes/no). For the second set, the panelists were shown the added concentrations of propolis in each sample; then, a short summary on 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

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.

Evaluating the survivors using selective recovery media showed that propolis caused sub-lethal injuries on the cytoplasmic membranes of most *L. monocytogenes* EGD-e after 1 h of incubation at pH 7.0 (Table 2): While 1.3 $\log_{10}$ cycles of cells were inactivated, 3.7 extra $\log_{10}$ cell cycles were sub-lethally injured. Injured cells were finally inactivated by propolis after 24 h of incubation at 20 ºC. In contrast, none of the *E. coli* O157:H7 Sakai cells were sub-lethally injured at the cytoplasmic or outer membranes after 24 h of incubation. The higher resistance among Gram-negative bacteria to certain antimicrobial compounds has been attributed to the presence of an outer membrane, which acts as a barrier to lipophilic compounds (Burt, 2004). This outer membrane avoids the action of propolis against the sensitive cytoplasmic membrane. To the best of our knowledge, no attempts to evaluate sub-lethal injury in cell membranes of propolis-treated cells using the selective medium plating technique have been carried out before.

As shown, propolis might perform as an effective antimicrobial against *L. monocytogenes* EGD-e at very low doses (0.2 mg/mL) and at both treatment medium's pH; however, *E. coli* O157:H7 Sakai was hardly affected under the same treatment conditions. Since higher doses of propolis might not be suitable for application by the food industry, from a sensorial and economical point of view, our goal was to investigate the application of low doses of propolis in combination with mild heat, in order to design a new combined process to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 Sakai. In previous studies, combinations of mild heat and low doses (0.2 µL/mL) of EOs and EO constituents were investigated, and synergistic effects against *E. coli* O157:H7 were described in both laboratory media and fruit juices (Ait-Ouazzou et al., 2012; Ait-Ouazzou et al., 2013; Espina et al., 2014; Espina et al., 2012). For instance, the combination of mild heat and carvacrol, which is also a constituent of some propolis

extracts (Segueni et al., 2010), was effective against *E. coli* O157:H7 suspended in mango, orange, apple, and tomato juices (Ait-Ouazzou et al., 2013). This combined process allowed for a significant reduction in the heat treatment intensity, which avoids undesirable effects on food quality. Therefore, the first step was to demonstrate the synergism between heat and propolis in laboratory media, and later its persistence in apple juice as a food matrix.

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

To evaluate the synergistic lethal effect of heat and propolis on *E. coli* O157:H7 Sakai, the action of each hurdle acting alone was firstly investigated. The experiments were performed using a McIlvaine buffer of pH 4.0, close to that of fruit juices, in order to more deeply investigate the mechanisms and kinetics of inactivation.

Fig. 1 shows the survival curves for a heat treatment of 51 ºC by recovering the survivors on the non-selective TSAYE medium and the selective TSAYE-SC and TSAYE-BS media. As linear and concave downward survival curves were observed, a mathematical model based on the Weibull distribution (Mafart et al., 2002) was used to fit the curves obtained in TSAYE ($R^2 \geq 0.92$) and to calculate the time needed to inactivate up to 5 $\log_{10}$ cell cycles (Table 3). The thermal treatment at 51 ºC inactivated 5 $\log_{10}$ cycles of microorganisms in approximately 44 min. Nevertheless, the inactivation kinetics in the non-selective medium were not linear because a 20 min "shoulder" was observed, adjusting the ρ value to 2.34. On the other hand, at 20 ºC, the population of *E. coli* O157:H7 suspended in a buffer of pH 4.0 with propolis (0.2 mg/mL) added hardly decreased 0.3 $\log_{10}$ cycles after 45 min (data not shown). A dose of 0.2 mg/mL was chosen for comparing the efficacy of this antimicrobial with others, such as EOs and their constituents, which were previously tested at the same

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

mild heat and propolis might be proposed for alternative food preservation treatments or even as a cleaning and disinfection method.

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

Microbial heat resistance usually is higher in food than in buffers of the same pH (Manas and Pagán, 2005). Food components such as salts, sugars, proteins, and fats might help to protect cells against heat damage. Synergism might also be influenced by the interaction of food constituents with barriers or microorganisms.

Our results demonstrated that the time needed to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 by heat at 51 ºC, when suspended in apple juice, increased from 44 (in buffer of pH 4.0) to 61 min (in apple juice) (Fig. 2; Table 3). The kinetics of the inactivation also showed a pronounced shoulder in apple juice; similarly, the survival curves obtained in TSAYE media required 30% extra time to reach the 5 $\log_{10}$ cycles of inactivation. The synergism was also reduced, since the time required to achieve the 5 $\log_{10}$ cycles of inactivation increased from 1 (in buffer of pH 4.0) to 9.8 min (in apple juice) (Table 3). Despite the partial loss of synergism between heat and propolis, the time to inactivate 5 $\log_{10}$ cycles of *E. coli* O157:H7 using the combined treatment was 6.25 times shorter than that required when applying heat alone. Thus, the synergism's effectiveness between heat and propolis extract was similar to that observed when applying mild heat and citral to apple juice (Espina et al., 2010) or mild heat and lemon EO or limonene to orange juice (Espina et al., 2014).

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

Because sensory evaluation is the key to ensuring compliance with the quality and marketability requirements of food products, this study aimed to determine an acceptable threshold concentration of propolis extract in apple juice through a sensory test. This experiment was carried out before exploring the influence of the treatment temperature in the efficacy of the combined treatment, in order to evaluate this relevant aspect with a tolerable propolis dose.

Fig. 3 shows the box-and-whiskers plots corresponding to the hedonic data collected from the sensory tests when the panelists were not revealed the concentrations of propolis in each sample. Since not all of the hedonic data could be fitted to a normal distribution, Kruskal–Wallis tests were performed to compare the results from the sensory tests. No statistically significant differences were found between the control and the samples with 0.05 and 0.1 mg/mL of propolis added. On the contrary, the sample with 0.2 mg/mL of propolis added was significantly less appreciated than the rest of the samples ($p < 0.05$). As a conclusion, the apple juice treated with heat and up to 0.1 mg/mL of propolis would be, in terms of hedonic evaluation, as acceptable as a sample with no propolis extract added ($p > 0.05$). Moreover, the purchase intention of panelists did not decrease after knowing the content of propolis in each sample ($p > 0.05$); on the contrary, the buying intention for apple juice with 0.05 mg/mL of propolis increased by 22%. This could indicate that the health and pharmaceutical benefits of propolis could account for the commercialization of propolis-enriched apple juice as a functional food with good sensory properties, meeting consumers' demands for healthy, nutritious, and tasty food.

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

Once the synergism of the combined process had been characterized at 51 ºC and the maximum acceptable hedonic concentration was chosen, the final step was to elucidate whether the effectiveness of the synergistic effect would be maintained when reducing the concentration of propolis extract and approaching pasteurization temperatures. For this purpose, thermal death time (TDT) curves were obtained in apple juice that was heat treated at 51–63 ºC and with 0.2 mg/mL (reference concentration) and 0.1 mg/mL (maximum acceptable hedonic concentration) of propolis extract added (Fig. 4).

Fig. 4 shows the TDT curves obtained from plotting the $\log_{10}$ values of the times to inactivate 5 $\log_{10}$ cycles for each temperature. This inactivation level matches FDA's recommendation for the hygienization of acidic fruit juices (FDA, 2001). The TDT curves were described by the following equations:

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

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

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

where t is the time to inactivate 5 $\log_{10}$ of the initial population of *E. coli* O157:H7 and T is the temperature of the control thermal treatment (Eq. 2) and the thermal treatments in the presence of 0.1 mg/mL (Eq. 3) or 0.2 mg/mL propolis (Eq. 4).

As shown in Fig. 4, the synergism observed at 51° C in the presence of the reference concentration (0.2 mg/mL) of propolis was maintained or even slightly increased when increasing the thermal treatment up to 57 °C ($p \leq 0.05$). The reduction of propolis concentration from 0.2 to 0.1 mg/mL (maximum acceptable hedonic concentration) caused a significant reduction in the effectiveness of the combined process. The synergistic effect observed at 51 °C in the presence of 0.1 mg/mL of

propolis caused a reduction of 75% in the treatment time, compared to the thermal treatment acting alone. The synergism was constant in the presence of 0.1 mg/mL of propolis, when increasing the thermal treatment up to 60 °C, because no statistically significant differences were observed between the slopes of the TDT curves defined by Eq. 3 (z = 7.0± 0.7 ºC) and Eq. 2 (z = 6.7 ± 0.2 ºC) (p > 0.05). From these equations, it can be observed that, at 60 ºC, the combined process also caused a reduction in the treatment time by 4 times, as observed at 51 ºC. As a consequence, the application of these combined processes at higher temperatures, such as those used during the current LTLT (low temperatures–long time) (60 – 65 ºC) pasteurization processes, would likely result in a similar synergistic effect. The same conclusion was drawn from the investigation of the effect of temperature on the synergistic inactivation of *E. coli* O157:H7 by heat and lemon EO in apple juice (Espina et al., 2012), as well as on orange EO and limonene in orange juice (Espina et al., 2014). As in those natural products, the antimicrobial constituents of propolis seem to be resistant to heat denaturation under the treatment conditions assayed.

Fig. 4 suggests two possibilities for reducing the intensity of thermal treatments. As the treatment time required to inactivate 5 $\log_{10}$ cycles was reduced by 4 times in the presence of propolis, the amount of processed apple juice could be increased by more than four time with the same equipment, with regards to heat treatments without propolis. On the other hand, the same microbial inactivation levels achieved after 2.8 min of treatment at 60 ºC with no antimicrobials (Eq. 2) were reached in combination with 0.1 mg/mL of propolis at 57 ºC (Eq. 3)—3 ºC lower. Thus, this decrease in the treatment temperature to achieve the same inactivation levels is expected to have a positive impact on the nutritional and organoleptic properties of apple juice (Vikram et 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.

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References

Ait-Ouazzou, A., Loran, S., Bakkali, M., Laglaoui, A., Rota, C., Herrera, A., Pagán, R., Conchello, P., 2011. Chemical composition and antimicrobial activity of essential oils of *Thymus algeriensis*, *Eucalyptus globulus* and *Rosmarinus officinalis* from Morocco. Journal of the Science of Food and Agriculture, 91, 2643-2651.

Ait-Ouazzou, A., Espina, L., García-Gonzalo, D., Pagán, R., 2013. Synergistic combination of physical treatments and carvacrol for *Escherichia coli* O157:H7

562 inactivation in apple, mango, orange, and tomato juices. Food Control, 32, 159-
563 167.
564 Ait-Ouazzou, A., Lorán, S., Arakrak, A., Laglaoui, A., Rota, C., Herrera, A., Pagán, R.,
565 Conchello, P., 2012. Evaluation of the chemical composition and antimicrobial
566 activity of *Mentha pulegium*, *Juniperus phoenicea*, and *Cyperus longus* essential
567 oils from Morocco. Food Research International, 45, 313-319.
568 Burdock, G.A., 1998. Review of the biological properties and toxicity of bee propolis
569 (propolis). Food and Chemical Toxicology, 36, 347-363.
570 Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in
571 foods—a review. International Journal of Food Microbiology, 94, 223-253.
572 Condón, S., Arrizubieta, M.J., Sala, F.J., 1993. Microbial heat-resistance determinations
573 by the multipoint system with the thermoresistometer TR-SC - Improvement of
574 this methodology. Journal of Microbiological Methods 18, 357-366.
575 Condón, S., Palop, A., Raso, J., Sala, F.J., 1996. Influence of the incubation temperature
576 after heat treatment upon the estimated heat resistance values of spores of
577 *Bacillus subtilis*. Letters in Applied Microbiology, 22, 149-152.
578 Cunha, I., Sawaya, A.C.H.F., Caetano, F.M., Shimizu, M.T., Marcucci, M.C., Drezza,
579 F.T., Carvalho, P.O., 2004. Factors that influence the yield and composition of
580 Brazilian propolis extracts. Journal of the Brazilian Chemical Society, 15, 964-
581 970.
582 Dobrowolski, J.W., Vohora, S.B., Sharma, K., Shah, S.A, Naqvi, S.A.H., Dandiya, P.C.,
583 1991. Antibacterial, antifungal, antiamoebic, antiinflammatory and antipyretic
584 studies on propolis bee products. Journal of Ethnopharmacology, 35, 77-82.
585 Espina, L., Somolinos, M., Loran, S., Conchello, P., Garcia, D., Pagan, R., 2011.
586 Chemical composition of commercial citrus fruit essential oils and evaluation of

their antimicrobial activity acting alone or in combined processes. Food Control, 22, 896-902.

Espina, L., Somolinos, M., Pagán, R., García-Gonzalo, D., 2010. Effect of citral on the thermal inactivation of *Escherichia coli* O157:H7 in citrate phosphate buffer and apple juice. Journal of Food Protection, 73, 2189-2196.

Espina, L., Condón, S., Pagán, R., García-Gonzalo, D., 2014. Synergistic effect of orange essential oil or (+)-limonene with heat rreatments to inactivate *Escherichia coli* O157:H7 in orange juice at lower intensities while maintaining hedonic acceptability. Food and Bioprocess Technology, 2, 471-481.

Espina, L., Somolinos, M., Ait-Ouazzou, A., Condón, S., García-Gonzalo, D., Pagán, R., 2012. Inactivation of *Escherichia coli* O157:H7 in fruit juices by combined treatments of citrus fruit essential oils and heat. International Journal of Food Microbiology, 159, 9-16.

FDA, 2001. Hazard analysis and critical control point (HACCP): procedures for the safe and sanitary processing and importing of juice: final rule (21 CFR Part 120). Fed. Regist, vol. 66. U.S. Food and Drug Administration,Washington, D. C., pp. 6137–6202.

Hansen, N.H., Rieman, H., 1963. Factors affecting the heat resistance of nonsporing organisms. Journal of Applied Bacteriology, 26, 314-333.

He, S., Yan, X., 2013. From resveratrol to its derivatives: new sources of natural antioxidant. Current Medicinal Chemistry, 20, 1005-1017.

Jug, M., Končić, M.Z., Kosalec, I., 2014. Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. LWT-Food Science and Technology, 57, 530-537.

611 Kalogeropoulos, N., Konteles, S.J, Troullidou, E., Mourtzinos, I., Karathanos, V.T.,
612 2009. Chemical composition, antioxidant activity and antimicrobial properties of
613 propolis extracts from Greece and Cyprus. Food Chemistry, 116, 452-461.
614 Kim, Y-H., Kim, S-A., Chung, H-J., 2014. Synergistic effect of propolis and heat
615 treatment leading to increased injury to *Escherichia coli* O157:H7 in Ground
616 Pork. Journal of Food Safety, 34, 1-8.
617 Koc, A.N., Silici, S., Mutlu-Sariguzel, F., Sagdic, O., 2007. Antifungal activity of
618 propolis in four different fruit juices. Food Technology and Biotechnology, 45,
619 57-61.
620 Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S.,
621 1999. Antibacterial, antifungal and antiviral activity of propolis of different
622 geographic origin. Journal of Ethnopharmacology, 64, 235-240.
623 Kumazawa, S., Bonvehí, J.S, Torres, C., Mok-Ryeon, A., Bermejo, F.J.O., 2013.
624 Chemical and functional characterisation of propolis collected from East
625 Andalusia (southern Spain). Phytochemical Analysis, 24, 608-615.
626 Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of
627 various geographic origins. Food Chemistry, 84, 329-339.
628 Leistner, L., Gorris, L.G.M., 1995. Food preservation by hurdle technology. Trends in
629 Food Science & Technology, 6, 41-46.
630 Mackey, B.M., 2000. Injured bacteria. In: Lund, B.M., Baird-Parker, T.C., Gould, G.W.
631 (Eds.), The Microbiological Safety and Quality of Food. Aspen Publisher, Inc.,
632 Gaithersburg, pp. 315–341.
633 Mafart, P., Couvert, O., Gaillard, S., Leguerinel, I., 2002. On calculating sterility in
634 thermal preservation methods: application of the Weibull frequency distribution
635 model. International Journal of Food Microbiology, 72, 107-113.

636 Manas, P., Pagán, R., 2005. Microbial inactivation by new technologies of food
637 preservation. Journal of Applied Microbiology, 98, 1387-1399.
638 Marcucci, M.C., 1995. Propolis: chemical composition, biological properties and
639 therapeutic activity. Apidologie, 26, 83-99.
640 Mazzotta, A.S., 2001. Thermal inactivation of stationary-phase and acid-adapted
641 *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit
642 juices. Journal of Food Protection, 64, 315-320.
643 Miguel, M.G., Nunes, S., Dandlen, S.A., Cavaco, A.M., Antunes, M.D., 2010. Phenols
644 and antioxidant activity of hydro-alcoholic extracts of propolis from Algarve,
645 South of Portugal. Food and Chemical Toxicology, 48, 3418-3423.
646 Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A.,
647 Yanagawa, H., 1999. Massive outbreak of *Escherichia coli* O157:H7 infection
648 in schoolchildren in Sakai city, Japan, associated with the comsuption of white
649 radish sprouts. American Journal of Epidemiology, 150, 787-796.
650 Mishima, S., Inoh, Y., Narita, Y., Ohta, S., Sakamoto, T., Araki, Y., Suzuki, K.M.,
651 Akao, Y., Nozawa, Y., 2005. Identification of caffeoylquinic acid derivatives
652 from Brazilian propolis as constituents involved in induction of granulocytic
653 differentiation of HL-60 cells. Bioorganic & Medicinal Chemistry, 13, 5814-
654 5818.
655 Moreira, L., Dias, L.G, Pereira, J.A., Estevinho, L., 2008. Antioxidant properties, total
656 phenols and pollen analysis of propolis samples from Portugal. Food and
657 Chemical Toxicology, 46, 3482-3485.
658 Parish, M.E., 2009. Food safety issues and the microbiology of fruit beverages and
659 bottled water. In: Heredia, N., Vesley, I., García, S. (Eds.), Microbiologically
660 Safe Foods, pp. 291–304.

661 Popova, M., Bankova, V., Butovska, D., Petkov, V., Nikolova-Damyanova, B.,
662 Sabatini, A. G., Marcazzan, G.L., Bogdanov, S., 2004. Validated methods for
663 the quantification of biologically active constituents of poplar-type propolis.
664 Phytochemical Analysis, 15, 235-240.
665 Popova, M., Trusheva, B., Antonova, D., Cutajar, S., Mifsud, D., Farrugia, C.,
666 Tsvetkova, I., Hristo, N., Bankova, V., 2011. The specific chemical profile of
667 Mediterranean propolis from Malta. Food Chemistry, 126, 1431-1435.
668 Popova, M., Silici, S., Kaftanoglu, O., Bankova, V., 2005. Antibacterial activity of
669 Turkish propolis and its qualitative and quantitative chemical composition.
670 Phytomedicine, 12, 221-228.
671 Prakash, B., Kedia, A., Mishra, P.K., Dubey, N.K., 2015. Plant essential oils as food
672 preservatives to control moulds, mycotoxin contamination and oxidative
673 deterioration of agri-food commodities–Potentials and challenges. Food Control,
674 47, 381-391.
675 Segueni, N., Khadraoui, F., Moussaoui, F., Zellagui, A., Gherraf, N., Lahouel, M.,
676 Rhouati, S., 2010. Volatile constituents of Algerian propolis. Annals of
677 Biological Research, 1, 103-107.
678 Silva, J.C., Rodrigues, S., Feás, X., Estevinho, L.M., 2012. Antimicrobial activity,
679 phenolic profile and role in the inflammation of propolis. Food and Chemical
680 Toxicology, 50, 1790-1795.
681 Somolinos, M., García, D., Condón, S., Mackey, B., Pagán, R., 2010. Inactivation of
682 *Escherichia coli* by citral. Journal of Applied Microbiology, 108, 1928–1939.
683 Vikram, V.B., Ramesh, M.N., Prapulla, S.G., 2005. Thermal degradation kinetics of
684 nutrients in orange juice heated by electromagnetic and conventional methods.
685 Journal of Food Engineering, 69, 31-40.

686

687 **Figure Legends**

688

689 **Fig. 1.** Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration:
690 3 x 10^7 CFU/mL) to a heat treatment at 51 ºC in citrate-phosphate buffer of pH 4.0, and
691 recovered in TSAYE (○), TSAYE-SC (□), TSAYE-BS (◊), or recovered in TSAYE
692 after a combined treatment of heat and propolis (0.2 mg/mL) (●). Data represent the
693 means ± standard error of the mean (error bars) of at least three independent
694 experiments.

695

696 **Fig. 2.** Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration:
697 3 x 10^7 CFU/mL) to a heat treatment at 51 ºC in apple juice, and recovered in TSAYE
698 (○), TSAYE-SC (□), TSAYE-BS (◊), or recovered in TSAYE after a combined
699 treatment of heat and of propolis (0.2 mg/mL) (●). Data represent the means ± standard
700 error of the mean (error bars) of at least three independent experiments.

701

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

707

708 **Fig. 4.** Log_{10} times (min) for inactivation of 5 log_{10} cycles of *Escherichia coli*
709 O157:H7 (initial concentration, 3 x 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.

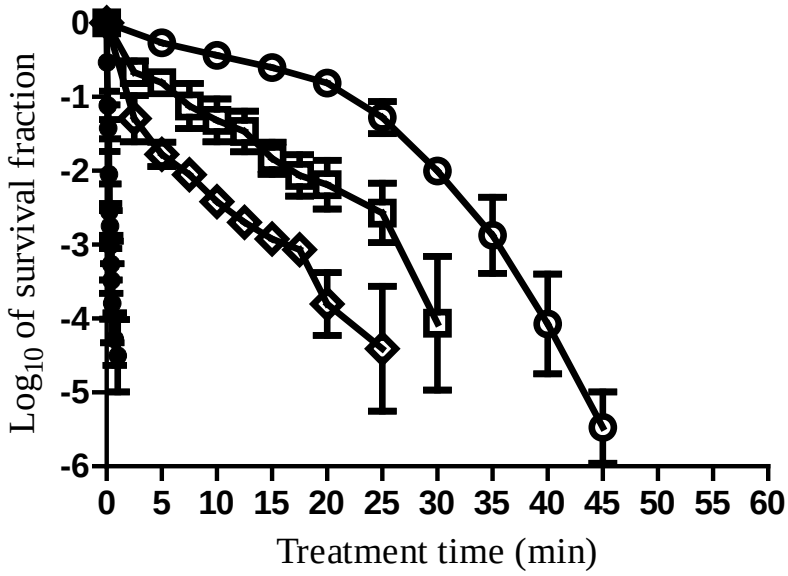
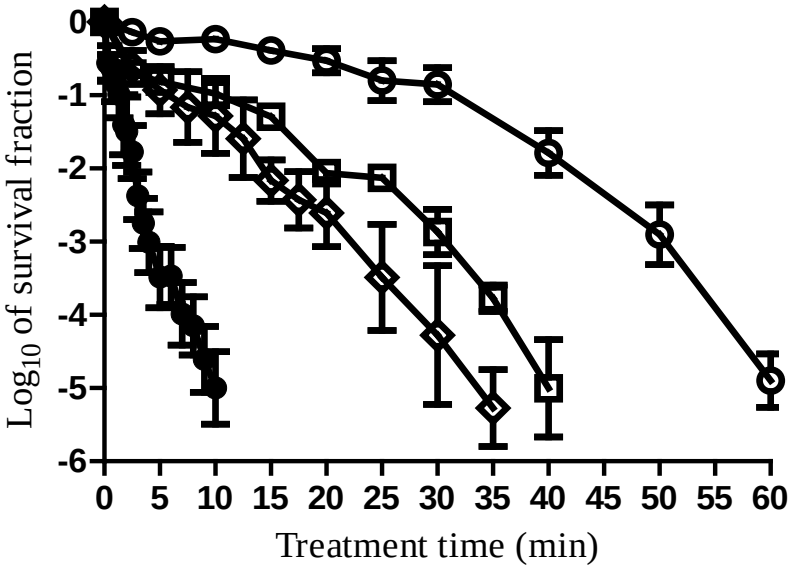

0
-1
-2
-3
-4
-5
-6
Log10 of survival fraction
0 5 10 15 20 25 30 35 40 45 50 55 60
Treatment time (min)

Fig.1.


0
-1
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-6
Log10 of survival fraction
0 5 10 15 20 25 30 35 40 45 50 55 60
Treatment time (min)

Fig.2.

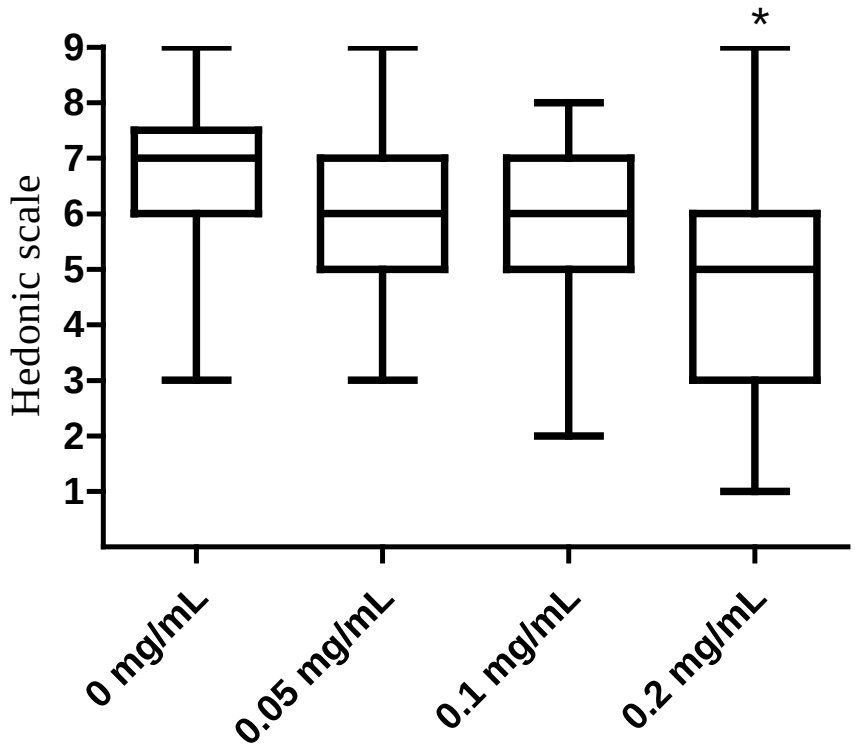

Fig.3.

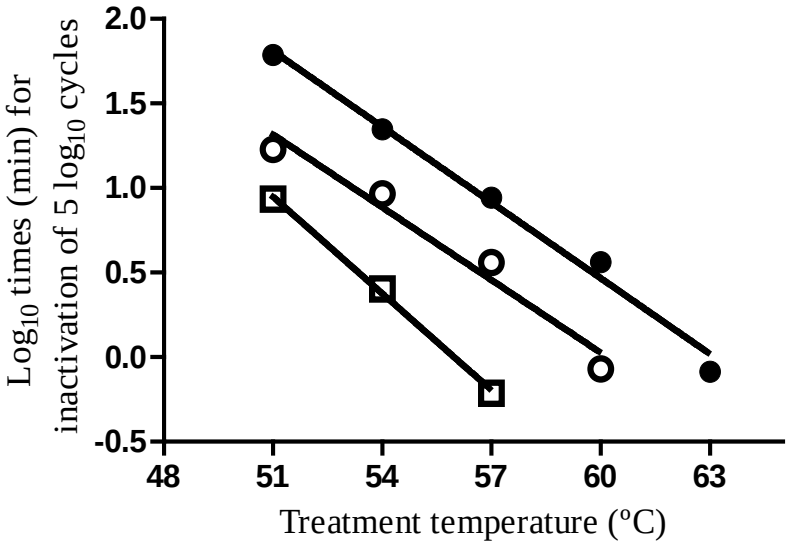
2.0
1.5
1.0
0.5
0.0
-0.5
48
51
54
57
60
63
Log10 times (min) for inactivation of 5 log10 cycles
Treatment temperature (°C)

Fig.4.

Table 1. Minimum inhibitory concentration (MIC) and minimum 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

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 ºC. 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			

Table 3. Regression parameters (δ, time to inactivate 5 $\log_{10}$ cell cycles, and ρ) and the goodness of fit (R^2 and Root Mean Square Error (RMSE)) estimated from the fit of equation 1 to experimental data of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 x 10^7 CFU/mL) heat-treated at 51 ºC and recovered in TSAYE. CI: 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