

1 **Honeybee-collected pollen for human consumption: impact of post-harvest conditioning**
2 **on the microbiota**

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4 RUNNING TITLE: Honeybee-collected pollen for human consumption

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6 MICHELA PALLA¹, ALESSANDRA TURRINI^{1,2}, CRISTIANA SBRANA³, FRANCESCA
7 SIGNORINI⁴, CRISTIANO NICOLELLA⁵, GIOVANNI BENELLI¹, ANGELO CANALE^{1,2},
8 MANUELA GIOVANNETTI^{1,2}, MONICA AGNOLUCCI^{1,2*}

9

10 ¹ Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto
11 80, 56124 Pisa, Italy

12 ² Interdepartmental Research Center “Nutraceuticals and Food for Health”, University of Pisa,
13 Pisa, Italy

14 ³ Institute of Agricultural Biology and Biotechnology, CNR, UOS Pisa, Italy

15 ⁴ Consorzio Polo Tecnologico Magona, via Magona snc, Cecina, Livorno, Italy

16 ⁵ Dipartimento di Ingegneria Civile e Industriale, Università di Pisa, Largo Lucio Lazzarino 2,
17 Pisa, Italy

18

19 *Corresponding author: Monica Agnolucci, Department of Agriculture, Food and
20 Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

21 e-mail: monica.agnolucci@unipi.it

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23 **SUMMARY**

24 Bee pollen is gaining attention as functional food for human consumption. However, scanty
25 information is available on the effects of post-harvest conditioning methods on microbial

26 populations associated to bee pollen. Here, we assessed the microbiological quality and safety
27 of bee-collected chestnut and willow pollen processed by different treatments, such as
28 conventional, freeze- and microwave-assisted drying. Conventional drying of chestnut pollen
29 significantly reduced the abundance of aerobic mesophilic bacteria and the contamination by
30 enterobacteria and yeasts. No impact of freeze-drying and microwave-assisted conditioning
31 was found on hygiene indicators. In chestnut pollen, microwave-assisted drying effectively
32 reduced aerobic sporeforming bacteria, while all conditioning treatments strongly decreased
33 coagulase-positive staphylococci. None of the conditioning methods allowed the reduction of
34 moulds contamination and the abundance of sulphite-reducing clostridia. Our findings stress
35 the importance of studying the microbiota of bee-collected pollen for human consumption, in
36 order to process safe pollen with high microbiological quality.

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38 *Keywords: honeybee-collected pollen; pollen freeze-drying; pollen microbiota; pollen*
39 *microwave-assisted conditioning; pollen post-harvest processing*

40

41 INTRODUCTION. - Honeybee-collected pollen is gaining increasing attention as functional food
42 for human consumption, due to antiproliferative, anti-allergic, antibiotic, antidiarrheic and
43 antioxidant activities (MARGHITAS *et al.*, 2009; GRAIKOU *et al.*, 2011). Its healthy potential
44 mainly depends on the high content of bioactive compounds, such as essential amino acids,
45 antioxidants, vitamins and lipids (CAMPOS *et al.*, 2008; SOARES DE ARRUDA *et al.*, 2013;
46 MĂRGĂOAN *et al.*, 2014; KRYSZYJAN *et al.*, 2015; ALMEIDA *et al.*, 2016). Among the different
47 bioactive molecules, flavonoids have been reported as powerful antioxidant and antiradical
48 compounds (LEJA *et al.*, 2007). Furthermore, it has been recently pointed out that a high level
49 of omega-6 fatty acids characterized chestnut pollen, while willow pollen showed a high
50 concentration of omega-3 fatty acids and carotenoids (CONTE *et al.*, 2016).

51 On the other hand, while basic knowledge on pollen biochemical and nutritional
52 properties is growing, little has been reported on the microbial contaminants potentially
53 present in this functional food and on its microbiological safety (BELHADJ *et al.*, 2012;
54 OBERSTEINER *et al.*, 2016). EC Regulation 178/2002 reported that chemical, physical and
55 biological agents could contaminate bee-collected pollen. Chemical agents may include
56 agricultural pesticides, environmental contaminants (e.g., heavy metals), toxic substances
57 from the plants visited by bees, with special reference to alkaloids, as well as detergents and
58 disinfectants. Pollen is also affected by atmospheric contamination with radionuclides, closely
59 reflecting air quality (BRUSA *et al.*, 2011). Lastly, biological contaminants may include
60 mycotoxins (CAMPOS *et al.*, 2008), bee young instars and their fragments, as well as parasitic
61 mites and other arthropods living in beehives (CANALE *et al.*, 2014a, b), and microbial
62 contaminants.

63 Concerning microbial contamination, recent studies have pointed out that bee-
64 collected pollen can be contaminated by bacteria (GILLIAM, 1979a; BELHADJ *et al.*, 2012),
65 yeasts (GILLIAM, 1979b; DE MELO *et al.*, 2015), and fungi (BRINDZA *et al.*, 2010; ESTEVINHO
66 *et al.*, 2012; FEÁS *et al.*, 2012). The latter are of particular interest for food safety, due to the
67 ability of several species to produce mycotoxins and to trigger allergic reactions (RYZHKIN *et al.*,
68 2002). The most critical phase for pollen contamination is represented by the persistence
69 time of bee pollen in the traps positioned on beehives (GONZÁLEZ *et al.*, 2005). Notably, there
70 are no official criteria for microbiological safety of bee-collected pollen, both at the European
71 Community and international level (BOGDANOV, 2004; CAMPOS *et al.*, 2008). To overcome
72 this important gap, CAMPOS *et al.* (2008) proposed several microbiological standards for bee-
73 collected pollen (Table 1).

74 Fresh honeybee-collected pollen contains a relatively high amount of water (*i.e.* from
75 15 to 30%, w:w), which should be reduced in order to improve its physico-chemical stability

76 and to lower the chances of microbial growth (CANALE *et al.*, 2016). Classic pollen-drying
77 processes should be carried out at low temperatures, with short exposure times, lowering the
78 risk of Maillard's compounds formation (COLLIN *et al.*, 1995). In addition, drying processes
79 conducted at high temperatures and/or with scarcely standardized methods may play a
80 detrimental role on the content of polyphenols and flavonoids, leading to a decrease of the
81 food functional value (SERRA BONVEHÌ *et al.*, 2001). Therefore, novel and reliable
82 technologies are urgently needed in order to boost pollen shelf life and its high nutraceutical
83 quality. In this scenario, CANALE *et al.* (2016) recently showed that microwave-assisted
84 drying offers important advantages for the conservation of bee-collected pollen. Moreover,
85 also freeze-drying can be successfully used to reduce water content in honeybee-collected
86 pollen for human consumption, with negligible impact on polyunsaturated lipids (CONTE *et*
87 *al.*, 2016).

88 Scanty information is available about how post-harvest conditioning methods can
89 affect possible microbial contaminants associated to honeybee-collected pollen. In this work,
90 we investigated the microbiological quality of untreated bee-collected pollen from two
91 botanical species of high economic importance widespread in central Italy, *Castanea sativa*
92 (chestnut) and *Salix alba* (willow), by microbiological analyses, assessing colony forming
93 units (CFU). In particular, we assessed the occurrence and abundance of possible microbial
94 contaminants representing indicators of food hygiene and safety. Furthermore, we evaluated
95 the impact of several post-harvest pollen conditioning methods, including conventional
96 drying, freeze-drying and microwave-assisted drying, on key microbial contaminants.

97

98 MATERIALS AND METHODS. - *Chestnut and willow pollen samples.* - Honeybee-collected
99 chestnut pollen was harvested by a beekeeper in July 2015 in a natural chestnut forest located
100 in Castelnuovo Garfagnana (44° 06' 22.7"N 10° 24' 02.7"E, Lucca, Italy), using a pollen trap

101 (A. Metalori, Italy). Honeybee-collected willow pollen was harvested by a beekeeper in April
102 2015 in natural willow plantations located in Massa Macinaia (43° 47' 45.6"N 10° 32'
103 03.2"E, Capannori, Lucca, Italy), using the pollen trap mentioned above. Chestnut and willow
104 pollen samples were immediately frozen (-20 °C) and transferred to the laboratory within 2 h
105 (control). For both bee-collected pollens, their floral origin was checked by light microscopy
106 examination (400x magnification) and identified using pollen atlas databases (ERDTMAN,
107 1969; MARGHITAS *et al.*, 2009). Results showed that more than 80 % of the pollen grains
108 belonged to the two botanical species. Post-conditioning, all analytical results were compared
109 with the untreated pollen samples. All chestnut and willow pollen samples were analysed in
110 three replicates per pollen species and treatment, to evaluate the effect of conventional
111 drying [i.e. bee-pollen dried at 32 °C for 24 h in a Northwest Technology (Italy) cool-air
112 dryer, <http://www.northwest-technology.com/>] (CONTE *et al.*, 2016), freeze- and microwave-
113 drying.

114 *Freeze-drying and microwave-assisted drying.* - Pollen freeze-drying was carried out
115 using a lyophiliser Heto PowerDry® LL1500 (Thermo Fisher Scientific, Waltham,
116 Massachusetts, USA) following the method recently described by CONTE *et al.* (2016).
117 During the whole freeze-drying process, the temperature inside the condensation chamber was
118 -115 °C, with full vacuum. The samples, at the end of the treatment, were sealed and stored at
119 a temperature of -20 °C for subsequent analysis. Samples were stored in freezer at -20 °C
120 until analysed.

121 Microwave-assisted drying was carried out following the method described by
122 CANALE *et al.* (2016). Experiments were carried out at the absolute pressure of 50 mbar. For
123 both pollen types, microwave power was 150 W and the exposure time was 30 min. At the
124 end of the treatment, pollen samples were weighed and their temperature measured with a K-
125 type thermocouple. The pollen was transferred into an airtight container and stored at 20°C

126 until analysed. Thermo-gravimetric analysis (TGA) carried out as described by CANALE *et al.*,
127 (2016) was used to evaluate the water content in untreated chestnut and willow pollen, as well
128 as freeze-dried and microwave-dried pollen samples of both species.

129 *Microbiological analyses.* - Pollen samples submitted to different treatments were
130 used to assess standard microbiological quality parameters and safety indicators. Different
131 indicators of hygiene and contamination after processing were enumerated, i.e. aerobic
132 microorganisms, assessed on Plate Count Agar (PCA, Oxoid, Milan, Italy, based on BS EN
133 ISO 4833:2003), yeasts and moulds, assessed on Oxytetracycline Glucose Yeast Extract
134 (OGYE Agar, Oxoid, Milan, Italy, based on ISO 6611:2004), *Enterobacteriaceae*, assessed
135 on Violet Red Bile Glucose (VRBG Agar, Oxoid, Milan, Italy, based on ISO 21528-2:2004).
136 Contamination due to spore-forming bacteria was evaluated on pasteurised samples (80 °C for
137 15 min), enumerating both sulphite-reducing clostridia, assessed on Iron Sulphite Agar (ISA,
138 Oxoid, Milan, Italy, based on BS ISO 15213:2003), and aerobic sporeformers, assessed on
139 Tryptone Soya Agar (TSA, Oxoid, Milan, Italy). Coagulase-positive staphylococci, including
140 *Staphylococcus aureus* and other species, were enumerated on Bair Parker Agar (BPA,
141 Oxoid, Milan, Italy, based on ISO 6888-1:1999). Pollen samples were also used to start
142 enrichment cultures for the assessment of safety indicators, such as *Salmonella* spp. (based on
143 ISO 6579: 2002) and *Escherichia coli* (based on ISO 16654:2001) (Oxoid, Milan, Italy).

144 *Statistical analysis.* - SPSS version 23 (IBM Corp., Armonk, NY, USA) was used for
145 statistical analyses of microbial abundance expressed as Log₁₀ cfu g⁻¹. Data were subjected to
146 one-way analysis of variance (ANOVA) followed by Tukey's HSD test at a 95% confidence
147 level to evaluate the effect of different pollen treatments on the abundance of microbial
148 agents. When data did not fit ANOVA assumptions, Welch robust test of equality of means or
149 Kruskal-Wallis test were performed.

150

151 RESULTS AND DISCUSSION. - *Microbial contaminants of untreated bee pollen.* - TGA
152 carried out to evaluate the water content in untreated chestnut and willow pollen showed
153 values of 14.87 % and 22.90 %, respectively. After freeze-drying for 540 min, TGA analysis
154 showed that the residual water content in chestnut and willow pollen was 6.02 % and 6.25 %,
155 respectively. After microwave-assisted drying, TGA conducted at 120 °C showed that the
156 residual water content was 6.44 % for chestnut pollen and 10.33 % for willow pollen.

157 Microbiological analyses carried out on bee-collected chestnut and willow pollen
158 showed large abundance of aerobic mesophilic microorganisms, *Enterobacteriaceae*, yeasts
159 and moulds (Tables 2-3). Values observed for the aerobic mesophilic microorganisms
160 ($F_{3,8}=969.22$) and for *Enterobacteriaceae* ($F_{3,8}=21.26$) in chestnut were significantly higher
161 than those detected in willow pollen ($P<0.01$). Previous studies detected low numbers of
162 coliform bacteria in untreated pollen samples (FEÁS *et al.*, 2012), whereas values ranging
163 from 4.18 to 7.67 Log cfu g⁻¹ of *Enterobacteriaceae* were found in commercial pollen
164 (BELHADJ *et al.*, 2014). We support the choice of *Enterobacteriaceae* as indicator, in place of
165 coliforms, since the method used allows the detection and enumeration of both lactose-
166 fermenting and important non-lactose fermenting organisms, such as salmonellas. Previous
167 works on untreated or commercial bee pollen samples of different geographic origins reported
168 lower or similar counts, with maximum values of 5.49 Log cfu g⁻¹ for aerobic microflora in
169 commercial dry samples and 5.8 Log cfu g⁻¹ in untreated samples (FEÁS *et al.*, 2012;
170 NOGUEIRA *et al.*, 2012; BELHADJ *et al.*, 2012, 2014). The numbers of yeasts and moulds (8.75
171 and 8.65 Log cfu g⁻¹) in chestnut and willow pollen, respectively, were high, compared with
172 previous data (up to 6.99 Log cfu g⁻¹) obtained from commercial dry pollen (FEÁS *et al.*,
173 2012; HANI *et al.*, 2012; NOGUEIRA *et al.*, 2012; BELHADJ *et al.*, 2012, 2014). Previous
174 qualitative studies carried out on honeybee-collected pollen samples of different geographic
175 origins showed the occurrence of yeasts and moulds belonging to the genera *Candida*,

176 *Debaryomyces*, *Rhodotorula*, *Torulopsis* and *Zygosaccharomyces* (GILLIAM, 1979b; DE MELO
177 *et al.*, 2015), and *Aspergillus*, *Alternaria*, *Cladosporium*, *Mucor*, *Rhizopus*, *Paecilomyces* and
178 *Penicillium* (BRINDZA *et al.*, 2010; ESTEVINHO *et al.*, 2012; BELHADJ *et al.*, 2014; NARDONI *et*
179 *al.*, 2016), respectively.

180 Aerobic sporeformers showed similar low values in chestnut and willow pollen,
181 (2.02 ± 0.30 and 2.63 ± 0.02 Log cfu g⁻¹), while anaerobic sporeformers, assayed by sulphite-
182 reducing clostridia detection, had values of 0.44 ± 0.22 and 1.33 ± 0.6 Log cfu g⁻¹, respectively.
183 Our data agree with previous evaluations of Spanish pollen, which detected maximum
184 *Bacillus* counts of 1 Log cfu g⁻¹, and with data from different pollen samples, reporting the
185 absence or low counts (1 Log ufc g⁻¹) of sulphite-reducing clostridia (SERRA BONVEHÌ and
186 ESCOLÀ JORDA, 1997; ESTEVINHO *et al.*, 2012; FEÁS *et al.*, 2012; NOGUEIRA *et al.*, 2012). By
187 contrast, high levels of total anaerobes (5.77 Log cfu g⁻¹) were detected in untreated pollen
188 samples from different Algerian regions (BELHADJ *et al.*, 2012).

189 As to safety indicators, contamination due to presumptive *S. aureus*, estimated by
190 analysing coagulase-positive staphylococci, was low in chestnut and willow pollen, showing
191 values of 0.67 ± 0.01 and 1.49 ± 0.76 Log cfu g⁻¹, respectively, lower than those detected in both
192 untreated and commercial dry Algerian pollen samples (BELHADJ *et al.*, 2012, 2014).
193 Noteworthy, *S. aureus* was absent from Spanish and Portuguese pollen samples (NOGUEIRA
194 *et al.*, 2012). Both *Salmonella* spp. and *E. coli* were absent from all chestnut and willow
195 pollen samples analysed in this study. Previous works reported the absence of such food
196 safety indicators from pollen samples of different origins (SERRA BONVEHÌ and ESCOLÀ
197 JORDA, 1997; FEÁS *et al.*, 2012; NOGUEIRA *et al.*, 2012; BELHADJ *et al.*, 2012, 2014), except
198 for 7 out of 15 samples of commercial dry pollen analysed by BELHADJ *et al.* (2014).

199 In this work, the high microbial loads of hygiene indicators in untreated pollen did not
200 conform to the microbiological standards suggested by CAMPOS *et al.* (2008) (Table 4). On

201 the other hand, safety indicators were absent or very low. No guidelines are available for
202 sporeformer bacteria, despite the important toxigenic activities of several *Bacillus* and
203 *Clostridium* species.

204 *Impact of post-harvest conditioning on microbial hygiene and safety indicators. -*

205 Several studies highlighted that water content is the key factor for the development of
206 undesirable microorganisms in pollen for human consumption (SERRA BONVEHÌ and ESCOLÀ
207 JORDA, 1997; ESTEVINHO *et al.*, 2012). In our assays, conventional drying led to a significant
208 decrease in the abundance of aerobic mesophilic bacteria (-51%) in chestnut pollen, and it
209 reduced contamination by enterobacteria (absent in both pollen types) and yeasts in chestnut
210 (-48%) and willow (-50%) pollen (Tables 2-3). The significant reduction of enterobacteria
211 after conventional drying is an important finding, as high values, ranging from 4.18 to 7.67 Log
212 cfu g⁻¹, were reported for *Enterobacteriaceae* in commercial dried pollen samples of different
213 geographic origin (BELHADJ *et al.*, 2014).

214 No impact of freeze-drying and microwave-assisted conditioning was detected on
215 hygiene indicators for pollen originating from both plant species (Tables 2-3). The only data
216 available reported inconsistent quantities for pollen samples after oven-drying or freeze-
217 drying, varying depending on sample collection season (DE MELO *et al.*, 2016).

218 None of the conditioning methods tested allowed the reduction of mould
219 contamination in chestnut and pollen samples, confirming previous data on the abundance of
220 yeasts and moulds (up to 6.99 Log cfu g⁻¹) in commercial dry pollen (FEÁS *et al.*, 2012; HANI
221 *et al.*, 2012; NOGUEIRA *et al.*, 2012; BELHADJ *et al.*, 2014). Mould contamination may
222 represent an important hazard source, since bee pollen is considered a good substrate able to
223 induce mycotoxins production (MEDINA *et al.*, 2004) and could be reduced by frequent pollen
224 collection from beehive traps and optimal storage conditions.

225 Aerobic sporeforming bacterial contamination was significantly affected by
226 microwave-based treatment of chestnut pollen (-60%), compared with the untreated control
227 ($F_{3,8}=134.6$, $P<0.001$), whereas such contaminants were undetectable in willow pollen
228 submitted to conventional drying. Anaerobic sporeformers analyses of conditioned pollen
229 showed that no treatments were able to reduce the abundance of sulphite-reducing clostridia
230 in samples of both botanical origin (data not shown). Indeed, the resistance of bacterial spores
231 to drying and heating is well-known, and safety concerns may be raised from the occurrence
232 of both aerobic and anaerobic sporeformers, since many species and isolates in these groups
233 are able to produce toxins, causing gastroenteritis or neuroparalytic illness. Unfortunately,
234 despite their importance for human health, no guidelines have been proposed for their control
235 in pollen.

236 Concerning the occurrence of foodborne pathogenic bacteria, no changes were
237 observed in the occurrence of the safety indicators *Salmonella* spp. and *E. coli*, which were
238 absent both in control and treated pollen samples, consistently with the proposed guidelines
239 (Table 1). Coagulase-positive staphylococci were no more detectable in all treated chestnut
240 pollen samples, whereas no significant differences were detected among differentially treated
241 and control willow pollen samples, showing values ranging from undetectability (microwave-
242 assisted drying) to 1.7 ± 0.8 Log ufc g⁻¹ (freeze-drying). In a previous survey, a wide variability
243 of values has been reported for presumptive *S. aureus* contamination in commercial dried
244 pollen from different North African and Chinese regions, ranging from undetectability to a
245 maximum of 8.32 Log cfu g⁻¹ (BELHADJ *et al.*, 2014). Further studies should assess coagulase-
246 positive staphylococcal contamination in European commercial pollen, although such a
247 microbial indicator is not strictly limited to pathogenic *S. aureus*.

248

249 CONCLUSIONS. - The present study shows that conventional drying is effective in
250 decreasing the number of microbial hygiene indicators and, for the first time, that microwave-

251 assisted conditioning affects microbial contaminants of honeybee-collected pollen for human
252 consumption. Microwave-assisted conditioning was able to strongly reduce coagulase-
253 positive staphylococci and aerobic sporeformer populations in willow and chestnut pollen,
254 respectively, compared with conventional and freeze-drying. Bee-collected pollen quality may
255 be further boosted by such a treatment, which was recently reported to preserve the content of
256 plant secondary metabolites with nutraceutical properties (CANALE *et al.*, 2016).

257 Only few countries apply microbiological criteria to commercial dehydrated bee-
258 pollen, while the International Honey Commission (IHC) has proposed guidelines including
259 limits for total aerobic microorganisms, yeasts and moulds, *Enterobacteriaceae*, *E. coli*,
260 *Salmonella* spp. and *S. aureus* (CAMPOS *et al.*, 2008). Our findings, together with those of DE-
261 MELO *et al.* (2016), reporting variable responses of bee pollen microbiota to conditioning
262 treatments, stress the importance of studies on the microbiota of bee-collected pollen for
263 human consumption, in order to process pollen with high microbiological quality, possibly
264 conforming to the proposed guidelines and to additional key indicators relevant for food
265 quality and safety.

266

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REFERENCES

275

276 ALMEIDA J.F., DE FLORIO ALMEIDA J., SOARES DOS REIS A., SERAFINI HELDT L.F., PEREIRA
277 D., BIANCHIN M., et al.: Lyophilized bee pollen extract: A natural antioxidant source
278 to prevent lipid oxidation in refrigerated sausages. *LWT – Food Sci. Technol.* 76, 299-
279 305 (2016).

280 BELHADJ H., BOUMRA D., DAHAMNA S., HARZALLAH D., GHADBANE M., KHENNOUF S.:
281 Microbiological sanitary aspects of pollen. *Adv. Environ. Biol.* 6, 1415-1420 (2012).

282 BELHADJ H., HARZALLAH D., DAHAMNA S., KHENNOUF S.: Microbiological quality control of
283 marketed pollen. *Pharm. Lett.* 6, 37-42 (2014).

284 BOGDANOV S.: Quality and standards of pollen and beeswax. *Apiacta* 38, 334-341 (2004).

285 BRINDZA J., GRÓF J., BACIGÁLOVÁ K., FERIANC P., TÓTH D.: Pollen microbial colonization
286 and food safety. *Acta Chim. Slov.* 3, 95-102 (2010).

287 BRUSA B., GUARALDO P., PALMA A., RINALDI G., ROSSO A., MOGLIOTTI P.: Bees as
288 bioindicators to guarantee healthy products for the consumer. *Ital. J. Food Saf.* 25,
289 67111-91 (2011).

290 CAMPOS M.G., BOGDANOV S., DE ALMEIDA-MURADIAN L.G., SZCZESNA T., MANCEBO Y.,
291 FRIGERIO C., FERREIRA F.: Pollen composition and standardization of analytical
292 methods. *J. Apicult. Res.* 47, 156-136 (2008).

293 CANALE A., CANOVAI R., COSCI F., GIANNOTTI P., BENELLI G.: Survey of Italian honeys for
294 the presence of foreign matter using the filth test. *Food Addit. Contam. Part A* 31,
295 905-909 (2014a).

296 CANALE A., COSCI F., CANOVAI R., GIANNOTTI P., BENELLI G.: Foreign matter contaminating
297 ethanolic extract of propolis: a filth-test survey comparing products from small
298 beekeeping farms and industrial producers. *Food Addit. Contam. Part A* 31, 2022-
299 2025 (2014b).

300 CANALE A., BENELLI G., CASTAGNA A., SGHERRI C., POLI P., SERRA A., et al.: Microwave-
301 assisted drying for the conservation of honeybee pollen. *Materials* 9, 363 (2016).
302 doi:10.3390/ma9050363

303 COLLIN S., VANHAVRE T., ODART E., BOUSETA A.: Heat treatment of pollens: impact on their
304 volatile flavor constituents. *J. Agr. Food Chem.* 43, 444-448 (1995).

305 CONTE G., BENELLI G., SERRA A., SIGNORINI F., BIENTINESI M., NICOLELLA C., et al.: Lipid
306 characterization of chestnut and willow honeybee-collected pollen: impact of freeze-
307 drying and microwave-assisted drying. *J. Food Compos. Anal.* 55, 12-19 (2016).

308 DE MELO A.A.M., ESTEVINHO L.M., ALMEIDA-MURADIAN L.B.: A diagnosis of the
309 microbiological quality of dehydrated bee-pollen produced in Brazil. *Lett. Appl.*
310 *Microbiol.* 61, 477-483 (2015).

311 DE MELO A.A.M., ESTEVINHO L.M., ALMEIDA-MURADIAN L.B., SATTLER J.A.G., SOUZA B.R.,
312 FREITAS A.S., BARTH M.O.: Effects of processing condition in characteristic of
313 dehydrated bee-pollen and correlation between quality parameters. *Food Sci. Technol.*
314 65, 808-815 (2016).

315 ERDTMAN G.: *Handbook of palynology – An introduction to the study of pollen grains and*
316 *spores.* Munksgaard, Copenhagen (1969).

317 ESTEVINHO L.M., RODRIGUES S., PEREIRA A.P., FEÁS X.: Portuguese bee pollen: palynological
318 study, nutritional and microbiological evaluation. *Int. J. Food Sci. Tech.* 47, 429-435
319 (2012).

320 FEÁS X., PILAR VÁZQUEZ-TATO M., ESTEVINHO L., SEIJAS J.A., IGLESIAS A.: Organic bee
321 pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity
322 and microbiological quality. *Molecules* 17, 8359-8377 (2012).

323 GILLIAM M.: Microbiology of pollen and bee bread: the genus *Bacillus*. *Apidologie* 10, 269-
324 274 (1979a).

325 GILLIAM M.: Microbiology of pollen and bee bread: the yeasts. *Apidologie* 10, 43-53 (1979b).

326 GONZÁLEZ G., HINOJO M.J., MATEO R., MEDINA A., JIMÉNEZ M.: Occurrence of mycotoxin
327 producing fungi in bee pollen. *Int. J. Food Microb.* 105, 1-9 (2005).

328 GRAIKOU K., KAPETA S., ALIGIANNIS N., SOTIROUDIS G., CHONDROGIANNI N., GONOS E.,
329 CHINOU I.: Chemical analysis of Greek pollen – antioxidant, antimicrobial and
330 proteasome activation. *Chem. Cent. J.* 5, 3 (2011).

331 HANI B., DALILA B., SALIHA D., DAOUND H., MOULOUND G., SEDDIK K.: Microbiological
332 sanitary aspects of pollen. *Adv. Environ. Biol.* 6, 1415-1420 (2012).

333 KRZYSTYJAN M., GUMUL D., ZIOBRO R., KORUS A.: The fortification of biscuits with bee pollen
334 and its effect on physicochemical and antioxidant properties in biscuits. *LWT – Food*
335 *Sci. Technol.* 63, 640-646 (2015).

336 LEJA M., MARECZEK A., WYŻGOLIK G., KLEPACZ-BANIAK J., CZEKOŃSKA K.: Antioxidative
337 properties of bee pollen in selected plant species. *Food Chem.* 100, 237-240 (2007).

338 MĂRGĂOAN R., MĂRGHITAS L.A., DEZMIREAN D.S., DULF F.V., BUNEA A., SOCACI S.A.,
339 BOBIȘ O.: Predominant and secondary pollen botanical origins influence the
340 carotenoid and fatty acid profile in fresh honeybee-collected pollen. *J. Agr. Food*
341 *Chem.* 62, 6306-6316 (2014).

342 MARGHITAS L.A., STANCIU O.G., DEZMIREAN D.S., BOBIȘ O., POPESCU O., BOGDANOV S.,
343 CAMPOS M.G.: In vitro antioxidant capacity of honeybee-collected pollen of selected
344 floral origin harvested from Romania. *Food Chem.* 115, 878-883 (2009).

345 MEDINA A., GONZÁLEZ G., SÁEZ J.M., MATEO R., JIMÉNEZ M.: Bee pollen, a substrate that
346 stimulates Ochratoxin A production by *Aspergillus ochraceus* Wilh. *Syst. App.*
347 *Microbiol.* 27, 261-267 (2004).

348 NARDONI S., D'ASCENZI C., ROCCHIGIANI G., MORETTI V., MANCIANTI F.: Occurrence of
349 moulds from bee pollen in Central Italy – A preliminary study. *Ann. Agric. Environ.*
350 *Med.* 23, 103-105 (2016).

351 NOGUEIRA C., IGLESIAS A., FEÁS X., ESTEVINHO L.M.: Commercial bee pollen with different
352 geographical origins: a comprehensive approach. *Int. J. Mol. Sci.* 13, 11173-11187
353 (2012).

354 OBERSTEINER A., GILLES S., FRANK U., BECK I., HÄRING F., ERNST D. et al.: Pollen-associated
355 microbiome correlates with pollution parameters and the allergenicity of pollen. *PLoS*
356 *ONE*, 11, e0149545 (2016).

357 RYZHKIN D.V., ELANSKIJ S.N., ZHIJOLTIKOVA T.M.: Monitoring koncentracii spor gribov
358 *Cladosporium* i *Alternaria* v atmosferom vozduhe g. Moskvy. *Atmosfera.*
359 *Pul'monologija i Alergologija* 2, 30-31 (2002).

360 SERRA BONVEHÌ J., ESCOLÀ JORDA R.: Nutrient composition and microbiological quality of
361 honeybee-collected pollen in Spain. *J. Agric. Food Chem.* 45, 725-732 (1997).

362 SERRA BONVEHÌ J., SOLIVA TORRENTO M., CENTELLES LORENTE E.: Evaluation of
363 Polyphenolic and Flavonoid Compounds in Honeybee-Collected Pollen Produced in
364 Spain. *J. Agric. Food Chem.* 49, 1848-1853 (2001).

365 SOARES DE ARRUDA V.A., SANTOS PEREIRA A.A., SILVA DE FREITAS A., BARTH O.M., BICUDO
366 DE ALMEIDA-MURADIAN L.: Dried bee pollen: B complex vitamins, physicochemical
367 and botanical composition. *J. Food Compos. Anal.* 29, 100-105 (2013).

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370 TABLE 1. - *Microbiological standards suggested for bee-collected pollen (CAMPOS et al.,*
 371 *2008).*

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Markers of	Parameter	Threshold
Microbiological quality	Total mesophilic bacteria	$<10^5$ cfu g ⁻¹
	Yeasts	$<5 \times 10^4$ cfu g ⁻¹
	Fungi	$<5 \times 10^4$ cfu g ⁻¹
	<i>Enterobacteriaceae</i>	$<10^2$ cfu g ⁻¹
Microbiological safety	<i>Escherichia coli</i>	Absent 1g ⁻¹
	<i>Salmonella</i> spp.	Absent 10g ⁻¹
	<i>Staphylococcus aureus</i>	Absent 1g ⁻¹

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376 TABLE 2. - *Impact of conventional drying, freeze-drying and microwave-assisted conditioning on microbial hygiene indicators in chestnut*
 377 *pollen for human consumption. Values indicate mean Log₁₀ cfu g⁻¹ ± SE. In columns, different letters indicate significant differences*
 378 *(ANOVA, Tukey's HSD test, P<0.05).*

379

Treatment	Aerobic mesophilic microorganisms	Enterobacteria	Yeasts	Moulds
Control	6.28±0.04 a	4.11±0.05 a	4.05±0.02 a	4.70±0.05 a
Freeze-drying	6.26±0.02 a	3.83±0.10 a	3.91±0.10 a	4.56±0.03 a
Conventional drying	3.07±0.44 b	0.00±0.00 *	2.12±0.68 b	4.08±0.08 a
Microwave-assisted conditioning	5.75±0.02 a	3.92±0.05 a	4.14±0.06 a	4.75±0.03 a
	Welch Sig. 0.001 F _{3,4} =106.199, P=0.001	F _{2,6} =4.019, P=0.078	F _{3,8} =7.659, P=0.01	F _{3,8} =37.478, P<0.001

380 * Values not included in ANOVA.

381 TABLE 3. - *Impact of conventional drying, freeze-drying and microwave-assisted conditioning on microbial hygiene indicators in willow*
 382 *pollen for human consumption. Values indicate mean Log₁₀ cfu g⁻¹ ± SE. In columns, different letters indicate significant differences*
 383 *(ANOVA, Tukey's HSD test, P<0.05).*

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Treatment	Aerobic mesophilic microorganisms	Enterobacteria	Yeasts	Moulds
Control	5.09±0.01 ab	1.09±0.65 b	4.75±0.11 a	3.90±0.10 a
Freeze-drying	5.26±0.03 a	3.36±0.27 a	4.84±0.05 a	3.85±0.05 a
Conventional drying	4.01±0.54 b	0.00±0.00 *	2.38±0.73 b	3.88±0.15 a
Microwave-assisted conditioning	5.01±0.06 ab	2.42±0.34 ab	4.71±0.02 a	3.98±0.02 a
	Welch Sig. 0.029 F _{3,4} =4.28, P=0.044	F _{2,6} =6.304, P=0.034	F _{3,8} =10.326, P=0.004	F _{3,8} =0.335, P=0.801

385 * Values not included in ANOVA.

386 TABLE 4. - *Microbiological contaminants found in untreated Italian bee-collected*
 387 *pollen for human consumption. Values in bold highlighted lacking compliance to the*
 388 *microbiological safety standards suggested by CAMPOS et al. (2008).*
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Microbial contaminants	Chestnut pollen	Willow pollen
Aerobic mesophilic bacteria	6.28 log cfu g⁻¹±0.04	5.09 log cfu g⁻¹±0.01
<i>Enterobacteriaceae</i>	4.11 log cfu g⁻¹±0.05	1.09 log cfu g ⁻¹ ±0.65
Yeasts	4.05 log cfu g ⁻¹ ±0.02	4.75 log cfu g⁻¹±0.11
Molds	4.70 log cfu g ⁻¹ ±0.05	3.90 log cfu g ⁻¹ ±0.10
Coagulase-positive staphylococci	6.67 log cfu g⁻¹±0.00	1.49 log cfu g⁻¹±0.76
<i>Escherichia coli</i>	Absent 1g ⁻¹	Absent 1g ⁻¹
<i>Salmonella</i> spp.	Absent 10 g ⁻¹	Absent 10 g ⁻¹

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