

1 **Title**

2 **An alternative encapsulation approach for production of active chitosan - propolis**
3 **beads**

4

5 **Running title**

6 **Chitosan-propolis beads as active device**

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19 **Summary**

20 Encapsulation is a promising technology to carry natural active substances, preventing
21 their loss and maintaining their stability until use. Beads of chitosan containing propolis
22 have been prepared using a mono-pore filter device, which permits the encapsulation of
23 natural polyphenols avoiding heat treatments, high shear rates and the use of toxic
24 solvents. Beads proved to be active against *Bacillus cereus*, *Escherichia coli*, *Listeria*
25 *innocua*, *Pseudomonas fluorescens*, *Yarrowia lipolytica*, and three moulds strains; the

26 highest effect was found against *Staphylococcus aureus* (MIC 0.8 mg beads/mL).
27 Results in liquid cultures of *S. aureus* evidenced that beads were able to release the
28 flavonoids from propolis: the diffusion of the active compounds is a key factor in the
29 exploitation of the microbial activity. The obtained chitosan-propolis beads represent an
30 example of natural antimicrobial delivery system that could be used to prevent the
31 growth of pathogenic/spoilage bacteria in food applications.

32

33 **Keywords:** propolis, chitosan, membrane encapsulation, active device, food packaging

34

35 **Introduction**

36 Propolis, a natural brownish resinous substance collected by honeybees (*Apis mellifera*)
37 (Lu et al., 2005; Burdock, 1998; Salomao et al., 2004), is largely used in the
38 pharmaceutical fields and recent studies have paved the way for potential applications
39 of propolis also within the food and food packaging fields, to control primary factors of
40 antimicrobial degradation and oxidation especially thanks to its great polyphenols
41 content (Pastor et al., 2010, Guo et al., 2011; Tosi et al., 2007). Nowadays there is also a
42 growing interest on employing natural additives with packaging technology designed to
43 keep produce fresh, optimizing its shelf life (Weiss et al., 2009; Cutter et al., 2006).
44 However, as propolis is a strongly adhesive, resinous mixture of insoluble or slightly
45 soluble substances with bitter taste and no standard composition, the application of
46 propolis in the food area has been limited (Sforcin and Bankova, 2011). In this
47 perspective, encapsulation of propolis could be a promising technology to create
48 standardized active delivery systems able to maintain propolis polyphenols active until
49 use.

50 Not all the conventional encapsulating techniques are applicable due to propolis heat
51 sensibility to temperatures below the room temperature and above 120° C for more than
52 one minute (Gonzales et al., 2009). The membrane encapsulating technique could be a
53 suitable technique, in fact one of the main advantages of this encapsulating technique
54 respect to the conventional methods (Munin and Edwards-Lévy, 2011; Nedovic et al.,
55 2011; Zhang et al., 2010), is the use of low energy density avoiding high shear rates and
56 temperature; this helps to maintain quality and functionality of labile molecules, such as
57 propolis polyphenols. Moreover, the encapsulation filter process employed is based on a
58 membrane emulsification technique, which allows homogeneity of the matrix, the easy
59 control of droplet sizes and size distribution of the obtained beads, by choosing suitable
60 membranes and focusing on some operating process parameters (Piacentini et al., 2010).
61 In particular, one of the main advantages of the membrane emulsification technique is
62 the formation of active beads through the use of lower energy density and room
63 temperature, in comparison with other techniques like extrusion blending, freeze drying
64 or spray drying. In fact, in these conventional techniques, the high shear rates and the
65 high variation of the process temperatures could have negative effects on sensitive
66 active components.

67 One of the biopolymer that is largely used for encapsulation is chitosan. Chitosan is
68 nowadays used for biomedical applications, drug delivery systems, coatings and tissue
69 engineering, as well as applications in food, cosmetics and agricultural industries (Dutta
70 et al., 2009; Senel and McClure, 2004). Even if it is not soluble in pure water, chitosan
71 needs to be cross-linked in order to increase stability in contact with a lot of media
72 (acidic solutions, oil/water emulsion, etc). One of the natural cross-linkers that is
73 already used to cross-link biopolymers, to control swelling ratio and mechanical

74 properties, is genipin (Chen et al., 2004; Jin et al., 2004; Mi et al., 2001, 2005; Yuan et
75 al., 2007; Liang et al, 2009).

76 In the present work, beads of chitosan and propolis, with genipin as cross-linker, were
77 produced using the membrane process concept to create an example of delivery system
78 stable during storage time and active against pathogen and spoilage food
79 microorganisms.

80

81 **Material and methods**

82

83 **Materials**

84 Propolis as hydroalcoholic extract (60 wt % ethanol) was used. The extract was
85 obtained with a patented method of purification, starting from propolis of italian
86 regions, that permits to eliminate waxes and resins to have a Dewaxed Hydrodispersible
87 Propolis Extract (EPID). It is characterized by a standardized polyphenolic profile,
88 determined by LC-DAD-MS analysis, reported in Table 2. The propolis extract was
89 kindly supplied by Specchiasol (Bussolengo, Verona – Italy). Chitosan (Medium
90 Molecular Weight) was purchased by Sigma Aldrich. Genipin, an aglycone derived
91 from an iridoid glycoside named geniposide and extracted from the plant *Gardenia*
92 *jasminoides Ellis*, was supplied by Wako Chemicals GmbH (Germany). All other
93 reagents were of analytical grade.

94

95 **Microrganisms and culture conditions**

96 Several microbial strains were tested, in particular eight bacteria, five yeasts and three
97 moulds, as follows: *Bacillus cereus* MIM 71 (MIM: Microbiologia Industriale Milano),
98 *Enterobacter agglomerans* ATCC 29904 (ATCC: American Type Culture Collection),

99 *Enterococcus faecalis* MIM 109, *Escherichia coli* CECT 434 (CECT: Spanish Type
100 Culture Collection), *Listeria innocua* DSMZ 20649 (DSMZ: Deutsche Sammlung von
101 Mikroorganismen und Zellkulturen), *Pseudomonas fluorescens* MIM C20 and MIM S9,
102 *Staphylococcus aureus* MIM 178, *Candida kefyr* CCY 29810 (CCY: Czechoslovak
103 Collection of Yeast), *Yarrowia lipolytica* CCY 29.26.5, *Kluyveromyces bulgaricus* IMI
104 LT (IMI: International Mycological Institute), *Kluyveromyces marxianus* var. *lactis* IMI
105 C1 69, *Rhodotorula mucilaginosa* IMAP 6484 (IMAP: Istituto Microbiologia Agraria
106 Perugia), *Aspergillus niger* NRRL 565 (NRRL: Agricultural Research Service Culture
107 Collection), *Penicillium notatum* MIM 29, *Cladosporium cladosporioides* MIM 259.

108 Yeasts and moulds were grown on MEA (Malt Extract Agar) medium, of the following
109 composition (g/l): malt extract (Costantino, Favria, Turin) 20, soybean peptone 2
110 (Costantino), agar 15, glucose 20, pH 5.8, sterilisation at 118°C for 20 min. Bacterial
111 strains were grown on TSA medium (Tryptic Soy Agar, Scharlau Chemie - Barcelona),
112 incubated at 30 °C for 24-72 h. Cultures were maintained as frozen stocks at - 20 °C in
113 the above mentioned media in liquid form added with glycerol (10 % w/v), and
114 propagated twice before use in experiments.

115

116 **Beads preparation**

117 Beads were produced using the membrane process concept previously explored by some
118 of the same authors (Figoli et al., 2007, Lakshmi et al, 2012). In this work, chitosan
119 powder was added (2% wt.) to an aqueous solution containing 1% wt. of acetic acid.
120 After complete solubilisation of chitosan, the solution was purged into a cylindrical
121 Teflon module tank (50 ml volume) containing a polyethylene (PE) mono-pore film,
122 produced by using a micro-driller of a local goldsmith shop, obtaining a pore-size
123 dimension of 600 micron and thickness of about 1 mm. The chitosan solution, passing

124 through the mono-pore film by gravity, formed the droplets in the air-gap phase
125 (distance between the film and the aqueous continuous solution of about 10 cm) and
126 entered in contact with the continuous phase forming the chitosan beads. The
127 continuous aqueous solution phase was made of an acetic acid solution (1% wt.) with
128 propolis concentration of 0%, 2% and 10% wt., and genipin as cross linker (0.023%
129 wt.). Wet beads were left in the mixture for reticulation and propolis encapsulation,
130 under stirring (100 rpm, ARE, VELP Scientific, Italy) for 24 h, then recovered using a
131 filter paper (wet beads) (Fig. 1). The natural cross-linker genipin was used with the aim
132 of stabilizing the beads and to permit chitosan future swelling without its solubilisation
133 in contact with different means. The beads were finally set in a climate chamber
134 (Angelantoni E301, Italy) at 30°C for 24 h upon drying (dry beads) and stored at least
135 for 30 days.

136

137 **Beads investigation**

138 Diameter of wet beads and dry beads was determined using a digital micrometer (Carl
139 Mahr D 7300 Esslingen A.N.) and an optical microscope (Olympus MIC-D). The
140 morphology of chitosan beads was evaluated employing Scanning Electron Microscopy
141 (SEM) at 20 kV (Cambridge Instruments Stereoscan 360). The efficiency of the cross-
142 linking process was tested: chitosan beads, before and after cross-linking, were added to
143 the distilled water and their solubility was evaluated as percentage of weight loss.
144 Encapsulation efficiency was qualitatively evaluated by the identification of propolis
145 polyphenols. A total extraction of polyphenols from beads was done putting in contact
146 the beads with an ethanol solution (water:ethanol 50:50) for 24 hours under stirring and
147 for 10 minutes of ultrasonic treatment. After filtration, the solutions were analysed by
148 the LC-DAD-MS instrument as reported by Gardana et al. (2007). The chromatographic

149 system consisted of an Alliance 2695 (Waters, Milford, MA) equipped with a model
150 2996 (Waters) photodiode array detector and a triple quadrupole mass spectrometer
151 mod. Quattromicro (Micromass, Beverly, MA). Identification of propolis polyphenols
152 was achieved from a mother solution prepared by dissolving 10 mg of standard
153 polyphenols (Sigma-Aldrich), in 10 mL methanol. The working solutions were
154 prepared in the range of 0.5-50 µg/mL of Caffeic acid (CA), Ferulic acid (FA),
155 3,4-dimethyl-caffeic acid (DMCA), *p*-coumaric acid (pC), Chrysin (C), Galangin
156 (G) from standard solutions while pinocembrin (P), pinobanksin (Pb) and
157 pinobanksin-5-methyl-ether (Pb5ME) were assayed using pinocembrin.

158

159 **Antimicrobial activity assay**

160 Propolis antimicrobial activity was tested before and after encapsulation in the beads
161 employing two liquid cultures, in particular TSB (Tryptic Soy Broth) for bacteria and
162 MEB (Malt Extract Broth) for yeasts and moulds. The media were aliquoted (5 mL) in
163 tubes and sterilized at 118°C for 20 min. Propolis, either in crude or as beads, was
164 added after sterilization in order to obtain concentrations variable in the range 0-1
165 mg/mL. To avoid propolis sedimentation and favour the contact with microorganisms,
166 cultures were subjected to magnetic stirring (150 rpm). Assays performed with moulds
167 were set up in 100 mL Erlenmeyer flasks, each containing 10 mL MEB culture medium,
168 maintaining the same propolis and beads concentrations range and stirring.

169 Microorganisms were inoculated (1% v/v) in form of a cell or spore suspensions, in the
170 same culture medium, having an Optical Density (OD) at 600 nm of 0.300 ± 0.010 .
171 Cultures were incubated at $30 \pm 1^\circ\text{C}$, up to 72 h for bacteria and yeasts and up to 14
172 days for moulds.

173 Control cultures (named Positive Control- PC) were always set up, without propolis. To
174 determine propolis contribution to the increase of absorbance, series of tubes containing
175 liquid medium and propolis at the tested concentrations (named Blank Propolis – BP)
176 were also prepared.

177 Microbial growth for bacteria and yeasts was determined evaluating the increase in
178 absorbance (A_c) (OD 600 nm) with the 6705 UV/Vis Spectrophotometer (Jenway). The
179 autozero was done with the same base medium; cultures stirring was stopped 5 min
180 before evaluation, to favor propolis but not microbial sedimentation. For each
181 microorganism, growth in terms of absorbance (A_{mo}) was calculated as follows:

$$182 \quad A_{mo} = A_c - A_{BP}$$

183 Where: A_c is the sample absorbance; A_{BP} is the absorbance of the liquid medium
184 containing only propolis.

185 The Minimal Inhibitory Concentration (MIC) was defined as the lowest amount of
186 crude propolis or beads that inhibited microbial growth, with A_{mo} values obtained lower
187 than the initial absorbance or without significant difference ($p > 0.05$) from each other
188 by the Least Significant Different (LSD) test (Williams and Abdi, 2010).

189 Subsequent trials performed with *Staphylococcus aureus* were set up as mentioned
190 before; culture samples were taken at appropriate intervals and microbial growth (log
191 cfu/ml) determined employing the plate count technique. The polyphenols components
192 were identified inside culture samples. After incubation, cultures were centrifuged to
193 separate cell mass from culture filtrates which were subjected to HPLC analysis as
194 reported in section 2.4.

195

196 **Results and Discussion**

197 **Beads characterization**

198 Wet chitosan cross-linked beads produced with and without propolis are shown in Fig.
199 2. The successful loading of different quantity of propolis in chitosan beads resulted in a
200 visible change in colour from yellow (without propolis), to dark brown: the darker the
201 colour, the higher the concentration of propolis (from 2% to 10% wt) in chitosan beads.
202 The efficacy of the cross-linking process was determined by the beads solubility in
203 water that was found less than 10% after 24 h of contact, whereas more than 50%
204 solubilisation was observed in the case of the chitosan beads without the cross-linker.
205 A series of Scanning Electron Microscopy (SEM) pictures of the surface of dry chitosan
206 beads, with and without the active compound, is shown in Fig. 3(a). These
207 characterizations confirm the spherical shape of the dry active devices and highlight that
208 the presence of propolis determine the formation of a rough surface, visible at high
209 magnification (X 60K). The result of the encapsulation process is biopolymeric beads in
210 which propolis is dispersed in the chitosan according to the model presented in Fig.
211 3(b).

212 Size distribution was found to be different in wet and dry beads with and without
213 propolis, as shown in Fig. 4. In particular, wet beads either with or without propolis,
214 evidenced similar average capsule size diameter ($1650 \pm 20 \mu\text{m}$), Fig 4A, due to the
215 chitosan swelling phenomena presents in both types of beads. Desiccation obviously
216 produced a reduction in size dimension and such modification resulted in different
217 extent for beads with and without propolis. In fact, 40% of dry chitosan beads without
218 propolis had a mean diameter of about $500 \mu\text{m}$, that increases to an average diameter of
219 600 and $700 \mu\text{m}$ for 2% wt. and 10% wt. of propolis concentration in solution during
220 encapsulation (Fig.4B). The absence of water and the residual presence of propolis
221 molecules respectively in the two types of dry beads (with and without propolis),

222 resulted in a different steric hindrance and so in a different size of the beads in dry
223 conditions.

224 To better understand which active propolis molecules are present in the beads, and so to
225 evaluate the encapsulation quality, the identification of propolis polyphenols in the
226 chitosan beads was done, by HPLC analysis. The chromatogram reported in Fig. 5
227 evidenced no significant presence of phenolic acids, whereas a significant presence of
228 the main propolis phenolic esters and flavonoids was detected; this suggests that the
229 phenolic acids are not encapsulated in the beads probably due their polar nature that
230 leads to a greater affinity of the acids to the aqueous solution compared to the cross-
231 linked chitosan. Contrariwise, the flavonoids and the esters, due to their amphiphilic
232 nature, are able to interact with chitosan occupying areas of the beads structure.

233

234 **Crude propolis and chitosan-propolis beads antimicrobial activities**

235 Preliminary experiments were carried out in order to evaluate antimicrobial spectrum of
236 activity of crude propolis. Trials were performed in liquid cultures, employing bacteria,
237 yeasts and fungi selected among the most commons spoilage and/or pathogen
238 microorganisms that might be present in fresh food products. Obtained results are
239 reported in Table 1. Propolis was found to be active against *Staphylococcus aureus*
240 (MIC 0.2 mg/mL), *Listeria innocua* (MIC 0.6 mg/mL) and *Enterococcus faecalis* (MIC
241 1 mg/mL), all Gram-positive bacteria, slightly against the yeast *Yarrowia lipolytica*
242 (MIC 1 mg/mL after 72 h) and the moulds *Penicillium* and *Cladosporium* (MIC < 1
243 mg/mL only in the first 5 days), but not against *Bacillus*, Gram-negatives and yeasts in
244 general. Similar results were reported in the literature, employing propolis of different
245 geographical origin with differences in chemical compositions (Stepanovic et al., 2003;
246 Koru et al., 2007; Kujumgiev et al. (1999). To be noted that the propolis used in this

247 work has been standardised by applying a patented method of purification: this assures a
248 precise polyphenols profiles and permits to correlate microbiological activity to the pool
249 type of polyphenols and to their relative percentages. It is in fact well known that
250 different substance combinations are essential for propolis biological activity
251 (Stepanovic et al., 2003) (Table 2).

252 Considering that propolis concentration inside the dry beads, and consequently its
253 polyphenols content, is lower than in the crude propolis samples, it is possible to say
254 that beads generally present a similar spectrum of action respect to crude propolis
255 (Table 1). This means that propolis antimicrobial activity is maintained also after
256 encapsulation, drying and storage. Results also evidenced that propolis-chitosan
257 combination increased propolis spectrum of activity, with the contribution of chitosan
258 against Gram-negative bacteria (*Pseudomonas* and *E. coli*). This behaviour has also
259 been evidenced in literature with chitosan and other natural antimicrobials (Dutta et al.,
260 2009; Scazzocchio et al., 2006, Rodriguez-Nunez et al., 2012).

261

262 **Antimicrobial activity against *S. aureus* liquid cultures**

263 In view of the importance of *S. aureus* in food poisoning, this microorganism was
264 chosen for the prosecution of the research. Beads were added (different concentration
265 comparatively) in liquid TSB cultures immediately after the inoculum, and microbial
266 growth monitored at appropriate intervals in terms of total viable count (log CFU/mL).
267 Table 3 reports the obtained results. In control samples without beads, *S. aureus*
268 population was found to increase from 6.6 to 10.2 log cfu/mL in about 24 h incubation.
269 The presence of chitosan beads without propolis did not produce a statistically
270 significant growth reduction. Instead, propolis beads added even at low concentration
271 (0.7 mg/mL culture) were found to inhibit *S. aureus* growth, and after 30 h incubation

272 microbial population remained at the same level or even lower than the initial inoculum
273 ($5.9 \pm 0.3 - 6.9 \pm 0.4 \log \text{ cfu/mL}$).

274 Filtrates obtained by centrifugation of culture samples at 30 h incubation were analysed
275 through HPLC. The presence of peaks with the characteristic Retention Times (Rt) of
276 the most representative flavonoids (Chrysin, Pinobanksin and Galangin) of the crude
277 propolis, and their absence in control cultures with beads without propolis, indicates
278 that flavonoids are effectively released in the surrounding media, leading to the
279 hypothesis that only in this way they can play their antimicrobial activity. The
280 antimicrobial activity can thus be attributed to the presence of flavonoids in the beads
281 that are the polyphenolic components most represented also in samples of crude
282 propolis.

283

284 **Conclusions**

285 Obtained results evidenced that the membrane emulsification technique is a promising
286 encapsulation technology to create dry active devices. These devices are stable during
287 their storage time and maintain their activity until use when they release in a controlled
288 way. Moreover, the laboratory membrane technology applied in this work, that can be
289 considered as precursor of the membrane emulsification process, can be considered a
290 promising encapsulation technique for propolis, thanks to the possibility of using mild
291 (room temperature and no shear stress) and “green” (no toxic solvent) process
292 conditions on a resinous complex matrix that is not water soluble.

293 Propolis-chitosan beads were found to inhibit the growth of several microbial strains
294 selected among the most commons spoilage and/or pathogen microorganisms that might
295 be present in fresh food products. Beads were found to inhibit microbial growth of *S.*

296 *aureus* in liquid culture, and flavonoids were found to be more responsible of this
297 activity respect to phenolic acids that are not encapsulated.

298 The obtained chitosan-propolis beads represent an example for the creations of an
299 innovative antimicrobial delivery system to prevent the growth of pathogenic and
300 sometimes also spoilage bacteria in food applications. Beads should be posed in direct
301 contact with the surrounding mean to be active. This study, that describes the efficacy of
302 a device based on propolis, could be completed undergoing the device to a risk
303 assessment procedure. This could permit to consider the active solution by potential
304 users for a real food application, for example on the internal surface of trays or bottles.

305

306 **Acknowledgments**

307 Authors wish to thank Fondo Sociale Europeo, Regione Lombardia (Dote Ricerca) for
308 economical support; Dott.ssa Martina Scaglianti of Specchiasol S.r.l for technical
309 support.

310

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406 Table 1: Antimicrobial activity of crude propolis and chitosan-propolis beads obtained

407 with a chitosan solution containing 10% of propolis, and MIC (Minimum Inhibitory

408 Concentration, mg/mL) determination. Assays carried out in liquid culture.

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Microrganism	MIC crude propolis (mg/mL)	MIC chitosan-propolis beads (mg/mL)
<i>Bacillus cereus</i>	> 1.2	1
<i>Enterobacter agglomerans</i>	> 1.2	> 2
<i>Enterococcus faecalis</i>	1	> 2
<i>Escherichia coli</i>	> 1.2	1
<i>Listeria innocua</i>	0.6	1
<i>Ps. fluorescens</i> MIM 151	> 1.2	1
<i>Ps. fluorescens</i> MIM 153	> 1.2	1
<i>Staphylococcus aureus</i>	0.2	0.8
<i>Candida kefir</i>	> 1.2	> 2
<i>Yarrowia lipolytica</i>	0.2 (1 after 72 h)	1
<i>Kluyveromyces bulgaricus</i>	> 1.2	> 2
<i>Kluyver. marxianus</i> var. <i>lactis</i>	> 1.2	> 2
<i>Rhodotorula mucilaginosa</i>	> 1.2	> 2
<i>Aspergillus niger</i>	>1, different morphology	1, different morphology
<i>Penicillium notatum</i>	0.6 (> 1 after 5 days)	1.2
<i>Cladosporium cladosporioides</i>	0.6 (> 1 after 5 days)	1.2

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417 Table 2: Propolis composition in crude propolis dry extract as phenolic acid end esters
418 (no bold), and flavonoids (Bold) (polyphenols correspond to 23% of the crude propolis).
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Component	%
Caffeic acid	0.53
P-coumaric acid	0.44
Ferulic acid	0.44
Isoferulic acid	0.85
Caffeic acid dimethyl ether (DMCA)	1.02
Cinnamic Acid	0.26
Caffeic acid phenethyl ester (CAPE)	0.89
Chrysin	3.54
Pinocembrin	4.72
Pinobanksin-acetate	2.53
Pinobanksin-5-Methyl ether	1.68
Pinobanksin	3.62
Galangin	2.83

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422 Table 3. Time course of *S. aureus* growth (expressed as log cfu/mL) in liquid cultures in
 423 absence and presence of different concentration of beads.

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Beads added (mg/ml)	Log cfu/ml					
	0	6	7	24	26	30
0 (control)	6.6 ± 0.2	7.0 ± 0.5	7.3 ± 0.4	10.2 ± 0.7	10.3 ± 0.6	10.2 ± 0.8
+ 0.4	6.4 ± 0.3	6.9 ± 0.3	7.1 ± 0.2	8.3 ± 0.4	8.7 ± 0.4	9.4 ± 0.3
+ 0.7	6.5 ± 0.2	6.0 ± 0.4	6.5 ± 0.2	6.4 ± 0.3	6.7 ± 0.2	6.9 ± 0.4
+ 1.4	6.6 ± 0.4	6.5 ± 0.2	6.5 ± 0.5	6.2 ± 0.5	6.4 ± 0.5	6.3 ± 0.2
+ 2.8	6.4 ± 0.3	6.4 ± 0.2	6.1 ± 0.4	5.9 ± 0.3	5.9 ± 0.3	5.9 ± 0.3
+ 2.8 w/o*	6.4 ± 0.3	7.1 ± 0.3	7.7 ± 0.4	9.5 ± 0.5	9.3 ± 0.4	9.3 ± 0.5

425 * w/o: beads without propolis

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