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Additional Information

Evidence of viable *Helicobacter pylori* and other bacteria of public health interest associated with free-living amoebae in lettuce samples by next generation sequencing and other molecular techniques

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Abstract

Vegetables are one of the sources from which *Helicobacter pylori* can be acquired. This bacterium infects more than 50% of the global population and is a recognized type I human carcinogen. *H. pylori* enters into the viable but non-culturable state when it is in the environment, and therefore the use of molecular techniques is much convenient for its detection. Free-living amoebae (FLA) are protozoans found in vegetables. They are transmission vehicles for amoeba-resistant bacteria, among which *H. pylori* is included. The aim of this study is to study the occurrence and viability of *H. pylori* from lettuce samples*, H. pylori* internalized into FLA and the microbiome of FLA isolated from these samples. Special focus was pointed to human pathogenic bacteria.

H. pylori was not directly detected in any lettuce sample by means of molecular techniques and neither by culture. However, intra-amoebic *H. pylori* DNA was detected by means of PMA-qPCR in 55% of the samples and viable intra-amoebic *H. pylori* cells in 25% of the samples by means of DVC-FISH technique. When FLA microbiome was studied, 21 bacterial genera were part of FLA microbiome in all samples. *Helicobacter* genus was detected as part of the FLA microbiome in two samples. Other bacteria of public health interest such as *Aeromonas* sp., *Arcobacter* sp., *Legionella* sp., *Mycobacterium* sp., *Pseudomonas* sp. and *Salmonella* sp. were detected as part of FLA microbiome along the analysed samples.

This study demonstrates for the first time that *H. pylori* is internalized as well as alive inside FLA isolated from vegetables. Moreover, this study shows that FLA promote *H. pylori* detection in environmental samples. In addition, as far as we are aware, this is the first study which studies the microbiome of FLA isolated from vegetables. Among the FLA microbiome, bacteria of public health interest were detected, pointing out that FLA are carriers of these pathogens which can reach humans and cause a public health concern.

46 Keywords: lettuce; free-living amoebae; *Helicobacter pylori*; microbiome; metagenomics;
47 amoeba-resistant bacteria

48

49 **1. Introduction**

50 *Helicobacter pylori* is a microaerophilic Gram-negative bacteria which is estimated to infect
51 more than 50% of the global population. However, its prevalence is widely variable and depends
52 not only on the country or region, but also on the level of development, sanitation, access to
53 clean water and socioeconomic status of the population (Hooi et al., 2017). Infection in humans
54 can lead to the development of chronic gastritis, peptic ulcer, gastric lymphoma and gastric
55 cancer (Marshall, 2002). In fact, this bacterium was recognized in 1994 by the WHO as a type I
56 human carcinogen.

57 The transmission route of *H. pylori* has been strongly debated and is not yet clearly established
58 (Eusebi et al., 2014; Percival and Thomas, 2009). It is suggested that it can be acquired by person
59 to person contact through faecal-oral (Mladenova et al., 2006), oral-oral (Cellini et al., 2010) and
60 gastric-oral routes and also by water (Bai et al., 2016; Santiago et al., 2015) and food
61 consumption (Atapoor et al., 2014: Yahaghi et al., 2014). Among food, vegetables have been
62 extensively studied as vehicles for *H. pylori* transmission. Atapoor et al. (2014) analysed 460
63 samples of vegetables, such as basil, spinach or lettuce, and detected *H. pylori* by means of qPCR
64 and culture in 10.86% and 9.56% of the samples, respectively. Moreover, Yahaghi et al. (2014)
65 analysed 430 samples and showed the presence of *H. pylori* by culture in 13.68% of vegetables
66 and in 14% of commercial salads.

67 When in adverse environmental conditions, *H. pylori* enters into the viable but non-culturable
68 (VBNC) state, which leads to metabolic, morphological and growth behavioural changes
69 (Azevedo et al., 2007; Nilsson et al., 2002). This is believed to be a survival strategy that
70 contributes to maintain an important pathogenic bacteria reservoir in the environment, which
71 could pose a significant public health risk (Lleò et al., 2007). Different authors have shown that
72 VBNC cells of *H. pylori* are able to revert their state and grow on synthetic media again, thus
73 proving their infectivity (Andersen et al., 1997; Cellini et al., 1998; Kurokawa et al., 1999). More

74 recently, Richards et al. (2011) quantified RNA and *cdrA* gene levels, which are related to cellular
75 division, to show the resuscitation of VBNC cells of *H. pylori*. In VBNC state, *H. pylori* is not able
76 to grow on culture media although it keeps its viability and pathogenicity (Signoretto et al.,
77 2000). Therefore, it cannot be detected by conventional culture methods and molecular
78 techniques such as quantitative polymerase chain reaction (qPCR) or fluorescent *in situ*
79 hybridization (FISH) should be used instead. However, since the bacterium has been detected
80 by culture from environmental samples, it has been suggested that its survival may be enhanced
81 by other factors, such as being part of biofilms or associated with Free-Living Amoebae (FLA) (Ng
82 et al., 2017; Sarem and Corti, 2015; Winiecka-Krusnell et al., 2002).

83 FLA are ubiquitous protozoa which are proven transmission vehicles for a wide range of
84 pathogens, such as *Legionella* spp. (Moreno et al., 2019) or *Campylobacter jejuni* (Olofsson et
85 al., 2013) among others. In fact, they are called "Trojan horses" for these amoeba-resistant
86 bacteria (ARB) (Barker and Brown, 1994).

87 The presence of FLA in vegetables has not been as widely studied as, for example, their presence
88 in water. However, FLA have been detected in lettuce and other vegetables (Chavatte et al.,
89 2016; Vaerewijck et al., 2011). Interestingly, Vaerewijck et al. (2010) showed that FLA were
90 present in domestic refrigerators, especially in vegetable trays, where they found FLA more
91 frequently and with the highest diversity. This is an indicator that both FLA are present in
92 vegetables and they can be transferred to surfaces.

93 On the other hand, the relationship between *H. pylori* and FLA has been previously studied by
94 our group *in vitro* (Moreno-Mesonero et al., 2016) and in wastewater and drinking water
95 samples (Moreno-Mesonero et al., 2017). Nevertheless, it has never been studied in vegetable
96 samples.

97 Finally, there is only one study about the composition of FLA microbiome. Delafont et al. (2013)
98 isolated FLA from drinking water sources and analysed their microbiome by next generation

99 sequencing (NGS) technology. They established the bacterial genera which were part of the FLA
100 microbiome in their samples and also described genera which had not been previously related
101 to FLA. Nowadays, the NGS technology commonly used to characterize the microbial diversity is
102 the amplicon-based metagenomics, in which the hypervariable regions of the *16S rRNA* gene are
103 amplified and sequenced. *16S rRNA* gene consists of hypervariable regions flanked by highly
104 conserved regions. The former allow differentiating between different microorganisms and
105 classify them taxonomically, and the latter are useful for the design of primers (Huse et al., 2008;
106 Sharpton, 2014).

107 Taking all this into account, the aim of this investigation was to study the presence of *H. pylori*
108 in vegetable food samples, the occurrence and viability of *H. pylori* internalized into FLA isolated
109 from vegetables and to establish the microbiome of these FLA. Special focus was pointed to
110 human pathogenic bacteria.

2. Material and methods

2.1. Samples

A total of 20 lettuces (*Lactuca sativa*) were bought from different local shops located at Eastern Spain. They were individually processed within an hour and analyzed to study the presence of *H. pylori*, *H. pylori* associated with FLA and bacterial microbiome of FLA.

2.2. Detection of *H. pylori*

Ten grams of the outer leaves of each lettuce sample were placed in sterile stomacher bags which contained 90 ml of Brucella broth (Biolife Italiana, Italy). Samples were homogenized for 1 minute at maximum speed. Then, the presence of *H. pylori* in the homogenate was analyzed by means of qPCR, DVC-FISH and culture.

2.2.1. qPCR

DNA was extracted from a 1 ml aliquot of each homogenized sample using the GeneJet™ genomic DNA purification kit (ThermoScientific, Germany), following the Gram-negative bacteria protocol. The final elution step was performed using 50 µl of elution buffer.

H. pylori qPCR based on SYBR®Green I fluorescence dye was subsequently performed using VacA primers to amplify a 372 bp fragment (Nilsson et al., 2002) in LightCycler® 2.0 Instrument (Roche Applied Science, Spain). The final reaction volume of 20 µl contained: 2 µl of LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science, Spain), 1.6 µl of $MgCl_2$ (25 mM stock solution), 0.5 µl of each primer (20 µM stock solution) and 2 µl of DNA template. The amplification consisted of an initial DNA denaturalization step at 95°C for 10 min, followed by 40 cycles of: 95°C for 10 s, 62°C for 5 s and 72°C for 16 s; and finally, one cycle at 72°C for 15 s and one at 40°C for 30 s (Santiago et al., 2015). A positive control with *H. pylori* DNA and a control of external contamination (qPCR mix without DNA) were added to each qPCR analysis. Samples

135 were analysed in duplicate. The quantification cycle (Cq) value along with a standard curve were
136 used to calculate the quantification of the number of DNA copies (genomic units, GU) of *H.*
137 *pylori*, as previously described by Santiago et al. (2015).

138 **2.2.2. DVC-FISH**

139 DVC-FISH analysis was performed according to Piqueres et al. (2006) from a 1 ml aliquot of each
140 homogenized sample. Each aliquot was incubated for 24 hours at 37°C under microaerophilic
141 conditions (5% O_2, 10% CO_2 and 85% N_2) in 9 ml of DVC broth (Brucella broth supplemented with
142 5% fetal bovine serum (FBS) and 0.5 mg/l of Novobiocin antibiotic). After the incubation, DVC
143 tubes were centrifuged at 8000 rpm for 8 min and resuspended in 1 ml of PBS.

144 Afterwards, samples were immediately fixed with 4% paraformaldehyde for 3 h at 4°C, washed
145 with PBS and resuspended in ethanol and PBS (v/v 1:1). Slides were dehydrated by successive
146 immersions in 50%, 80% and 100% ethanol for 3 min each. Then, each well was covered with
147 10 ml of hybridization buffer (0.9 M NaCl, 20 mM HCl-Tris, 0.01% SDS and 40% formamide, pH
148 7.5) containing 50 ng of each probe. The reaction was carried out under darkness at 46 °C for
149 1.5 h.

150 A combination of three EUB338 probes, complementary to a region of the *16S rRNA* Eubacteria
151 domain was used as a positive control (Moreno et al., 2003). For the specific detection of *H.*
152 *pylori*, a LNA probe was used: LNA-HPY: 5’- CTG GAG AGA C+ TA AGC CC+ T CC-3’ (Piqueres et
153 al., 2006).

154 After hybridization, slides were washed under darkness at 48°C for 15 min with 50 ml of washing
155 solution (0.10 M NaCl, 0.02 M HCl-Tris, 0.01% SDS and 0.005 M EDTA). Finally, they were washed
156 with distilled water and air-dried under darkness. Slides were mounted with FluoroGuard
157 Antifade Reagent (Bio-Rad, Spain) between the coverslip and the slide. They were visualized
158 using an Olympus BX 50 fluorescence microscope with the filters U-MWB, U-MWIB and U-

159 MWIG. Photographs were taken with an Olympus DP-12 camera. A pure culture of DVC-
160 incubated *H. pylori* cells was used as a positive control of the reaction.

161 **2.2.3. Culture**

162 In order to isolate culturable *H. pylori* cells, 100 µl of each homogenized sample were spread in
163 Agar Dent plates (AD; Campylobacter selective agar (Merck, Spain) containing 10% (v/v)
164 defibrinated horse blood (Oxoid, United Kingdom) and *Helicobacter pylori* selective supplement
165 Dent (Oxoid, United Kingdom)). Plates were incubated for 3-5 days under microaerophilic
166 conditions (5% O_2, 10% CO_2 and 85% N_2). Suspicious *H. pylori* colonies were picked and
167 suspended in 200 µl PBS. Subsequently, DNA was extracted using the GeneJet™ genomic DNA
168 purification kit (ThermoScientific, Germany) following the Gram-negative bacteria protocol and
169 qPCR was performed as explained in section 2.2.1.

170

171 **2.3. Study of the presence of *H. pylori* associated with FLA in lettuces**

172 A total of 10 g of the outer leaves of each lettuce sample were homogenized in stomacher for 1
173 minute at maximum speed with 90 ml of Page's amoeba saline medium (PAS). Homogenates
174 were then filtered through nitrocellulose filters with 3 µm of pore size (Whatman, Maidstone,
175 England). Filters were aseptically transferred to a non-nutrient agar plate (NNA, bacteriological
176 agar dissolved in PAS solution (Iovieno et al., 2010)), which were incubated at 28°C. After 24 h,
177 filters were removed and plates were kept at 28°C for up to 30 days. NNA plates were monitored
178 daily by inverted and phase contrast microscopy until FLA growth was observed.

179 Once FLA growth was observed, NNA plate's content was collected adding PAS and using a sterile
180 cell scraper. Content was centrifuged at 500 g for 3 min and resuspended in 500 µl of phosphate
181 buffer saline (PBS). Thereafter, a 100 ppm sodium hypochlorite solution was added to each
182 sample and kept for 1 h in order to kill extra-amoebic bacteria. After this time, sodium

183 hypochlorite was removed by washing the samples three times by means of centrifugation at
184 500 g for 3 min (Moreno-Mesonero et al., 2016). Finally, sediments were resuspended in 1 ml of
185 PBS, and the solution was used to detect *H. pylori* from inside any FLA present in the samples by
186 means of PMA-qPCR, DVC-FISH and culture.

187 **2.3.1. PMA-qPCR**

188 In order to detect intra-amoebic *H. pylori* cells, 500 µl of the hypochlorite-treated samples
189 resuspended in PBS were treated with propidium monoazide (PMA) to prevent amplification
190 signals from non-internalized non-viable *H. pylori* DNA (Moreno-Mesonero et al., 2016). PMA
191 was dissolved in PCR grade water following the manufacturer's instructions, to obtain a stock
192 concentration of 2 mM. To reach a final concentration of 50 µM, 12.5 µl of PMA were added to
193 each aliquot. Samples were incubated for 10 min under darkness with occasional mixing to allow
194 better reagent penetration into cells with damaged membranes. Thereafter, samples were
195 exposed to high power LED light for 15 min using the photo activation system PhAST Blue
196 (GenIUL, Spain). Samples were then centrifuged at 14,000 rpm for 5 min and resuspended in 200
197 µl of PBS (Agustí et al., 2010).

198 Afterwards, DNA was extracted using the GeneJet™ genomic DNA purification kit
199 (ThermoScientific, Germany) following the mammalian tissue protocol instructions, with the
200 exception of the incubation time at 56°C, which was increased from 10 min to 30 min (Moreno-
201 Mesonero et al., 2016).

202 The subsequent *H. pylori*-specific qPCR was performed as explained in 2.2.1 section.

203 **2.3.2. DVC-FISH**

204 To detect viable intra-amoebic viable *H. pylori* cells, DVC-FISH analysis was performed according
205 to Piqueres et al. (2006) to 400 µl of the hypochlorite-treated samples. Aliquots were incubated
206 for 24 hours at 37°C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) in 3.6 ml of

207 DVC broth previously described. After the incubation, DVC tubes were centrifuged at 8000 rpm
208 for 8 min and resuspended in 1 ml of PBS. Thereafter, samples were fixed, hybridized and
209 visualized as explained in 2.2.2 section.

210 **2.3.3. Culture**

211 One hundred microlitres of the hypochlorite-treated samples were spread in AD plates in order
212 to detect the presence of living culturable *H. pylori* cells present inside FLA. Samples were
213 incubated for 3-5 days at 37°C under the *H. pylori* specific microaerophilic conditions detailed
214 above. Suspicious *H. pylori* colonies were picked and suspended in 200 µl of PBS. Subsequently,
215 DNA was extracted using the GeneJet™ genomic DNA purification kit (ThermoScientific,
216 Germany) following the Gram-negative bacteria protocol and qPCR was performed as explained
217 in section 2.2.1.

218

219 **2.4. Study of the bacterial microbiome of FLA**

220 Bacterial microbiome of FLA was investigated in six of the processed samples. To do this,
221 extracted DNA from FLA-positive cultures (section 2.3.1) was sequenced by FISABIO Sequencing
222 and Bioinformatics Services (Valencia, Spain) by means of Illumina sequencing platform. The
223 amplicon library was prepared following the 16S Metagenomic Sequencing Library Preparation
224 (Part # 15044223 Rev. B) using the recommended set of primers, which target the *16S rRNA* V3-
225 V4 regions and amplify a single amplicon of around 460 bp (Klindworth et al., 2013). After the
226 amplification, amplicons were sequenced on a MiSeq sequencer using the automated cluster
227 generation and paired-end sequencing with dual indexes reads (2 × 300 bp run).

228 Illumina data were analyzed by using QIIME 1.9.1 (http://qiime.org; Caporaso et al., 2010),
229 applying additional scripts available in Microbiome Helper virtualbox (Comeau et al., 2017). As
230 a first step, forward and reverse reads were merged using PEAR v0.9.19 (Zhang et al., 2014).

231 FastQC (Andrews, 2010) was used to confirm that reads were correctly stitched. Subsequently,
232 stitched reads were filtered by length and quality score (reads with less than 200 bp or a
233 minimum of Q30 over at least 90% of the reads were removed) using FASTX-Toolkit v0.0.14
234 (Gordon, 2009). Reads with any ambiguous bases ("N") were also filtered out. After an additional
235 examination with FastQC the low quality tails of the merged sequences were removed. Potential
236 chimeric sequences were screened out using VSEARCH v1.11.1. (Rognes et al., 2016). The
237 remaining sequences were processed using the QIIME's open reference script, applying the
238 methods SortMeRNA v2.0 (Kopylova et al., 2012) and SUMACLUST v1.0.00 (Mercier et al., 2013)
239 for the reference-based and *de novo* clustering steps, respectively. Operational Taxonomic Units
240 (OTUs) were defined at 97% genetic similarity cut-off. The SILVA v132 ribosomal database (Quast
241 et al., 2013) was used to perform the taxonomy assignation.

242

243 **3. Results**

244 **3.1. Occurrence of *H. pylori***

245 *H. pylori* presence was not detected in any lettuce sample by means of the molecular techniques
246 qPCR and DVC-FISH. No *H. pylori* colonies were either recovered from the culture media
247 (Table1).

248

249 **3.2. Occurrence of *H. pylori* internalized into FLA**

250 FLA were present in 100% of the processed lettuce samples (20/20). After carrying out the
251 hypochlorite disinfection treatment, intra-amoebic *H. pylori* DNA was detected in 55% of the
252 samples (11/20) by means of PMA-qPCR. When DVC-FISH method was applied, viable intra-
253 amoebic *H. pylori* cells were detected in 25% (5/20) of the lettuce samples. However, no intra-
254 amoebic *H. pylori* colony was recovered from any of the processed samples (Table 1).

255

256 **3.3. Bacterial microbiome of FLA**

257 The six analyzed samples were positive for intra-amoebic *H. pylori* detection by means of PMA-
258 qPCR (samples 1, 2, 5, 8, 9 and 10). A total of 237,266 raw sequences were obtained after
259 Illumina MiSeq sequencing procedure. Once quality filtering, trimming and chimeras detection
260 were carried out, a total of 201,740 high-quality reads remained, which were clustered into
261 2,165 OTUs, taxonomically assigned at 97% similarity threshold.

262 Most abundant bacterial phyla associated with FLA in the average of the samples were
263 Proteobacteria (59.75%) and Bacteroidetes (38.69%), which represented 98.44% of the total
264 bacterial microbiome of FLA. When analysed individually, the most abundant phyla of each
265 lettuce sample were also Proteobacteria and Bacteroidetes, but at different relative abundances

266 (Figure 1). Bacteria belonging to Epsilonbacteraeota phylum were only detected in samples 1
267 and 8 at relative abundances of 0,003 and 0,032%, respectively.

268 The most abundant bacterial genera present in FLA in the average of all analysed samples were
269 *Massilia* (13.25%), *Fluviicola* (10.33%), *Flavobacterium* (10.08%), *Siphonobacter* (8.44%),
270 *Pseudorhodoferax* (6.76%), *Stenotrophomonas* (5.98%), *Achromobacter* (5.26%), *Delftia* (3.64%)
271 and *Pseudoxanthomonas* (3.22%), which represented 66.96% of the total FLA microbiota. The
272 relative abundances of the most abundant genus (> 2%) along each individual sample are
273 represented in figure 2. The most abundant genus in sample 1 was *Siphonobacter* sp.; in samples
274 2, 5 and 8 it was *Massilia* sp.; in sample 9, it was *Flavobacterium* sp., and in sample 10, the most
275 abundant genus was *Fluviicola* sp. Among all detected genera, 21 of them were present in all
276 samples: *Achromobacter* sp., *Acidovorax* sp., *Aquabacterium* sp., *Comamonas* sp., *Cupriavidus*
277 sp., *Delftia* sp., *Dyadobacter* sp., *Flaviaesturariibacter* sp., *Flavisolibacter* sp., *Massilia* sp.,
278 *Noviherbaspirillum* sp., *Pajaroellobacter* sp., *Parasegetibacter* sp., *Pigmentiphaga* sp.,
279 *Pseudomonas* sp., *Pseudorhodoferax* sp., *Pseudoxanthomonas* sp., *Rhizobacter* sp.,
280 *Stenotrophomonas* sp., *Taibaiella* sp. and *Variovorax* sp.

281 Relative abundances of bacteria of public health interest are detailed in table 2. Sample 10 had
282 the greatest amount of these bacteria (0.934%) followed by sample 9 (0.814%). *Helicobacter*
283 genus was only detected in samples 1 and 8 at low relative abundances, 0.003% and 0.011%,
284 respectively, despite it had been previously detected in all samples by means of qPCR.

285

286 **4. Discussion**

287 Vegetables which are eaten raw are potential vehicles for the transmission of any type of
288 pathogen able to cause human illnesses (Lynch et al., 2009). In this study, lettuce samples were
289 analysed in order to determine the occurrence and viability of *H. pylori* in the samples, *H. pylori*
290 internalized into FLA and the microbiome of FLA isolated from this type of samples.

291 Among the bacterial pathogens found in vegetables, the presence of *H. pylori* has been
292 previously demonstrated (Atapoor et al., 2014; Yahaghi et al., 2014). In this study, the presence
293 of *H. pylori* in lettuce samples could not be demonstrated when samples were directly analysed,
294 neither by means of molecular techniques nor by culture (Table 1). Some authors have
295 demonstrated that *H. pylori* is able to form biofilms and micro-colonies on different vegetables
296 and thus prolong its survival, but this ability is strain and vegetable dependent (Ng et al., 2017).
297 The fact that no *H. pylori* was detected when lettuce samples were directly analyzed suggests
298 that in this case the bacteria is not forming biofilms to enhance its survival. However, when the
299 homogenate of lettuce samples was incubated into NNA plates to isolate FLA, it was possible to
300 detect *H. pylori* by means of qPCR and DVC-FISH as part of FLA microbiome. Amoebal enrichment
301 has been previously used by some authors to isolate bacteria that otherwise could not have
302 been isolated (Jacquier et al., 2013; Lienard and Greub, 2011). In this study, the FLA already
303 present in the samples may have helped to detect *H. pylori* by means of molecular techniques
304 after cultivation.

305 The processing method used in this study to isolate FLA allowed for performing the analysis of
306 10 g of lettuce samples, since all homogenized volume was filtered. Other authors generally use
307 less sample quantity to evaluate the presence of protozoa in vegetable samples. Vaerewijck et
308 al. (2011) homogenized a single lettuce leaf using stomacher in a nine-fold dilution of PAS and,
309 afterwards, they only used 1 ml of the homogenate to carry out the free-living protozoa
310 isolation. In the same study, authors also homogenized single lettuce leaves with 50 ml of PAS

311 in stomacher and only used 30 ml to isolate free-living protozoa. Chavatte et al. (2016) carried
312 out the same homogenization procedure as the one used in the present study, i.e.,
313 homogenization of 10 g of vegetables with 90 ml of PAS, in this case for 2 minutes, but they only
314 used 1 ml of the homogenate to isolate free-living protozoa from vegetable sprouts.
315 Furthermore, Gourabathini et al. (2008) washed lettuce and spinach leaves in a saline solution
316 to subsequently transfer aliquots into NNA to isolate FLA. The fact that in our study a large
317 quantity of each lettuce (10 g/sample) was analyzed could have been one of the reasons why all
318 samples turned out to be positive for FLA isolation.

319 Nowadays there are very few studies in which the presence of free-living protozoa, and more
320 specifically of FLA, is investigated in vegetable samples. Vaerewijck et al. (2011) analyzed the
321 presence of protozoa and FLA in lettuce samples. In their study, all samples contained free-living
322 protozoa after one week of incubation period. Gourabathini et al. (2008) studied the protozoa
323 prevalence in spinach and lettuce samples in order to investigate their interaction with the
324 pathogenic bacteria *Salmonella enterica*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*.
325 These authors found protozoa in 78% of the analyzed samples but with little abundance of FLA.
326 They proposed that FLA abundance was underrepresented, due to the fact that they tend to
327 strongly adhere to surfaces, what suggests that a vigorous homogenization step, such as
328 stomacher homogenization, is more suitable than the washing method used by the authors.
329 Chavatte et al. (2016) also analyzed the presence of free-living protozoa from vegetable sprouts
330 and found that 68% of the samples contained FLA.

331 The presence of intra-amoebic *H. pylori* was evaluated in all lettuce samples since the isolation
332 of FLA was positive in all of them. The techniques used in this study had previously proved to be
333 effective to assess the presence and viability of intra-amoebic *H. pylori* cells, both *in vitro*
334 (Moreno-Mesonero, 2016) and in water samples (Moreno-Mesonero, 2017).

335 qPCR technique was used instead of conventional PCR due to the fact that the former is much
336 faster and sensitive than the latter. However, the quantification of *H. pylori* DNA data obtained
337 by qPCR was of no use for determining the level of contamination of the samples, since a
338 cultivation (and thus, enrichment) step was carried out in NNA plates.

339 The viability of intra-amoebic *H. pylori* cells was confirmed by means of DVC-FISH technique in
340 5 out of the 11 samples which contained internalized cells of this bacterium. This is likely to be
341 due because not all *H. pylori* internalized cells are viable. However, although internalized *H.*
342 *pylori* cells viability was confirmed by the molecular technique DVC-FISH, no colonies were
343 recovered from culture media. This may be due because accompanying microbiota inhibits *H.*
344 *pylori* growth or because these bacterial cells are in their VBNC state.

345 To our knowledge, this is the first study in which the presence of a pathogenic bacteria from
346 inside FLA in vegetable samples is investigated. The obtained results demonstrate that *H. pylori*
347 is able to be inside as well as alive in the FLA present in lettuce samples, thus evading their
348 fagocitation mechanisms. In this study, it has been evidenced that the bacterium is able to
349 survive for long periods of time under aerobic conditions and keep its viability when protected
350 by FLA (Moreno-Mesonero et al., 2017). These results support the hypothesis that FLA could
351 play an important role in *H. pylori* transmission to human beings through the consumption of
352 raw vegetables that, in some cases, are poorly washed or disinfected.

353 Aside from studying internalized *H. pylori* cells into FLA, in this investigation it was carried out
354 the first approach of the FLA microbiome present in lettuce samples. In this study, the
355 microbiome of FLA present in 6 lettuce samples previously analysed for the presence of intra-
356 amoebic *H. pylori* was determined. The technique amplicon-based metagenomics along with a
357 set of primers and conditions able to detect this bacterium was used. All samples were analysed
358 using the same DNA as the one used to study the presence of intra-amoebic *H. pylori*. Therefore,
359 it is reasonable to infer that identified bacteria are probably part of FLA microbiome. However,

360 since amplicon-based metagenomics detects DNA, and in this case DNA from inside FLA, it could
361 also be identifying residual DNA of phagocytised bacteria, as suggested by other authors
362 (Delafont et al., 2013).

363 As far as we know, there is only one previous study about FLA microbiome in which
364 metagenomics methodology was used. However, in that case FLA were isolated from drinking
365 water samples and both metagenomics sequencing platform as well as primers were different
366 from the ones used in the present study (Delafont et al., 2013). They found that most of the
367 identified bacteria belonged to Proteobacteria and Bacteroidetes phyla, which coincides with
368 the results obtained in the present study at phylum level. Within the detected genera in all
369 samples, *Acidovorax, Pseudomonas* and *Stenotrophomonas* have been previously found to
370 interact with FLA (Corsaro et al., 2012; Maschio et al., 2015; Thomas et al., 2008).

371 Until recently, bacteria belonging to *Helicobacter* genus were classified under the phyla
372 Proteobacteria and the class Epsilonproteobacteria. While this class is a monophyletic group
373 which is stable within the bacterial life tree, different studies suggest that they do no affiliate in
374 a reproducible way with other Proteobacteria, thus implying that a taxonomic review at Phylum
375 level is needed (Di Rienzi et al., 2013; Hug et al., 2016; McLean et al., 2013; Rinke et al., 2013;
376 Wu et al., 2009; Yeoh et al., 2016; Zhang and Sievert, 2014). In fact, Waite et al. (2017) suggest
377 reclassifying the classes Epsilonproteobacteria and Desulfurellales as a new phylum,
378 Epsilonbacteraeota, which would lead to changes in their taxonomic classification in the lower
379 taxonomic levels. This proposal is reflected in the taxonomy of the latest version of the SILVA
380 database (v132), which was the one used in this study.

381 Although by means of PMA-qPCR *H. pylori* had been detected in all analysed samples, by means
382 of amplicon-based metagenomics it was only detected in two samples. This may be due to the
383 fact that the relative abundance of this bacterium is very low in each sample, and other more
384 abundant DNAs may have had a preferential amplification, since universal bacterial primers

385 were used. Therefore, in order to study the most abundant bacterial groups which are part of
386 FLA microbiome, amplicon-based metagenomics is an adequate technique. However, if the aim
387 is to study the presence of a specific bacterium, which is expected to be present at low
388 abundances, other molecular techniques, such as qPCR of FISH should be used.

389 Some of the identified genera in this study are of public health interest because they contain
390 species which are human pathogens (Table 2). Among all genera, *Pseudomonas* sp. had the
391 greatest relative abundance. Although by means of amplicon-based metagenomics the different
392 species of the *Pseudomonas* genus could not be identified, this genus contains pathogenic
393 species. One of these is *Pseudomonas aeruginosa*, which is an opportunistic human pathogen
394 able to adapt to a wide variety of environments (Gellatly and Hancock, 2013). Infections caused
395 by this bacterium are nosocomial and most of them are associated with compromised defense
396 hosts (Lyczak et al., 2000). *P. aeruginosa* has been previously related to FLA and identified as
397 ARB (Cengiz et al., 2000; Maschio et al., 2015). The other bacteria of public health concern had
398 lower relative abundances and were not present in all analyzed samples. The different species
399 of *Aeromonas* cause a wide range of illnesses in both humans and animals (Ghenghesh et al.,
400 2008). They have been associated with acute diarrheas in immunocompetent adults, chronic
401 diarrheas in children, elderly and immunocompromised individuals and with travelers' diarrhea
402 (Batra et al., 2016). Moreover, *Aeromonas* sp. has been also previously described as able to
403 interact *in vitro* with the FLA *A. castellanii* (Rahman et al., 2008; Yousuf et al., 2013). *Arcobacter*
404 sp. has been detected as part of the FLA microbiome in one sample. This genus contains 28
405 species, among which human and pathogenic species can be found. They are foodborne
406 pathogens which cause enteritis and bacteraemia in humans (Ferreira et al., 2019) and have also
407 been related to FLA (Medina et al., 2014; Villanueva et al., 2016). Bacteria of the genus *Legionella*
408 have been widely described as ARB and in this study they have been detected as part of the FLA
409 microbiome in one sample. This genus comprises more than 50 species, among which at least
410 20 are associated with human disease (Burstein et al., 2016). It is an opportunistic pathogen

411 which causes legionellosis to mainly immunocompromised individuals. *Mycobacterium* spp. was
412 found to be part of FLA microbiome in three samples. This genus includes species which cause
413 human illnesses with great morbidity and mortality worldwide, such as tuberculosis, leprosy or
414 mycobacteriosis. Moreover, the species *Mycobacterium avium* has been identified as ARB
415 (Samba-Louaka et al., 2018; White et al., 2010). Finally, *Salmonella* spp. has been detected in
416 two samples as part of FLA microbiome. It causes infections to humans which produces enteric
417 or typhoid fever and diarrheal disease (Gordon, 2008) and different species have been also
418 identified as ARB (Gaze et al., 2003; Tezcan-Merdol, 2004).

419 Obtained results in this study support Hsueh and Gibson (2015) hypothesis, who proposed that,
420 since FLA are frequently found in the environment and due to their established relationship with
421 foodborne pathogens, it is possible that FLA containing foodborne pathogens could enter into
422 fresh produce production supply at the point of production, harvest or processing. This way, an
423 adequate disinfection treatment able to remove them is essential to guarantee food safety.

424 To our knowledge this study demonstrates for the first time that *H. pylori* is internalized as well
425 as alive inside FLA isolated from vegetables. Moreover, this study shows that FLA promote *H.*
426 *pylori* detection in environmental samples. In addition, as far as we are aware this is the first
427 study which investigates the microbiome of FLA isolated from vegetables. Among the FLA
428 microbiome, bacteria of public health interest were detected, pointing out that FLA are carriers
429 of these pathogens which can reach humans and cause a public health concern.

430

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440 **Declaration of competing interest**

441 The authors declare that they do not have any competing interest.

442

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683 **TABLE LEGENDS:**

684 Table 1: Occurrence of *H. pylori* and *H. pylori* internalized into FLA isolated from lettuce samples

685 Table 2: Relative abundances of bacteria of Public Health interest in FLA microbiome

686

687 **FIGURE LEGENDS**

688 Figure 1: Relative abundances of the most abundant Phyla

689 Figure 2: Relative abundances of the most abundant genera