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Artificial diet delivery system for *Philaenus spumarius*, the European vector of *Xylella fastidiosa*

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1 **Abstract**

2 Artificial diets represent an essential tool for investigations on intimate
3 relationship between plant pathogens and their vectors. Previous research failed
4 in devising an artificial diet delivery system for the meadow spittlebug *Philaenus*
5 *spumarius*, to date considered the most important vector of the bacterium *Xylella*
6 *fastidiosa* in Europe. Here we describe a new delivery “tube-system” by which
7 we succeeded in artificial feeding of *P. spumarius* with holidic diets (one sucrose-
8 diet and two amino-acids diets). Spittlebug probing and feeding behavior on either
9 the tube-system, or a traditional “flat-system” realized out of a small Petri dish
10 filled with diet and covered with stretched Parafilm[®], was observed in real-time
11 by video-EPG (Electrical Penetration Graph), in order to assess the occurrence of
12 ingestion and excretion. Moreover, we evaluated *P. spumarius* survival on either
13 the tube-system filled with the two holidic diets that gave the best EPG results, or
14 an empty tube-system serving as control. Contrary to the flat-system, where just
15 brief stylet insertions through the Parafilm[®] were recorded, the spittlebug ingested
16 the artificial diets when delivered with the tube-system. Survival on the diets
17 provided with the tube-system was significantly greater than the control, with no
18 differences between the diets tested. Furthermore, the tube-system was suitable
19 also for another spittlebug species shown to be a competent vector of *X. fastidiosa*,
20 i.e. *Neophilaenus campestris*. The tool we devised opens new perspectives for
21 investigations on *X. fastidiosa*/spittlebugs interactions, as well as for the
22 functional analysis of mutant *X. fastidiosa* strains in respect to insect colonization
23 and transmission.

24

25 **Key words**

26 Vector-borne plant pathogens; insect vectors; spittlebugs; EPG; probing and
27 feeding behavior; artificial feeding.

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29 Introduction

30 As others vector-borne plant pathogens, the bacterium *Xylella fastidiosa* Wells
31 (1987) “lives in two worlds” (Chatterjee et al., 2008), being capable of explore
32 and exploit two different hosts, the plant and the insect vector. Consequently, the
33 set-up of a long-term sustainable bacterium control strategy requires a deep
34 understanding of the intimate bacterium-vector-host plant interactions. Artificial
35 diet systems are useful to study how plant pathogens interact with their respective
36 vectors, excluding host plant-vector interactions (Mitsuashi, 1979; Killiny and
37 Almeida, 2009). For example, essential data on leafhoppers probing behavior and
38 plant pathogen transmission mechanisms have been gathered through the
39 application of artificial diets (Carter, 1927; Severin and Swezy, 1928; Storey,
40 1932; Crane, 1971; Mitsuashi and Koyama, 1971; Kawabe and McLean, 1978;
41 Triplehorn et al., 1984; Joost et al., 2006; Killiny and Almeida, 2009). *X.*
42 *fastidiosa* is restricted to the xylem; xylem-sap feeding habit is apparently the only
43 characteristic shared by its vectors, namely sharpshooters (Hemiptera:
44 Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Cercopoidea) (Purcell,
45 1990; Redak et al., 2004; Esteves et al., 2018). Sharpshooters are considered the
46 main vectors of *X. fastidiosa* throughout the American continent and Taiwan
47 (Almeida et al., 2005; Tuan et al., 2015). On the contrary, spittlebugs are likely to
48 play the main role in bacterial epidemiology in Europe (Cornara et al., 2018a).
49 Indeed, the meadow spittlebug *Philaenus spumarius* L. (1758) (Hemiptera:
50 Aphrophoridae) proved to be the main vector of *X. fastidiosa* in olive orchards of
51 Southern Italy (Saponari et al., 2014; Cornara et al., 2017a; Cornara et al., 2017b).
52 Furthermore, data from surveys currently ongoing throughout Europe suggest its
53 possible involvement in all the European outbreaks reported so far (EFSA, 2018;
54 Morente et al., 2018a; Cruaud et al., 2018). Additionally, two other spittlebugs,
55 i.e. *Neophilaenus campestris* Fallen (1805) (Hemiptera: Aphrophoridae) and
56 *Philaenus italosignus* Drosopoulos & Remane (Hemiptera: Aphrophoridae), have

57 been shown to be competent vectors of the bacterium (EFSA, 2018).
58 Understanding the intimate spittlebug-bacterium interaction might open new
59 possibilities for disrupting the transmission process; however, as previously
60 remarked, artificial diets are an essential tool for such investigations.
61 Unfortunately, past attempts to artificially feed *P. spumarius* adults with
62 traditional “flat” systems such as the commonly used sachets and artificial
63 chambers were unsuccessful, independently on the diet used (Watson, 1999).
64 Watson (1999) and Ponder et al. (2002) achieved spittlebug’s artificial feeding by
65 using a stem perfusion system; nevertheless, stem perfusion requires a plant
66 portion through which the diet is injected, thus does not allow neither direct
67 observation of stylets activity during the probe, nor the complete exclusion of
68 plant effects on bacterium-insect interaction. The failure of artificially feeding *P.*
69 *spumarius* might be related to the lack of a proper stimulus required by the insect
70 to begin a probe. Indeed, according to Backus and McLean (1985), mechanical
71 stimuli are necessary for leafhoppers to initiate a probe, while chemical stimuli
72 are required for the probe to continue, and for prolonged ingestion to ensue. *P.*
73 *spumarius* usually prefers “rounded” tissues to “flat” ones; indeed, at least on
74 woody hosts, the spittlebug tends to settle on leaf petioles and stems (Cornara ,
75 pers. obs.), grabbing the tissue with the anterior two pairs of legs, and pressing
76 the tip of the stylet vertically down against the plant surface (Watson, 1999).
77 Accordingly, the reason underlying the failure of traditional “flat” systems for *P.*
78 *spumarius* artificial feeding would be their “non-resemblance” with a petiole or a
79 stem, thus the lack of a mechanical/tactile stimulus triggering the probe.

80 Therefore, setting up an artificial feeding system for *P. spumarius*, and more in
81 general for spittlebugs, represents a major challenge in research on *X. fastidiosa*
82 epidemics across Europe. In order to fill this knowledge gap, we tested if a new
83 concept of artificial diet delivery system, designed to mimic a plant stem or leaf
84 petiole, providing the insect with a more suitable surface to probe than a flat one,

85 would be feasible for *P. spumarius* artificial feeding. Furthermore, we tested the
86 applicability of this system for other spittlebugs by carrying out further
87 observations on *N. campestris*. The suitability of our feeding system versus a
88 traditional “flat” system derived from a Petri dish was assessed through feeding
89 behavioral observations performed with a combination of Electrical Penetration
90 Graph (EPG) technique and video recording.

91

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92 **Material and Methods**

93 **Spittlebug collection and rearing**

94 *P. spumarius* individuals used for the EPG recordings were collected, reared,
95 and maintained following the protocol illustrated by Cornara et al. (2018b)
96 slightly modified. Briefly, spittlebug nymphs were collected during spring, 2018
97 in Sierra de Aracena (Huelva Spain) on *Sonchus* sp. L., *Cirsium* sp. (Miller),
98 *Borago officinalis* L., *Calendula* sp. L., and *Scolymus hispanicus* L., and reared
99 on one month old *Sonchus oleraceus* L. plants until adulthood. Both nymphs and
100 adults were reared in the controlled-environmental facilities of Instituto de
101 Ciencias Agrarias-Consejo Superior de Investigaciones Cientificas (ICA-CSIC,
102 Madrid, Spain) in a walk-in growth chamber at 24:20°C day:night temperature,
103 humidity of ca. 60%, and photoperiod 14:10 light:dark. For colony maintenance,
104 adults were transferred in groups of ten per plant to one month old *S. oleraceus*
105 plants, which were replaced every two weeks. *N. campestris* were collected as
106 adults on *Bromus* sp. plants in an olive orchard in Morata de Tajuña (Madrid,
107 Spain) during fall 2017. The adults were maintained on three-week-old *Bromus*
108 sp. plants replaced every two weeks, in groups of ten per plant, at the same
109 conditions described above for *P. spumarius*. *S. oleraceus* and *Bromus* sp.
110 plants used for spittlebugs rearing were seedlings germinated and maintained in
111 a growth chamber (25:18 °C day:night temperature, 60% humidity, 16:8
112 light:dark photoperiod) in 5L pots filled with universal soil: vermiculite (2:1),
113 and water-fertilized every two days with a nutritional complex 20-20-20 (N:P:K)
114 of Nutrichem 60 fertilizer (Miller Chemical & Fertilizer. Hanover, PA, USA) (1
115 g/l).

116 **Artificial diet delivery systems**

117 For spittlebugs artificial feeding, we tested two delivery systems: the “Flat-
118 system” and the “Tube-system”. The Flat-system was similar to the one
119 described by Trebicki et al. (2012) for *Orosius orientalis* Matsumura

120 (Hemiptera: Cicadellidae). Briefly, an artificial diet–feeding platform was
121 constructed out of a small plastic Petri dish (1 x 3.5 cm); an EPG “diet”
122 electrode was inserted inside the Petri dish through a hole drilled at the bottom
123 of the dish, and sealed with hot-glue. The diet-electrode was connected to the
124 EPG by a clamp cable. A five cm plastic stick was glued to the bottom of the
125 dish, in order to secure the system with tape to a plastic holder. The bottom of
126 the dish was covered with a piece of green tape. The Petri dish was filled to
127 capacity with the diet, and a single layer of Parafilm[®] was stretched over the
128 chamber carefully to prevent the occurrence of air bubbles. The set-up of the
129 Flat-system is illustrated in Fig. 1.1. For the Tube-system (Fig. 1.2), two
130 rectangular windows (3x12 mm), 15 mm distant from each other, were carved
131 with a lancet blade on the surface of a 15 cm silicon tube (external diameter: 4
132 mm; internal diameter: 2 mm; wall thickness: 1 mm). The side opposite to the
133 window was covered with a green tape, without interfering with the openings.
134 The windows were then covered with two layers of stretched Parafilm[®]. The
135 tube was subsequently filled with the diet by using a syringe, avoiding the
136 formation of air bubbles; once filled, the tube was bent in a semi-circular shape,
137 and inserted in a 100ml Beaker containing the diet. Approximately five cm of
138 the tube protruded out of the Beaker; this portion was the one exposed to insect
139 feeding.

140 For both the delivery systems, we tested holidic diets used by other authors for
141 xylem-sap feeders: i) the sucrose-diet (Sucrose), used by Joost et al. (2006) for
142 *Homalodisca vitripennis* Germar (1821) (Hemiptera: Cicadellidae) (previously
143 *Homalodisca coagulata*); ii) the sharpshooter diet (SHPD), used by Killiny and
144 Almeida (2009) for *Graphocephala atropunctata* Signoret (1854) (Hemiptera:
145 Cicadellidae); iii) the XFM amino-acids diet (XFM), based on the amino-acids
146 fraction of the XFM medium for *X. fastidiosa* described by Killiny and Almeida
147 (2009). Holidic diets were chosen since they are easier to handle and

148 standardize in routinely laboratory activity compared to meridic diets.
149 Nevertheless, for the Flat-system, beside holidic diets, we also tested pure and
150 diluted olive xylem sap extracted with a Scholander pressure bomb (3005 Series
151 Plant Water Status Consoles, Soilmoisture Equipment Corp., Santa Barbara, CA,
152 U.S.A), following the protocol described by Alexou and Peuke (2012). The diets
153 used for the two systems, together with their compositions are reported in Tab.
154 1.

155 **Probing and feeding behavior observation**

156 The spittlebug probing and feeding behaviour on the two artificial systems was
157 observed and described through a combination of EPG and simultaneous video
158 recording. Flat- and Tube- systems (not tested contemporary) were assembled
159 inside a Faraday cage, in an acclimatized room ($23 \pm 2^\circ\text{C}$). *P. spumarius*
160 individuals were starved for three hours (1 hour for *N. campestris*; we observed
161 that this species does not withstand longer starvation periods) inside an aerated
162 Petri dish, then tethered with an $18 \mu\text{m}$ gold wire and connected to the EPG
163 probe as described by Cornara et al. (2018b). The substrate copper electrode was
164 inserted into the 100ml Beaker containing the diet. We recorded the probing
165 behaviour with a Giga 4-DC EPG (EPG-systems, Wageningen, The
166 Netherlands) at 1 Giga Ohm input resistance. Output from the EPG at 50x gain
167 was digitalized at a rate of 100 samples per sec. per channel, and recorded using
168 Stylet+ software (EPG-systems, Wageningen, The Netherlands). EPG
169 recordings were set and adjusted following the indications of Cornara et al.
170 (2018b). For *P. spumarius*, and for each combination delivery system/artificial
171 diet, we carried out five 3-hour long EPG-assisted observations, with one single
172 insect recorded per time, from 4 to 7 p.m. (thus a total of 15 hours of recording
173 per delivery system/diet combination, with three males and two females per
174 combination). During the EPG-recording, the activities of the tethered
175 spittlebugs were simultaneously observed through a 600X 4.3" 3.6MP LCD

176 Display Electronic Digital Video Portable LED Microscope R9N7 (KKmoon,
177 <https://www.kkmoon.com>) in order to: i) distinguish probing (stylet penetration)
178 from non-probing signals (e.g. crawling and wire-pulling); ii) observe
179 occurrence of excretions during feeding in artificial diets (we considered
180 excretion as occurring in case multiple watery drops were shed by the spittlebug
181 for an interval longer than 30sec). For *N. campestris*, we performed four 3-hour
182 long EPG-assisted observation of the spittlebug probing and feeding behavior on
183 the Tube-system filled with XFM-diet, following the same protocol used for *P.*
184 *spumarius*. The main aim was to assess whether a spittlebug other than *P.*
185 *spumarius* would feed from an artificial diet provided with the Tube-system.

186 **EPG data analysis**

187 The EPG waveforms obtained during artificial feeding were distinguished and
188 correlated with their possible biological meaning through simultaneous
189 observations and analysis of the video recorded, and by analogy to the ones
190 previously reported by Joost et al. (2006) and Cornara et al. (2018b). The main
191 goal of this work was to develop a suitable artificial diet delivery system for *P.*
192 *spumarius* and other spittlebugs; EPG and video recording were used to
193 discriminate probing from non-probing signals, and to verify the occurrence of
194 ingestion. A complete characterization of *P. spumarius* feeding behavior on
195 artificial diet, or a comparison of the diets used, were out of the purpose of this
196 research. Nevertheless, we performed a basic analysis of the EPG recordings
197 obtained from the different diets, in order to gather preliminary data for future
198 work on spittlebug artificial feeding. Therefore, after identifying the typical
199 waveform categories, we calculated a series of non-sequential and sequential
200 variables of the EPG recordings. The non-sequential variables were: i) n probes:
201 total number of probes performed by the insect; ii) n succ probes: number of
202 probes during which the spittlebug ingested the diet; iii) np WDI: total duration
203 of the non-probing phase per insect; iv) C WDI: total duration of the pathway

204 phase per insect; v) Xi WDI: total duration of the ingestion phase per insect; vi)
205 Xi WDEI: duration of the single ingestion event per insect; vii) Xi NWEI: total
206 number of ingestions performed per insect; viii) Xi>10min: occurrence of an
207 ingestion longer than 10 minutes. The sequential variables were: i) Time to first
208 C: time required by the spittlebug to start a probe from the beginning of the
209 recording; ii) Time to first Xi: time required by the spittlebug to start an
210 ingestion from the beginning of the recording; iii) Time from 1st C to Xi: time
211 required by the spittlebug to start an ingestion after the first absolute probe. EPG
212 data were elaborated with an Excel Workbook purposely developed for *P.*
213 *spumarius* by Antonio J. Alvarez (Universidad de Almeria, Spain) (Cornara et
214 al., 2018b).

215 Probing and feeding behavioral differences among the three holidic diets tested
216 were evaluated through Kruskal-Wallis test by ranks and Dunn test. Statistical
217 analysis was conducted with the software R (R Core Team, 2018); differences
218 were considered significant for $p < 0.1$.

219 **Survival test on the Tube system**

220 Finally, we performed a survival test of *P. spumarius* on the Tube-system under
221 non-choice conditions but without wiring the insect to the EPG device and
222 allowing free movement inside a cage. We assessed *P. spumarius* survivorship
223 on two diets that led to the best results during the EPG recordings, i.e. XFM and
224 Sucrose. The test was conducted under laboratory conditions ($T=24\pm 2^{\circ}\text{C}$,
225 $\text{HR}=40\%$, constant artificial light), with 12 replicates per diet (six males and six
226 females), plus six controls (three males and three females). Insects, caged inside
227 a plastic and mesh cage, were offered the artificial diets contained in the tubes;
228 the controls consisted of empty tubes not filled with diet (the setup is illustrated
229 in Fig. 1.3). *P. spumarius* used for the survival test were one-month old adults
230 obtained through indoor artificial rearing, following the protocol described by
231 Morente et al. (2018b). Differences in survival either between the diets and the

232 control, or between the XFM and the Sucrose were evaluated by Cox
233 Proportional-Hazards Model (Cox, 1972), with the statistical analysis performed
234 with the software R (R Core Team, 2018); differences were considered
235 significant for $p < 0.1$.

236

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237 Results

238 Except for a few very quick stylets insertion attempts as short as one or two
239 seconds (as observed by the help of the microscope video recorder), we achieved
240 no probing with the Flat-system, regardless of the type of diet used. On the
241 contrary, *P. spumarius* probed and fed readily from all the artificial diets provided
242 with the Tube-system. The EPG signals produced on the artificial diets were
243 distinguished in: i) non probing (np) signals, corresponding mainly to crawling
244 and wire pulling (Fig. 2); ii) pathway/non ingestion waveform (C) (Fig. 3); iii)
245 ingestion waveform (Xi) (Fig. 4). During one of the recordings on XFM we also
246 observed an interruption of the ingestion activity similar to the N waveform
247 described by Cornara et al. (2018b) (Fig. 4.f). The waveforms characteristics and
248 their likely biological meaning are reported in Tab. 2. We observed the longest
249 ingestion and a subsequent excretion of *P. spumarius* with the Tube-system
250 containing the sucrose-diet (multiple watery drops excreted by the spittlebug
251 during the occurrence of the ingestion waveform); excretion was not observed in
252 the rest of the *P. spumarius* recorded. A summary of the sequential and non-
253 sequential variables calculated for the three diets provided to the meadow
254 spittlebug with the Tube-system, calculated by pooling the recordings of the five
255 insects per diet, is reported in Tab. 3; raw data (all the variables calculated for
256 each one of the spittlebugs tested) are provided as supporting information
257 (SuppInfo). One insect on SHPD and one on XFM jumped away 30 and 20
258 minutes before the end of the recording, respectively (Tab. 3).

259 Considering just the rough dataset of EPG variables, and those that could be
260 important for artificial feeding applications aimed at *X. fastidiosa* acquisition, i.e.
261 number of total and successful probes, total duration of ingestion and total number
262 of ingestion events, SHPD was by far the least suitable of the diets tested. For
263 Sucrose and XFM, we observed an overall greater number of probes on the former
264 compared to the latter, although an opposite trend was evident considering the

265 number of probes during which ingestion occurred (defined as successful probes).
266 Furthermore, despite ingestion was longer on sucrose-diet, the ingestion events in
267 XFM were twice the number of those recorded on Sucrose.

268 The results of the Kruskal-Wallis test by ranks (χ^2) and Dunn test (z), confirmed
269 the overall better performance of the meadow spittlebug on XFM and Sucrose
270 compared to the SHPD. *P. spumarius* performed significantly more successful
271 probes (probes during which ingestion occurred) ($\chi^2=4.865$, $p=0.744$; $z=-2.161$,
272 $p=0.0922$), longer total ingestion ($\chi^2=5.232$, $p=0.073$; $z=-1.862$, $p=0.098$), and
273 greater number of ingestion events ($\chi^2=4.972$, $p=0.083$; $z=-2.197$, $p=0.084$) on
274 XFM compared to SHPD. The single ingestion events were longer on Sucrose
275 than on SHPD ($\chi^2=4.997$, $p=0.082$; $z=-2.227$, $p=0.077$). Finally, the spittlebug
276 performed the first absolute probe on XFM earlier than on Sucrose ($\chi^2=6.076$,
277 $p=0.048$; $z=2.371$, $p=0.053$).

278 Regarding the survival test, the survival time of *P. spumarius* on the diets
279 provided with the tube system and the control was 13.25 ± 1.14 hours (h) for XFM
280 (min=9 h, max=21 h), 14.17 ± 1.76 h for Sucrose (min= 6 h, max= 24 h), and
281 9 ± 1.46 h for the control (min= 4h, max= 13 h.). According to the Cox
282 Proportional-Hazards Model, survival on the diets provided with the tube-system
283 was statistically significantly longer than on the control, while no gender-related
284 difference was observed (diet vs control: $z= 2.141$, $p=0.0323$; gender: $z=-1.207$;
285 $p=0.227$). Moreover, the spittlebugs showed similar survival time on the two diets,
286 with no statistically significant difference neither diet- nor gender-related (diet:
287 $z=-0.358$ $p=0.720$; gender: $z=-1.047$; 0.295).

288 During the survival test, all the insects including the controls were observed
289 settling on the tube and probing through the Parafilm® membrane, or even
290 apparently introducing their stylets through the tube itself, multiple times.

291 We also successfully verified that our Tube-system was suitable for artificial
292 feeding of *N. campestris*. Indeed, two out of the four spittlebugs connected to the
293 EPG device were observed feeding on XFM diet (the only diet tested for *N.*

294 *campestris*) provided with the Tube-system. *N. campestris* produced clearly
295 distinguishable ingestion waveforms (Fig. 4.e) very similar to those produced by
296 *P. spumarius*.

297

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298 Discussion

299 Host selection by leafhoppers and planthoppers can be studied by analogy to an
300 input-output relationship, with a stimulus being the input, and the response as
301 output (Backus, 1985). *P. spumarius* bears a low number of antennal olfactory
302 sensilla; thus it can be inferred that olfactory cues might not be as important as
303 other stimuli (e.g. visual, tactile) during host plant location (Ranieri et al., 2016).
304 Given the results of our tests, we suggest that *P. spumarius* requires a tactile
305 stimulus to begin a probe. Indeed, as proven by the success of the Tube- versus
306 the Flat-system, the meadow spittlebug needs a rounded/tubular surface to grab
307 with the anterior four legs, in order to push the stylets through and start a probe.
308 The green tape covering the bottom of the tube could also have played a role in
309 triggering the spittlebug settlement. Mittler (1988) reported the use of green and
310 yellow light in order to encourage aphids feeding on artificial diets. For aphids,
311 as well as for other phytophagous insects, many investigations have addressed the
312 role of plant spectral quality as principle stimulus in alighting behavior (reviewed
313 in Fereres, 2016). On the contrary, except for few reports on attraction toward
314 sticky traps of different colors (Wilson and Shade, 1967) and post-embryonic
315 photoreceptors development (Keskinen and Meyer-Rochow, 2004), nothing is
316 known about the role of visual cues in *P. spumarius* host seeking behavior. The
317 study of visual and olfactory cues in this vector species may reveal important
318 features that can potentially explain host plant selection and could be exploited to
319 attract, collect and monitor more efficiently the spittlebug.

320 The main goal of this work was to devise a ready-to-use system to deliver artificial
321 diet to spittlebugs. For this scope, we were more oriented toward holidic diets,
322 which can be easily prepared and standardized in laboratory routinely activity
323 compared to meridic diets. *P. spumarius* did not ingest holidic diets provided with
324 the Flat-system, and only very brief stylets insertions were recorded. In order to
325 rule out the hypothesis that absence of ingestion was related to the diet rather than
326 to the system itself, we additionally tested the Flat-system with meridic diets, i.e.

327 pure and diluted xylem sap. The further failure of such attempt supports our initial
328 hypothesis about the need for spittlebugs of a tactile cue triggering the probe.
329 EPG and video observations were used as supports to verify mainly the
330 occurrence and duration of ingestion and watery excretions. A deep and robust
331 characterization of EPG variables (sequential and non sequential) produced by the
332 spittlebugs on artificial diets, or a comparison among different artificial diets,
333 were out of the scope of this work. Nevertheless, the trends we observed in *P.*
334 *spumarius* probing behavior on the different diets (Tab. 3) should be taken into
335 account for further work on spittlebugs artificial feeding and transmission tests.
336 The diet devised by Killiny and Almeida (2009) for artificial acquisition of *X.*
337 *fastidiosa* by sharpshooters, i.e. SHPD, resulted to be the least acceptable for *P.*
338 *spumarius*, with a statistically significant shortest duration of the overall ingestion
339 and of the single ingestion events, and lowest number of successful probes and of
340 ingestion events compared to XFM and Sucrose. This might suggest a difference
341 between spittlebugs and sharpshooters in nutritional requirements or chemical
342 cues stimulating a sustained ingestion. The survival time of *P. spumarius* on XFM
343 and Sucrose was overall similar. The only statistically significant difference
344 detected between XFM and Sucrose was the time required to perform the first
345 absolute probe that resulted lower for the former compared to the latter diet.
346 However, looking at the rough dataset, we observed several differences between
347 XFM and Sucrose that could be relevant for experiments aimed at using the diets
348 for *X. fastidiosa* artificial acquisition. The greatest number of short non-ingesting
349 probes was recorded on the sucrose-diet, possibly indicating a low acceptability
350 of the medium (Crane, 1971). This is contrasting with the fact that one of the *P.*
351 *spumarius* feeding on the Sucrose showed the overall longest ingestion (almost
352 40 minutes) and the only observed excretion. Absence of excretion for the other
353 insects tested may be related to a condition of acute water stress due to the long
354 starvation (Crane, 1971), or just to ingestion not long enough to induce excretion.
355 Sucrose is the major phagostimulant component of aphid diets (Mittler and Dadd,

1963), and has been used also for sharpshooters artificial feeding (Joost et al., 2006). However, possible effects of sucrose on the viability of *X. fastidiosa* cells suspended in the diet should be carefully investigated prior to use a sucrose-diet for bacterium transmission tests. Moreover, considering the rough dataset, *P. spumarius* on XFM diet showed the greatest number of ingestion events, although their overall duration was reduced compared to Sucrose. According to Mitsuhashi (1979), a rich medium is not required for artificial acquisition of pathogens, since acquisition from artificial diets does not require a long ingestion. Therefore, considering our dataset, XFM could be the best candidate for *X. fastidiosa* artificial acquisition by *P. spumarius*. Given the results from the EPG-assisted feeding behavioral observation of the meadow spittlebug, we decided to choose XFM-diet to test Tube-system suitability for *N. campestris*. Assessment of nutritional requirements of *N. campestris*, or preference of this species for one diet over another, were out of the purpose of this work. The fact that also *N. campestris* fed on XFM-diet, suggests this diet could be a good candidate for further tests on spittlebugs, including *X. fastidiosa* transmission studies. However, as for Sucrose, bacterial cells viability in XFM diet should be accurately assessed prior to apply such a diet in transmission tests.

In the present work, we developed a functional system for artificial diet delivery to *P. spumarius*, that resulted to be suitable also for artificial feeding of another spittlebug, i.e. *N. campestris*. This tool opens new perspectives for investigations of *X. fastidiosa*/spittlebugs interactions and transmission mechanism. Furthermore, our Tube-delivery system could have an immediate applicability for behavioral and biological studies directly or indirectly related with the fastidious bacterium epidemiology and control strategies.

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382

383 **Author Contribution**

384

- 385 • DC and AF conceived research.
- 386 • DC, MR, MM, and EG conducted experiments.
- 387 • DC and MR wrote the manuscript.
- 388 • MM, EG, DB AM, and AF reviewed and edited the manuscript.
- 389 • DB, AM, and AF secured funding.
- 390 • All authors read and approved the manuscript.

391

392 **Data Availability Statement:** raw data (dataset containing all the variables
393 calculated for each one of the spittlebugs tested) are provided as supporting
394 information (SuppInfo).

395

396

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- 526

527 **Fig. 1: 1.1) Experimental setup of *P. spumarius* recording on artificial diet,**
528 **“Flat-system”; 1.2) Experimental setup of *P. spumarius* recording on**
529 **artificial diet, “Tube-system”; 1.3) Experimental setup of *P. spumarius***
530 **survival test on artificial diet “Tube-system”. a) plastic stick; b) Petri dish**
531 **with artificial diet, bottom covered with green tape; c) Parafilm® layer; d) diet-**
532 **electrode connected to the EPG through a clamp cable; e) insect electrode: brass**
533 **nail + copper wire + gold wire connected to *P. spumarius* with a drop of silver**
534 **glue; f) probe; g) Giga 4-DC EPG device; h) Beaker containing artificial diet**
535 **(~80 ml); i) tube filled with artificial diet; j) windows covered with stretched**
536 **Parafilm® layer, green tape covering the opposite side; k) copper “plant”**
537 **electrode; l) cotton-bed; m) conical cage; n) cage ceiling covered with net.**
538 **Original *P. spumarius* clipping derives from David O’Shea**
539 **(www.britishbugs.org.uk).**

540 **Fig 2. EPG recording for *P. spumarius* on artificial diet, non probing (np)**
541 **waveforms. 2.a, b) crawling phases; 2.c) regular signal produced during np**
542 **(stylets are out), insect abdomen touching the tube; 2.d-e) wire pulling; 2.f)**
543 **insect fallen, hanging on the wire and dangling.**

544 **Fig 3. EPG recording for *P. spumarius* on artificial diet, C waveform. 3.a, b,**
545 **d, e, f) waveform C; 3.c) brief probe.**

546 **Fig 4. EPG recording for *P. spumarius* on artificial diet, Xi waveform. 4.a, c,**
547 **d) waveform Xi; 4.b) Xi, high amplitude, corresponding to long ingestion phases**
548 **on sucrose-diet during which excretion was observed; 4.e) *N. campestris***
549 **ingestion waveform; 4.f) N during Xi.**

550

Tab. 1 Artificial diets tested for *P. spumarius*

| Artificial diet | Acronym | Delivery system | | pH | Composition | Concentration [g/l H ₂ O] | Molecular weight | Molarity [mM] | Reference |
|--------------------------------|----------------|-----------------|------|-----|--------------------------------|--------------------------------------|------------------|---------------|---|
| | | Flat | Tube | | | | | | |
| XFM amino-acids | XFM | X | x | 5.2 | L-asparagine | 10 | 132.12 | 75.69 | modified from Killiny and Almeida, 2009 |
| | | | | | L-cysteine | 5 | 121.16 | 41.27 | |
| | | | | | L-glutamine | 30 | 148.14 | 202.51 | |
| Sharpshooters diet | SHPD | X | x | 6.4 | L-asparagine | 0.0132 | 132.12 | 0.10 | Killiny and Almeida, 2009 |
| | | | | | L-glutamine | 0.1022 | 148.14 | 0.69 | |
| | | | | | tri-sodium citrate | 0.25 | 294.1 | 0.85 | |
| Sucrose | SUCROSE | X | x | 6.0 | Sucrose | 50 | 342.3 | 146.07 | Joost et al., 2006 |
| Pure olive xylem sap | | X | N/A | N/A | Pure olive xylem sap | N/A | N/A | N/A | Watson, 1999 |
| Diluted olive xylem sap | | X | N/A | N/A | Diluted (1:10) olive xylem sap | N/A | N/A | N/A | Watson, 1999 |

“Flat” and “Tube” refer to Flat-delivery system and Tube-delivery system, respectively.

Tab. 2 Waveforms characteristics of *P. spumarius* on artificial diets provided with the Tube system

| Waveforms characteristics | | | | | |
|---------------------------|------------------------------|----|------------------------|-----------|-------------------------------------|
| Waveform | Amplitude % [V] | | Frequency [Hz] | Excretion | Activity |
| np | 5 (1 – 20) | | mixed | no | non probing |
| | 200 ^s | | | | non probing - walking |
| | 100 | | | | non probing - wire pulling |
| C | 35.7 (10 - 100) | | mixed | no | Pathway |
| Xi | 25.7 (1 - 200 ^s) | | Waves: 1.4 (0.4 - 2.5) | yes | Ingestion |
| | | | Peaks: 1.4 (0.4 - 2.5) | | |
| N | First drop | N | mixed | no | Interruption during ingestion phase |
| | 48 | 16 | | | |

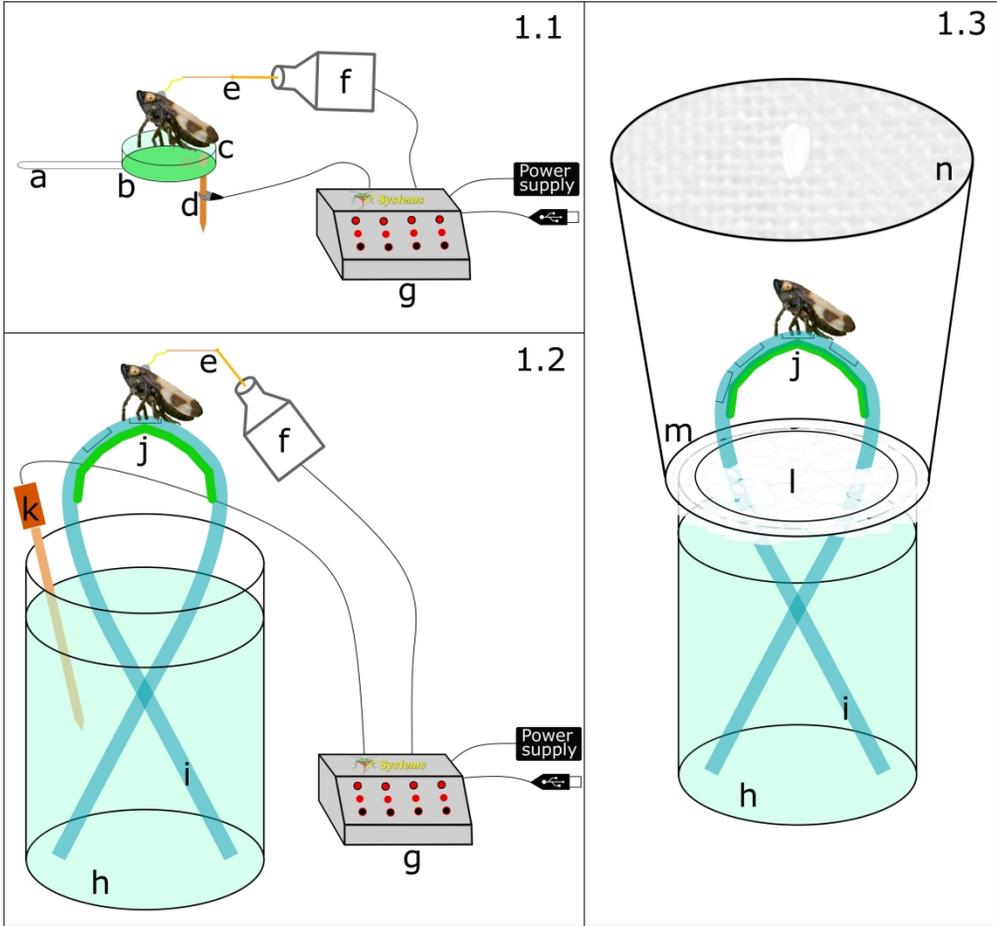
5V = 100% amplitude; 200^s indicates a 10V (from -5 to +5V) signal

Abbreviations: emf = electromotive force

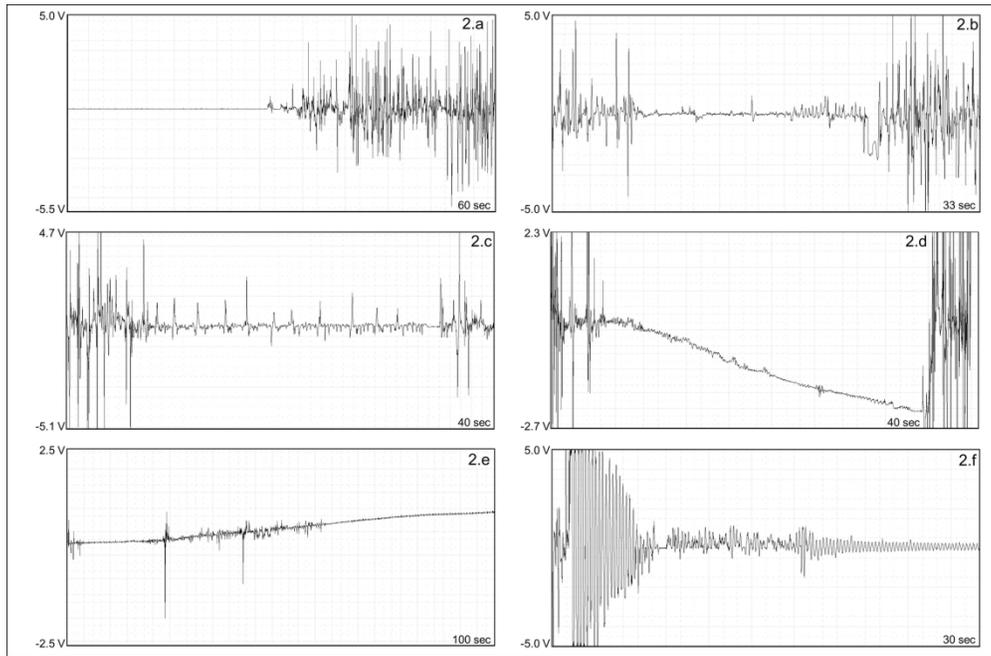
Tab. 3 *P. spumarius* probing behavior on artificial diets provided with the Tube-system: summary table EPG variables

| Total EPG time | n probes | n succ probes | np WDI | C WDI | Xi WDI | Xi WDEI | Xi NWEI | Xi>10 min | Time to 1st C | Time to 1st Xi | Time from 1st C to Xi | | | | |
|-----------------|---------------|---------------|--------------|---------------|-------------------------|-------------|-------------|------------|---------------|----------------|-----------------------|------------|--------------|--------------|-------------|
| SUCROSE | | | | | | | | | | | | | | | |
| TOTAL | 900 | 77 | 11 | 802.4 | 50.1 | 47.5 | 13 | yes | | | | | | | |
| MIN | | 1 | 0 | 136.2 | 0.4 | 0 | 0 | | | | | 1.1 | 4.1 | 0.4 | |
| MAX | | 44 | 6 | 179.6 | 27.8 | 39.9 | 39.9 | | | | | 8 | 54.2 | 53.2 | 3.4 |
| MEAN | | 15.4 | 2.2 | 160.34 | 10.0 2 | 9.5 | 3.65 | | | | | 2.6 | 24.86 | 24.75 | 1.95 |
| MEAN (%) | | | 89.15 | 5.57 | 5.28 | | | | | | | | | | |
| SHPD | | | | | | | | | | | | | | | |
| TOTAL | 868.63 | 21 | 2 | 844.13 | 23.6 | 0.9 | 2 | no | | | | | | | |
| MIN | | 2 | 0 | 140.33 | 0.7 | 0 | 0 | | | | | 0.5 | 47.4 | 43.5 | |
| MAX | | 9 | 2 | 179.3 | 12.8 | 0.9 | 0.45 | | | | | 2 | 59.7 | 47.4 | 43.5 |
| MEAN | | 4.2 | 0.4 | 168.82 | 4.72 | 0.18 | 0.09 | | | | | 0.4 | 15.5 | 47.4 | 43.5 |
| MEAN (%) | | | 97.17 | 2.71 | 0.12 | | | | | | | | | | |
| XFM | | | | | | | | | | | | | | | |
| TOTAL | 886.53 | 42 | 20 | 834.73 | 35.1 | 16.7 | 26 | no | | | | | | | |
| MIN | | 1 | 0 | 146.23 | 2 | 0 | 0 | | | | | 0.4 | 0.9 | 0.5 | |
| MAX | | 16 | 8 | 178 | 14.5 | 7.9 | 1.02 | | | | | 11 | 3.2 | 41.3 | 40.7 |
| MEAN | | 8.4 | 4 | 166.94 | 7.02 | 3.34 | 0.64 | | | | | 5.2 | 1.36 | 11.7 | 10.8 |
| MEAN (%) | | | 94.15 | 3.95 | 1.88 | | | | | | | | | | |

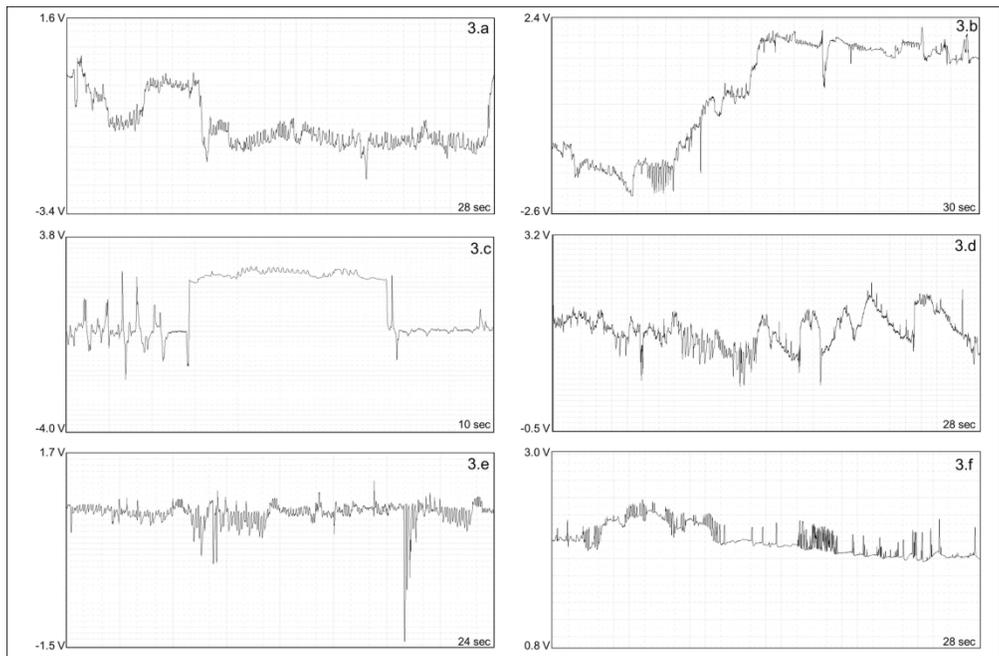
Total EPG time: total time the probing behavior of the spittlebug was recorded, calculated by pooling the recordings of the five spittlebugs tested per each diet. For SHPD and XFM one of the five replicates jumped away before the end of the 3 hours. **n probes:** total number of probes performed. **n succ probes:** number of probes during which the spittlebug ingested the diet. **np WDI:** total duration of the non-probing phase. **C WDI:** total duration of the pathway phase. **Xi WDI:** total duration of the ingestion. **Xi WDEI:** duration of the single ingestion events. **Xi NWEI:** total number of ingestions performed. **Xi>10min:** occurrence of an ingestion longer than 10 minutes. **Time to first C:** time required by the spittlebug to start a probe from the beginning of the recording. **Time to first Xi:** time required by the spittlebug to start an ingestion from the beginning of the recording. **Time from 1st C to Xi:** time required by the spittlebug to start an ingestion from the first absolute probe. All the values per each diet are calculated referring to the 15 hours recorded (5 spittlebugs/diet). Time is expressed in minutes.



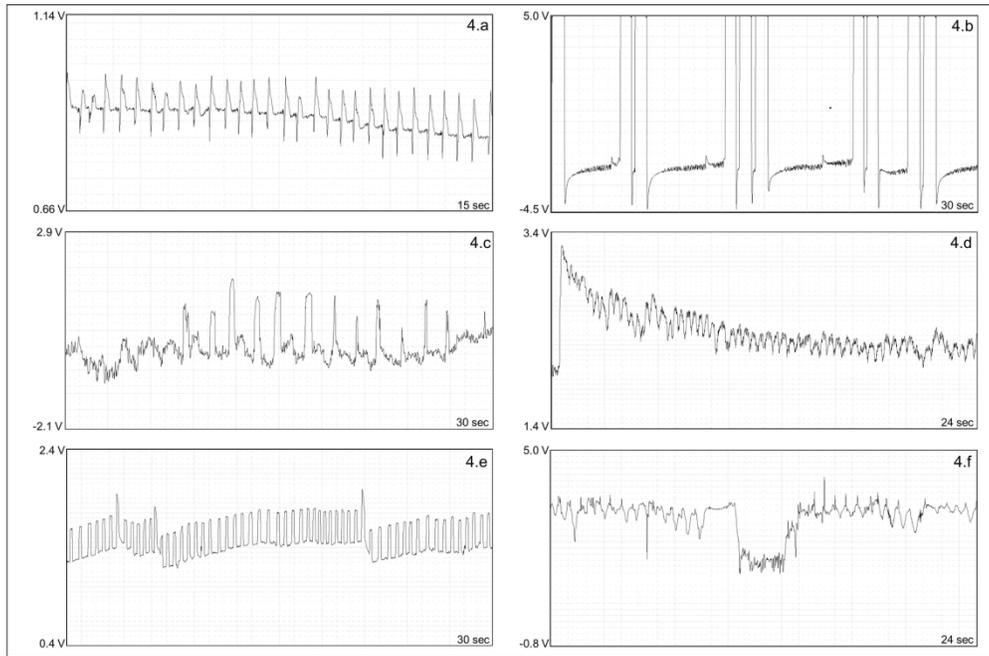
330x307mm (300 x 300 DPI)



179x118mm (300 x 300 DPI)



179x118mm (300 x 300 DPI)



180x118mm (300 x 300 DPI)