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Abstract

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

Key words

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

28

Introduction

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

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

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

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

Material and Methods

Spittlebug collection and rearing

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

Artificial diet delivery systems

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

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

For both the delivery systems, we tested holidic diets used by other authors for xylem-sap feeders: i) the sucrose-diet (Sucrose), used by Joost et al. (2006) for *Homalodisca vitripennis* Germar (1821) (Hemiptera: Cicadellidae) (previously *Homalodisca coagulata*); ii) the sharpshooter diet (SHPD), used by Killiny and Almeida (2009) for *Graphocephala atropunctata* Signoret (1854) (Hemiptera: Cicadellidae); iii) the XFM amino-acids diet (XFM), based on the amino-acids fraction of the XFM medium for *X. fastidiosa* described by Killiny and Almeida (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 ±2°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 µm 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

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

EPG data analysis

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

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

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

Survival test on the Tube system

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

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

Results

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

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

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

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

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

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

We also successfully verified that our Tube-system was suitable for artificial feeding of *N. campestris*. Indeed, two out of the four spittlebugs connected to the 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

Discussion

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

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

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

Author Contribution

- DC and AF conceived research.
- DC, MR, MM, and EG conducted experiments.
- DC and MR wrote the manuscript.
- MM, EG, DB AM, and AF reviewed and edited the manuscript.
- DB, AM, and AF secured funding.
- All authors read and approved the manuscript.

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

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

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

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

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

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

Artificial diet	Acronym	Delivery system		pH	Composition	Concentration [g/l H2O]	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§				non probing - walking
	100				non probing - wire pulling
C	35.7 (10 - 100)		mixed	no	Pathway
Xi	25.7 (1 - 200§)		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			

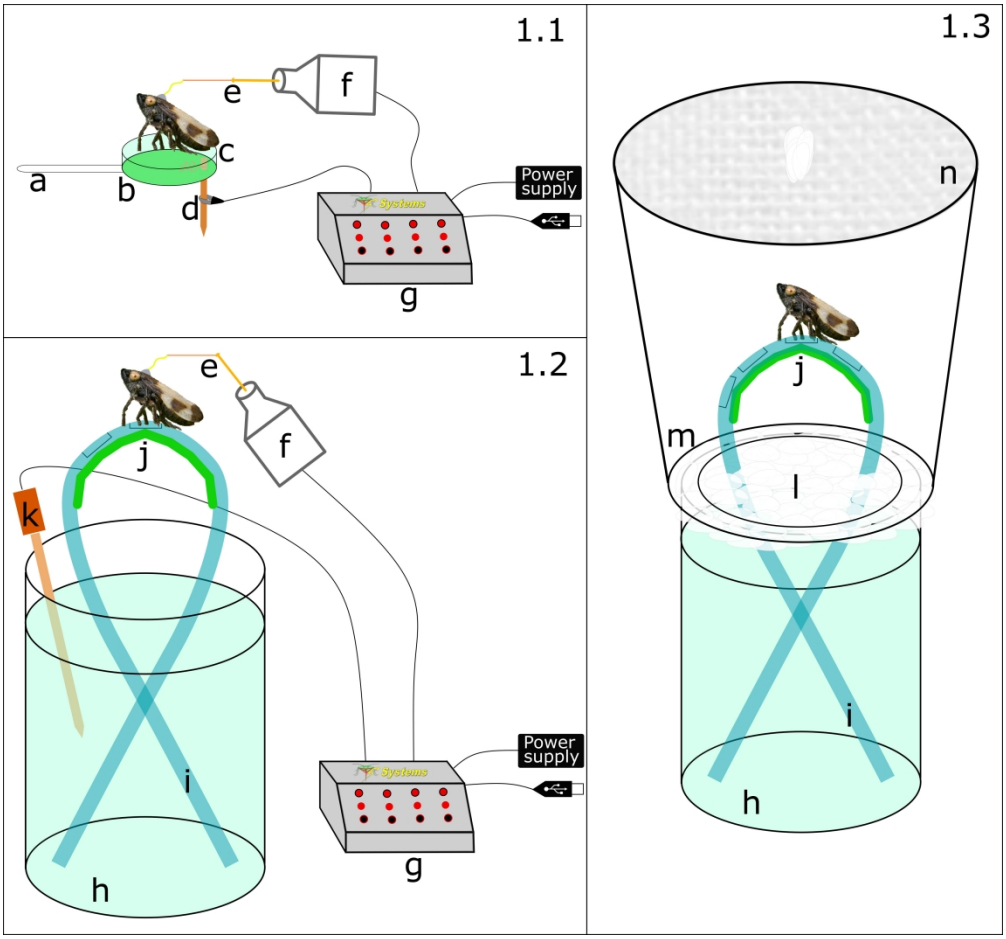
5V = 100% amplitude; 200§ indicates a 10V (from -5 to +5V) signal

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		77	11	802.4	50.1	47.5		13				
MIN		1	0	136.2	0.4	0	0	0		1.1	4.1	0.4
MAX	**900**	44	6	179.6	27.8	39.9	39.9	8	**yes**	54.2	53.2	3.4
MEAN		**15.4**	**2.2**	**160.34**	**10.02**	**9.5**	**3.65**	**2.6**		**24.86**	**24.75**	**1.95**
MEAN (%)				**89.15**	**5.57**	**5.28**						
SHPD												
TOTAL		21	2	844.13	23.6	0.9		2				
MIN		2	0	140.33	0.7	0	0	0		0.5	47.4	43.5
MAX	**868.63**	9	2	179.3	12.8	0.9	0.45	2	**no**	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		42	20	834.73	35.1	16.7		26				
MIN		1	0	146.23	2	0	0	0		0.4	0.9	0.5
MAX	**886.53**	16	8	178	14.5	7.9	1.02	11	**no**	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.



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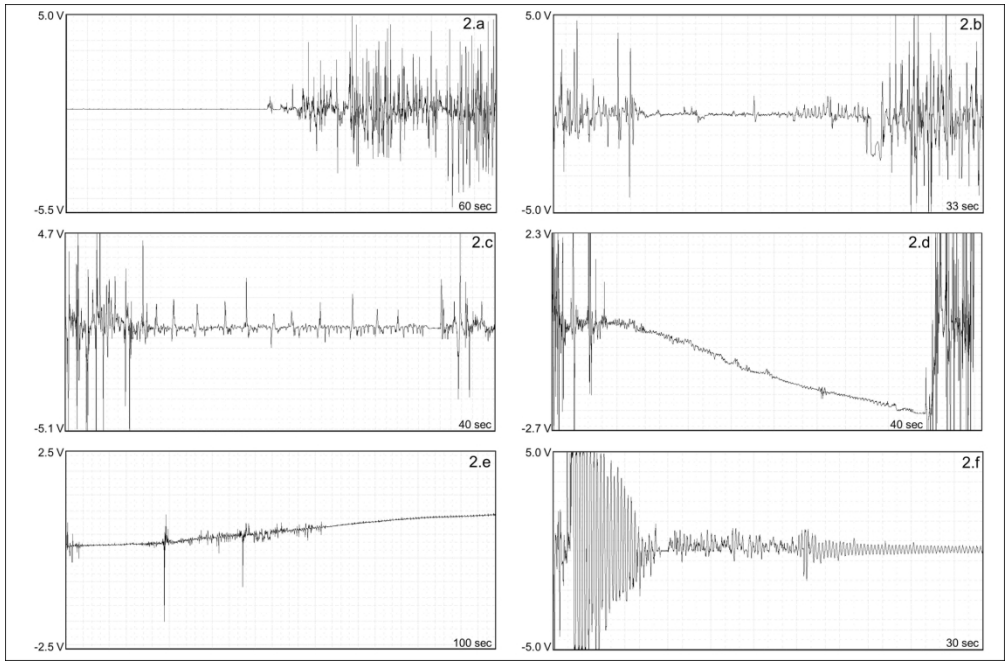

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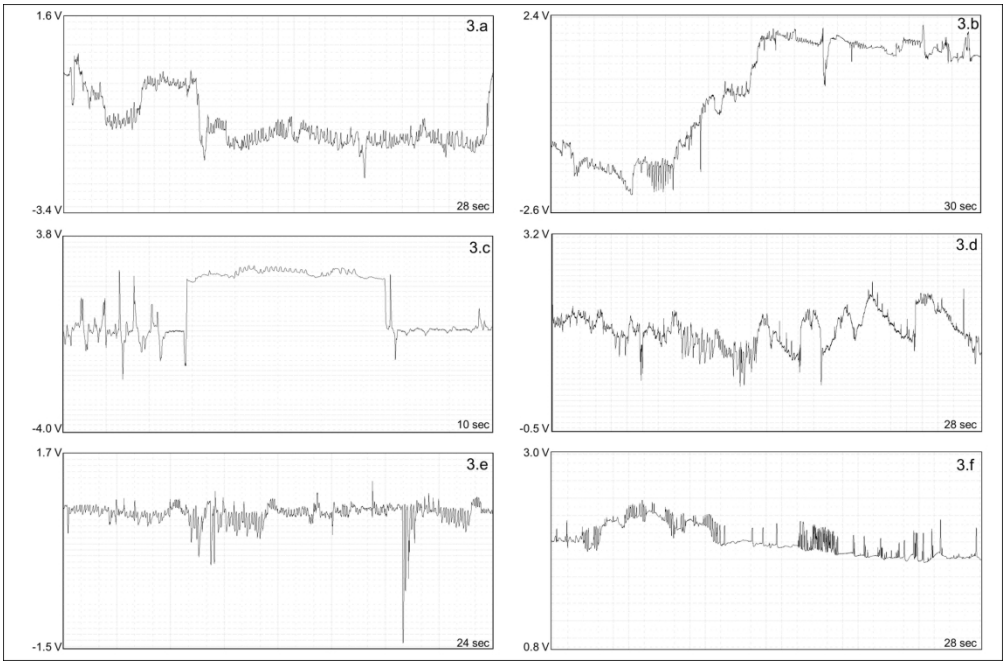

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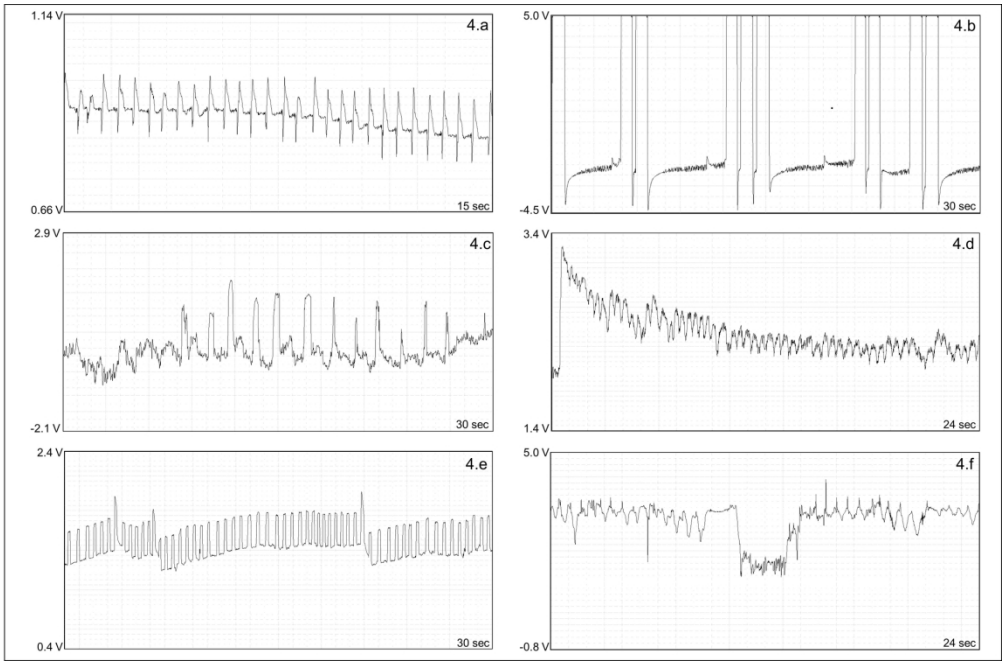

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