



Microbial dynamics in rearing trials of *Hermetia illucens* larvae fed coffee silverskin and microalgae

Andrea Osimani^a, Ilario Ferrocino^b, Maria Rita Corvaglia^b, Andrea Roncolini^a, Vesna Milanović^a, Cristiana Garofalo^a, Lucia Aquilanti^{a,*}, Paola Riolo^a, Sara Ruschioni^a, Elham Jamshidi^a, Nunzio Isidoro^a, Matteo Zarantonello^c, Luca Coccolin^b, Ike Olivotto^c, Francesca Clementi^a

^a Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy

^b Department of Agricultural, Forest, and Food Science, University of Turin, Largo Paolo Braccini 2, Grugliasco, Torino, Italy

^c Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy

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ABSTRACT

In the present study, *Hermetia illucens* larvae were reared on a main rearing substrate composed of a coffee roasting byproduct (coffee silverskin, Cs) enriched with microalgae (*Schizochytrium limacinum* or *Isochrysis galbana*) at various substitution levels. The microbial diversity of the rearing substrates, larvae, and frass (excrement from the larvae mixed with the substrate residue) were studied by the combination of microbial culturing on various growth media and metataxonomic analysis (Illumina sequencing). High counts of total mesophilic aerobes, bacterial spores, presumptive lactic acid bacteria, coagulase-positive cocci, and eumycetes were detected. Enterobacteriaceae counts were low in the rearing diets, whereas higher counts of this microbial family were observed in the larvae and frass. The microbiota of the rearing substrates was characterized by the presence of lactic acid bacteria, including the genera *Lactobacillus*, *Leuconostoc* and *Weissella*. The microbiota of the *H. illucens* larvae fed Cs was characterized by the dominance of *Paenibacillus*. *H. illucens* fed diets containing *I. galbana* were characterized by the presence of *Enterococcus*, *Lysinibacillus*, *Morganella*, and *Paenibacillus*, depending on the algae inclusion level, while *H. illucens* fed diets containing *S. limacinum* were characterized by high relative abundances of *Brevundimonas*, *Enterococcus*, *Paracoccus*, and *Paenibacillus*, depending on the algae inclusion level. *Brevundimonas* and *Alcaligenes* dominated in the frass from larvae fed *I. galbana*; the predominance of *Brevundimonas* was also observed in the frass from larvae fed *Schizochytrium*-enriched diets. Based on the results of the present study, an effect of algae nutrient bioactive substances (e.g. polysaccharides, high-unsaturated fatty acids, taurine, carotenoids) on the relative abundance of some of the bacterial taxa detected in larvae may be hypothesized, thus opening new intriguing perspectives for the control of the entomopathogenic species and foodborne human pathogens potentially occurring in edible insects. Further studies are needed to support this hypothesis. Finally, new information on the microbial diversity occurring in insect frass was also obtained.

1. Introduction

Since the introduction of Regulation (EU) 2015/2283 on novel foods and subsequent Regulation (EU) 2017/893 that allowed the use of insects as processed animal proteins (PAPs) in aquaculture, the exploitation of edible insects has been subjected to an increasing interest by the food and feed industry (Truzzi, Annibaldi, et al., 2020). To concretely exploit the potentialities of edible insects based on previous assessments performed by national authorities in Belgium (FASFC, 2014), the

Netherlands (NVWA, 2014) and France (ANSES, 2015), the European Food Safety Authority (EFSA) published in 2015 an opinion with a list of insect species that can potentially be used as food and feed in the European Union (EU) (EFSA, 2015). Taking into account the above-mentioned national risk assessments, as well as the EFSA opinion (EFSA, 2015), the following insect species were identified as those which fulfil the safety conditions for insect production for feed use: *Hermetia illucens*, *Musca domestica*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Acheta domesticus*, *Grylodes sigillatus*, and *Gryllus assimilis*

* Corresponding author.

E-mail address: l.aquilanti@univpm.it (L. Aquilanti).

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(Regulation (EU) 2017/893).

In Western countries, the human consumption of insects represents a novelty; however, in Asia, Africa, Latin America and Australia, insects are commonly consumed as a part of the daily diet (Garofalo, Milanović, Cardinali, Aquilanti, Clementi & Osimani, 2019). Numerous published studies have demonstrated suitability of the use of insects as feed to produce a high-quality source of nutrients for livestock (Cutrignelli et al., 2018; Gasco, Biancarosa, & Liland, 2020).

Certain most reared insects, including *T. molitor*, *A. diaperinus*, *A. domesticus*, and *H. illucens*, have been demonstrated to provide high value protein intended for use as food or feed.

H. illucens is a species that can act as a bioreactor capable of extracting energy from the most diverse substrates, including animal, vegetable, and urban wastes (Allegretti, Schmidt & Talamini, 2017), thus leading to efficient insect biomass production (Zarantoniello et al., 2019). Therefore, the real earning perspective deriving from the exploitation of *H. illucens* has resulted in the establishment of numerous companies that have to face issues related to the optimization of the rearing cycles and the safety of the end product (De Smet, Wynants, Cos & Van Campenhout, 2018; Wynants et al., 2019).

The massive production of edible insects relies on the use of the opportune substrates that should allow a proper development of the reared insects to obtain the end products (e.g., larvae) with suitable nutritional features (e.g., high protein and fat content) and compliance with high chemical and microbiological safety standards.

A recent review by Garofalo et al. (2019) concluded that edible insects are the vectors of a number of microbial species that can be present in their gut or on the external cuticle. Currently, commensals, spoilage or even human pathogens have been detected in edible insects (Garofalo et al., 2019; Osimani & Aquilanti, 2021). The biodiversity and dynamics of the microbiota occurring during the rearing of the insects is strongly influenced by numerous intrinsic and extrinsic factors, including: i) the transfer of the microorganisms from mother to offspring; ii) the natural microbial contamination of the rearing substrate; and iii) the hygiene of the rearing conditions (Bruno et al., 2019; Osimani et al., 2018). Recent studies carried out on the microbiota of the *H. illucens* larvae reared on various substrates, including food waste, cooked rice, calf forage and the so-called Gainesville housefly diet (a substrate containing alfalfa meal, wheat bran, corn meal, and pelleted peanut hulls) have reported the occurrence of a core microbiota and some other taxa that are likely influenced and thus modulated by biotic and abiotic factors, including the rearing substrates (De Smet et al., 2018; Wynants et al., 2019). Therefore, to obtain the insect-based ingredients suitable for feed or food production, the knowledge of microbial dynamics occurring during rearing is a key factor for the implementation of proper preventive or control strategies for microbiological risk management.

It should be noted that the rearing cycle of the edible insects can be a source of microbiological contamination of the environment, including frass (excrement from the larvae mixed with the substrate residues) highly contaminated by microbiota of the insect origin. The microbiology of frass is poorly understood; however, recent scientific studies highlighted a potential of this rearing waste for use as a soil amendment as reviewed by Schmitt & de Vries (2020), thus emphasizing the need for additional comprehensive microbiological data on this specific substrate.

In the present study, the *H. illucens* larvae were reared on a main rearing substrate composed of a coffee roasting by-product (coffee silverskin) enriched with various proportions of microalgae (*Schizochytrium limacinum* or *Isochrysis galbana*) as the source of polyunsaturated fatty acids (PUFAs) (Truzzi, Giorgini, et al., 2020). In more detail, *S. limacinum* are heterotrophic marine traustochytrids rich in docosahexaenoic acid (DHA) (35 g/100 g of their total fatty acids), whereas *I. galbana* are haptophyte microalgae characterized by a high content of DHA, stearidonic acid and alpha-linolenic acid (Truzzi, Giorgini, et al., 2020).

The microbial dynamics of the rearing substrates, larvae and frass

were then studied by the combination of microbial culture on various growth media and metataxonomic analysis (Illumina sequencing).

2. Materials and methods

2.1. Insect feeding substrates preparation

The main component of the experimental insect feeding substrates consisted of a coffee by-product (coffee silverskin, Cs) obtained from the roasting process of green coffee beans at Saccaria Caffè Srl (Marina di Montemarignano, Italy). Once collected, Cs was processed as already described by Zarantoniello et al. (2020). Briefly, the coffee by-product was grinded using an Ariete 1769 food blender (De Longhi Appliances Srl, Italy) up until particles of 0.4 ± 2 mm in diameter were obtained.

S. limacinum or *I. galbana*, added at different inclusion percentage to Cs, were provided in a lyophilized form by AlghItaly Società Agricola S. R.L. (Sommacampagna, VR, Italy).

Nine different feed blends were tested: 100% Cs; 95% Cs plus 5% *I. galbana* (CsI5); 90% Cs plus 10% *I. galbana* (CsI10); 80% Cs plus 20% *I. galbana* (CsI20); 75% Cs plus 25% *I. galbana* (CsI25); 95% Cs plus 5% *S. limacinum* (CsS5); 90% Cs plus 10% *S. limacinum* (CsS10); 80% Cs plus 20% *S. limacinum* (CsS20); 75% Cs plus 25% *S. limacinum* (CsS25).

Ingredients (Cs and microalgae) were mixed, homogenized, and kept 24 h at 4 °C, before use. All the experimental insect feeding substrates were moistened with distilled water to reach a final moisture of ~70% (Makkar, Tran, Heuzé & Ankers, 2014). Aliquots from diets were sampled and stored at 4 °C until analysis.

2.2. Insect rearing

Six-day-old larvae, purchased from Smart Bugs (Ponzano Veneto, Italy), were counted and divided into groups of 150 larvae per replicate ($n = 5$, for each of the tested feed blends) amounting to a total of 6750 specimens.

The rearing of larvae on the different feed blends was carried out in lid-covered plastic boxes (28 × 19 × 14 cm), screened with fine-mesh cotton gauze, and provided with a single ventilation hole. The boxes were placed into a climatic chamber maintained at a temperature of 27 ± 1 °C, relative humidity of $65 \pm 5\%$, and 24 h dark photoperiod (Zarantoniello et al., 2020). Larvae were collected as soon as they showed a change in tegument color from white to black, thus attesting that the prepupal stage was reached.

After collection, prepupae were stored at 4 °C until further analysis. Aliquots of frass from each treatment were also collected and stored at 4 °C up until analysis.

2.3. Viable counts

To estimate viable counts, 10 g of each sample (substrate, larvae and frass) was aseptically diluted in 90 mL of a sterile water solution of bacteriological peptone (1 g L^{-1}) and homogenized in a Stomacher 400 circulator apparatus (VWR International PBI, Milan, Italy). Ten-fold dilutions of the homogenates were then inoculated on suitable solid media for the counting of various microbial groups. Total mesophilic aerobes were counted in the standard plate count agar (PCA) (Oxoid, Basingstoke, UK) incubated at 30 °C for 48 h. Aerobic bacterial spores were counted after thermal treatment of the homogenates at 80 °C for 15 min followed by cooling in iced water for 5 min and plating in the standard plate count agar (PCA) (Oxoid,) incubated at 30 °C for 48 h. Presumptive lactic acid bacteria were counted on de Man, Rogosa and Sharp (MRS) agar (Oxoid) supplemented with cycloheximide (250 mg L^{-1}) and incubated at 37 °C for 72 h under anaerobic conditions. Coagulase-positive cocci were counted on the mannitol salt agar (MSA) (VWR ProLabo Chemicals, Leuven, Belgium) incubated at 37 °C for 24–48 h. Enterobacteriaceae were counted on violet red bile glucose agar (VRBGA) (VWR ProLabo Chemicals) incubated at 37 °C for 24 h.

Eumycetes were counted on the Wallerstein Laboratory Nutrient (WLN) agar (VWR ProLabo Chemicals) supplemented with chloramphenicol (100 mg L^{-1}) and incubated at 25°C for 72 h.

The presence of *Listeria monocytogenes* and *Salmonella* spp. was assessed in accordance with AFNOR BIO 12/11–03/04 and AFNOR BIO 12/16–09/05 standard methods, respectively.

For each of the rearing replicates ($n = 5$, for each of the feed blend) the analyses were carried out in duplicate and the data were expressed as the mean of log colony forming units (cfu) per gram \pm standard deviation of independent duplicate biological experiments.

2.4. DNA extraction

To obtain DNA extracts from the feed blends, larvae and frass, aliquots (1.5 mL) of each homogenate (dilution 10^{-1}) prepared as described in section 2.3 were centrifuged for 5 min at $16,000 \text{ g}$ and the supernatants were discarded. Total microbial DNA was extracted from the cell pellets with an E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instructions.

A Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the quantity and purity of the DNA extracts, which were then standardized to a concentration of $20 \text{ ng } \mu\text{L}^{-1}$ prior to further analysis. For each sample (diets, larvae, and frass), the DNA extracts obtained from each of the five rearing trials (replicates) were pooled to reduce the intersampling variability and were subjected to 16S rRNA gene sequencing.

2.5. Metataxonomic analysis

For each of the rearing trials ($n = 5$, for each of the feed blend), the extracted and pooled DNA standardized at $20 \text{ ng } \mu\text{L}^{-1}$ was used as a template in the PCR runs for the amplification of the V3-V4 region of the 16S rRNA gene according to the procedure described by Klindworth et al. (2013). Amplicons were purified and tagged according to the 16S metagenomic sequencing library preparation instruction from Illumina. The sequencing was performed using a MiSeq instrument (Illumina, San Diego, CA, USA) with V3 chemistry, which generated 250-bp paired-end reads, following the producer's instructions.

After sequencing, the reads were initially merged using FLASH v.1.2.11 (Magoč & Salzberg, 2011) with default parameters; chimera sequences were filtered using USEARCH v. 11 and then processed with QIIME1 (Caporaso et al., 2010) according to Ferrocino et al. (2017). Briefly, after quality filtering OTUs were clustered at 97% of similarity by using UCLUST clustering method, and representative sequences of each cluster were used to assign taxonomy using the Greengenes 16S rRNA gene database v2013, by means of the RDP Classifier, with a minimum confidence score of 0.80. The operational taxonomic unit (OTU) table was rarefied at the lowest number of sequences per sample (5000) by using QIIME1 and used to build the OTU tables. Sequences were manually blasted in order to confirm the taxonomic assignment. When genus level was not reached, OTUs belonging to family level were collapsed and identified as "Others". In the OTU tables, only OTUs with an incidence $\geq 1\%$ in at least one sample were shown.

Sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (PRJNA643230).

2.6. Statistical analysis

Shapiro–Wilk test was used to test the normality of the data (Mishra et al., 2019). The Tukey–Kramer's honest significant difference (HSD) test (level of significance 0.05) was used to evaluate the differences within viable counts of the samples by one-way analysis of variance (ANOVA). Tests were carried out using the JMP software version 11.0.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Viable counts

The results of viable counting performed on the rearing substrates are reported in Table 1.

Counts of total mesophilic aerobes were generally high from 8.3 to $7.8 \text{ log cfu g}^{-1}$ and there were no significant differences between the samples ($P > 0.05$). The counts of bacterial spores varied from 3.1 to $4.8 \text{ log cfu g}^{-1}$ and the highest values were recorded in the samples containing *I. galbana*; the lowest values were observed in the case of Cs. Presumptive lactic acid bacteria counts ranged from 8.0 to $8.6 \text{ log cfu g}^{-1}$ and Cs showed the highest values; the samples containing *I. galbana* had the lowest counts. In the case of coagulase-positive cocci, the values were from 4.0 to $6.6 \text{ log cfu g}^{-1}$; the Cs samples showed the lowest values and the samples containing *S. limacinum* had the highest counts. In all samples, Enterobacteriaceae counts were $< 1 \text{ log cfu g}^{-1}$. Finally, eumycetes counts ranged from 4.3 to $7.7 \text{ log cfu g}^{-1}$; the Cs samples showed the lowest values whereas the samples containing *S. limacinum* showed the highest values.

The results of viable counting performed on *H. illucens* larvae reared on various feed blends showed significant differences ($P < 0.05$) and are reported in Table 2.

In the case of total mesophilic aerobes, the counts were from 7.7 to $9.1 \text{ log cfu g}^{-1}$; the highest values were detected in the samples containing *I. galbana* and the lowest counts were in the samples containing *S. limacinum*. Bacterial spore counts ranged from 3.1 to $6.7 \text{ log cfu g}^{-1}$; the highest values were observed in the samples containing *I. galbana* and the lowest counts were in the Cs samples. The values of presumptive viable lactic acid bacteria were from 6.5 to $7.8 \text{ log cfu g}^{-1}$; the highest values were detected in the samples containing *I. galbana*. In the case of coagulase-positive cocci, viable counts ranged from 6.2 to $8.4 \text{ log cfu g}^{-1}$, with the highest values detected in the samples containing *I. galbana* and the lowest counts detected in the samples containing *S. limacinum*. Enterobacteriaceae counts were from 2.5 to $4.9 \text{ log cfu g}^{-1}$; the Cs samples showed the highest values whereas the samples containing *S. limacinum* had the lowest values. Values from 5.4 to $7.4 \text{ log cfu g}^{-1}$ were detected in the case of eumycetes, with the highest values found in the samples containing *I. galbana* and the lowest in the Cs samples.

The results of viable counting performed on frass from *H. illucens* larvae reared on the different feed blends are reported in Table 3.

The counts of total mesophilic aerobes were comprised between 9.3 and $9.9 \text{ log cfu g}^{-1}$, with no significant differences among samples. Bacterial spores ranged between 7.2 and $7.6 \text{ log cfu g}^{-1}$, with no significant differences among samples. As for presumptive lactic acid bacteria, values from 7.6 to $8.2 \text{ log cfu g}^{-1}$ were detected, with no significant differences among samples ($P > 0.05$). Coagulase-positive cocci were comprised between 7.0 and $7.3 \text{ log cfu g}^{-1}$, again with no significant differences among samples ($P > 0.05$). The counts of Enterobacteriaceae were comprised between 3.7 and $4.8 \text{ log cfu g}^{-1}$, with the highest values for the samples containing *I. galbana*. Finally, eumycetes counts ranged from 6.3 to $6.8 \text{ log cfu g}^{-1}$ and there were no significant differences between the samples.

Listeria monocytogenes and *Salmonella* spp. were never detected in 25 g sample.

3.2. 16S rRNA gene amplicon target sequencing

3.2.1. Microbiota of the rearing substrates

A total of 98,358 sequences were analyzed and the results showed that *Lactobacillus*, *Leuconostoc* and *Weissella* were stably detected in the basal diet (Cs), where they constitute the core microbiota (Fig. 1).

Diets with the inclusion of 5, 10, 20 or 25% of microalgae were clearly different compared to Cs (Fig. 1). Specifically, as *I. galbana* was added at 5, 10, 20, or 25% inclusion level, an increase in *Weissella* (57, 52, 62 and 62% of the relative abundance, respectively) was observed

Table 1

Viable counts (expressed as log cfu g⁻¹) of *Hermetia illucens* rearing substrates used to produce feed blends containing coffee silverskin (Cs) or different inclusion levels (5%, 10%, 20%, or 25%) of *Schyzochitrium limacinum* (S) or *Isochrysis galbana* (I).

| Rearing substrates | Total mesophilic aerobes | Bacterial spores | Presumptive lactic acid bacteria | Coagulase-positive cocci | Enterobacteriaceae | Eumycetes |
|--------------------|--------------------------|------------------------|----------------------------------|--------------------------|--------------------|------------------------|
| Cs | 8.0 ± 0.0 ^a | 3.1 ± 0.1 ^c | 8.4 ± 0.1 ^a | 4.0 ± 0.0 ^c | <1 ^a | 4.3 ± 0.0 ^c |
| CsI5 | 7.8 ± 0.1 ^a | 4.6 ± 0.0 ^a | 8.0 ± 0.1 ^b | 4.9 ± 0.1 ^b | <1 ^a | 4.6 ± 0.1 ^b |
| CsI10 | 8.0 ± 0.1 ^a | 4.8 ± 0.0 ^a | 8.1 ± 0.0 ^b | 4.9 ± 0.1 ^b | <1 ^a | 5.0 ± 0.0 ^b |
| CsI20 | 7.9 ± 0.0 ^a | 4.7 ± 0.1 ^a | 8.0 ± 0.0 ^b | 4.7 ± 0.1 ^b | <1 ^a | 4.9 ± 0.1 ^b |
| CsI25 | 7.8 ± 0.0 ^a | 4.8 ± 0.1 ^a | 8.0 ± 0.1 ^b | 5.0 ± 0.1 ^b | <1 ^a | 4.7 ± 0.1 ^b |
| CsS5 | 8.3 ± 0.1 ^a | 4.0 ± 0.0 ^b | 8.6 ± 0.0 ^a | 6.0 ± 0.1 ^a | <1 ^a | 7.6 ± 0.1 ^a |
| CsS10 | 8.3 ± 0.1 ^a | 4.3 ± 0.0 ^b | 8.6 ± 0.1 ^a | 6.6 ± 0.0 ^a | <1 ^a | 7.6 ± 0.1 ^a |
| CsS20 | 8.0 ± 0.0 ^a | 4.2 ± 0.1 ^b | 8.2 ± 0.0 ^{ab} | 6.2 ± 0.1 ^a | <1 ^a | 7.7 ± 0.1 ^a |
| CsS25 | 8.0 ± 0.1 ^a | 4.2 ± 0.1 ^b | 8.2 ± 0.1 ^{ab} | 6.0 ± 0.0 ^a | <1 ^a | 7.7 ± 0.0 ^a |

Counts are expressed as means ± standard deviation.

Within each column, means with different superscript letters are significantly different ($P < 0.05$).

Table 2

Viable counts (expressed as log cfu g⁻¹) of *Hermetia illucens* larvae (H) reared on the different substrates containing coffee silverskin (Cs) or different inclusion levels (5%, 10%, 20%, or 25%) of *Schyzochitrium limacinum* (S) or *Isochrysis galbana* (I).

| Larvae samples | Total mesophilic aerobes | Bacterial spores | Presumptive lactic acid bacteria | Coagulase-positive cocci | Enterobacteriaceae | Eumycetes |
|----------------|--------------------------|------------------------|----------------------------------|--------------------------|------------------------|------------------------|
| HCS | 8.4 ± 0.0 ^b | 3.1 ± 0.0 ^c | 6.8 ± 0.0 ^b | 7.3 ± 0.0 ^b | 4.9 ± 0.0 ^a | 5.4 ± 0.1 ^c |
| HCSI5 | 8.9 ± 0.1 ^a | 6.7 ± 0.0 ^a | 7.7 ± 0.0 ^a | 8.0 ± 0.1 ^a | 4.3 ± 0.0 ^b | 7.4 ± 0.0 ^a |
| HCSI10 | 8.8 ± 0.0 ^a | 6.7 ± 0.1 ^a | 7.6 ± 0.1 ^a | 8.0 ± 0.0 ^a | 4.1 ± 0.1 ^b | 7.3 ± 0.0 ^a |
| HCSI20 | 9.0 ± 0.1 ^a | 6.4 ± 0.1 ^a | 7.8 ± 0.1 ^a | 8.3 ± 0.0 ^a | 4.3 ± 0.0 ^b | 7.3 ± 0.1 ^a |
| HCSI25 | 9.1 ± 0.1 ^a | 6.7 ± 0.0 ^a | 7.8 ± 0.1 ^a | 8.4 ± 0.0 ^a | 4.3 ± 0.0 ^b | 7.2 ± 0.1 ^a |
| HCS5 | 7.8 ± 0.0 ^c | 3.9 ± 0.2 ^b | 7.0 ± 0.0 ^b | 6.2 ± 0.1 ^c | 2.9 ± 0.1 ^c | 5.9 ± 0.0 ^b |
| HCS10 | 8.0 ± 0.0 ^c | 4.2 ± 0.0 ^b | 6.5 ± 0.1 ^b | 6.3 ± 0.0 ^c | 3.2 ± 0.1 ^c | 6.2 ± 0.1 ^b |
| HCS20 | 7.7 ± 0.0 ^c | 3.9 ± 0.1 ^b | 6.7 ± 0.0 ^b | 6.8 ± 0.1 ^c | 3.2 ± 0.0 ^c | 6.2 ± 0.1 ^b |
| HCS25 | 8.0 ± 0.1 ^c | 3.7 ± 0.1 ^b | 6.5 ± 0.0 ^b | 6.7 ± 0.1 ^c | 3.2 ± 0.1 ^c | 5.9 ± 0.0 ^b |

Counts are expressed as means ± standard deviation.

Within each column, means with different superscript letters are significantly different ($P < 0.05$).

Table 3

Viable counts (expressed as log cfu g⁻¹) of frass (F) from *H. illucens* larvae reared on the different substrates containing coffee silverskin (Cs) or different inclusion levels (5%, 10%, 20%, or 25%) of *Schyzochitrium limacinum* (S) or *Isochrysis galbana* (I).

| Frass samples | Total mesophilic aerobes | Bacterial spores | Presumptive lactic acid bacteria | Coagulase-positive cocci | Enterobacteriaceae | Eumycetes |
|---------------|--------------------------|------------------------|----------------------------------|--------------------------|------------------------|------------------------|
| FCs | 9.4 ± 0.3 ^a | 7.2 ± 0.0 ^a | 8.1 ± 0.3 ^a | 7.0 ± 0.1 ^a | 4.1 ± 0.5 ^b | 6.4 ± 0.1 ^a |
| FCsI5 | 9.8 ± 0.3 ^a | 7.4 ± 0.2 ^a | 7.8 ± 0.3 ^a | 7.2 ± 0.4 ^a | 4.7 ± 0.6 ^a | 6.3 ± 0.2 ^a |
| FCsI10 | 9.7 ± 0.1 ^a | 7.6 ± 0.1 ^a | 8.2 ± 0.1 ^a | 7.1 ± 0.2 ^a | 4.8 ± 0.4 ^a | 6.5 ± 0.3 ^a |
| FCsI20 | 9.7 ± 0.1 ^a | 7.3 ± 0.1 ^a | 8.2 ± 0.1 ^a | 7.1 ± 0.2 ^a | 4.7 ± 0.4 ^a | 6.7 ± 0.2 ^a |
| FCsI25 | 9.9 ± 0.0 ^a | 7.4 ± 0.2 ^a | 8.0 ± 0.4 ^a | 7.3 ± 0.2 ^a | 5.0 ± 0.1 ^a | 6.7 ± 0.3 ^a |
| FCsS5 | 9.3 ± 0.2 ^a | 7.2 ± 0.0 ^a | 7.6 ± 0.5 ^a | 7.0 ± 0.1 ^a | 3.7 ± 0.2 ^b | 6.5 ± 0.1 ^a |
| FCsS10 | 9.4 ± 0.2 ^a | 7.2 ± 0.0 ^a | 7.6 ± 0.27 ^a | 7.1 ± 0.1 ^a | 4.1 ± 0.2 ^b | 6.7 ± 0.1 ^a |
| FCsS20 | 9.4 ± 0.1 ^a | 7.2 ± 0.1 ^a | 7.6 ± 0.2 ^a | 7.1 ± 0.1 ^a | 4.1 ± 0.0 ^b | 6.6 ± 0.4 ^a |
| FCsS25 | 9.5 ± 0.1 ^a | 7.2 ± 0.1 ^a | 8.1 ± 0.4 ^a | 7.1 ± 0.1 ^a | 4.2 ± 0.0 ^b | 6.8 ± 0.4 ^a |

Counts are expressed as means ± standard deviation.

Within each column, means with different superscript letters are significantly different ($P < 0.05$).

together with a reduction of *Lactobacillus* (from 33% of the Cs to about 2%). *Leuconostoc* was rarely observed (below 1%). The microbiota of the samples CsS5, CsS10 and CsS20 containing *S. limacinum* was characterized by the presence of *Leuconostoc* (with a relative abundance of 50, 28 and 57%, respectively) and *Lactobacillus* (11, 13 and 6%, respectively). Moreover, *Weissella* was stably detected in all samples (with a relative abundance of approximately 20%), reaching the maximum level on sample CsS25 (Fig. 1).

3.2.2. Microbiota of *H. illucens* larvae

The results of the 16S rRNA gene sequencing using 60,704 reads (Fig. 2) showed the presence of *Paenibacillus* (22% of the relative abundance) and *Lysinibacillus* (9%) in larvae fed Cs (HCs). In addition, *Solibacillus* and *Brevundimonas* were found at 7 and 4%, respectively, in the same samples.

Larvae samples HCsI5, HCsI10, and HCsI20 showed a high abundance of *Morganella* (about 9, 10 and 20%, respectively) (Fig. 2). *Paenibacillus* was stably detected in the same samples with a 13% relative

abundance, whereas *Lysinibacillus*, *Brevundimonas*, *Enterococcus*, *Paracoccus* and *Solibacillus* were found at a relative abundance <5%. In the HCsI25 larvae samples, *Lysinibacillus* (30%) and *Enterococcus* (9%) were detected, whereas the HCsS5, HCsS10, HCsS20 and HCsS25 samples showed high presence of *Brevundimonas* (with relative abundance of 14, 25, 15 and 14%, respectively). In the HCsS5, HCsS10 and HCsS20 samples, *Paenibacillus* was also identified with a relative abundance of 9, 5 and 13%, respectively, whereas the HCsS25 samples were characterized by the presence of *Enterococcus* (26%) and *Paracoccus* (8.5%).

A separation of the samples based on the microbiota composition emerged from the heat plot (Fig. 3). In more detail, the inclusion of *S. limacinum* was associated with the presence of *Bacillus*, *Brevundimonas*, *Devosia*, *Paenibacillus*, and *Paracoccus*, whereas the inclusion of *I. galbana* was associated with the presence of *Clostridium*, *Alcaligenes*, *Lysinibacillus*, and *Morganella*.

3.2.3. Microbiota of frass

The results of the 16S rRNA gene sequencing by using 70,881 reads

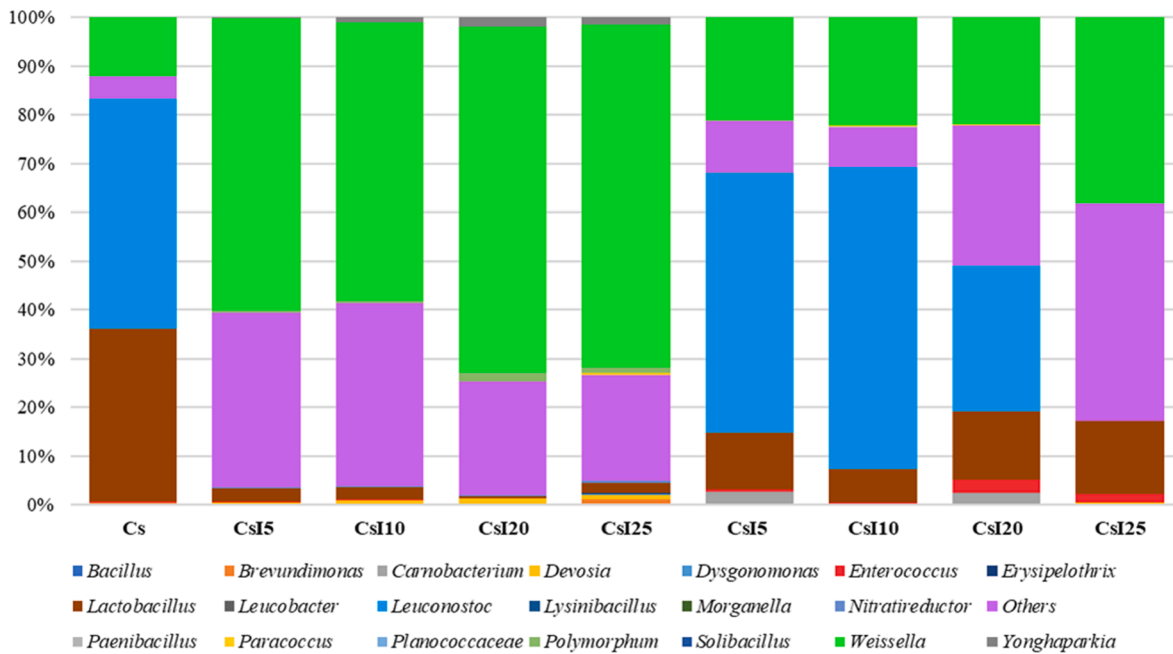


Fig. 1. Incidence of the major taxonomic groups detected by sequencing of the feed blends. Cs, 100% coffee silverskin; CsI5, 95% Cs plus 5% *Isochrysis galbana*; CsI10, 90% Cs plus 10% *I. galbana*; CsI20, 80% Cs plus 20% *I. galbana*; CsI25, 75% Cs plus 25% *I. galbana*; CsS5, 95% Cs plus 5% *Schizochytrium limacinum*; CsS10, 90% Cs plus 10% *S. limacinum*; CsS20, 80% Cs plus 20% *S. limacinum*; CsS25, 75% Cs plus 25% *S. limacinum*. Only OTUs with an incidence $\geq 1\%$ in at least one sample are shown. The OTUs with relative abundance below 1% are grouped together in “Others”. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

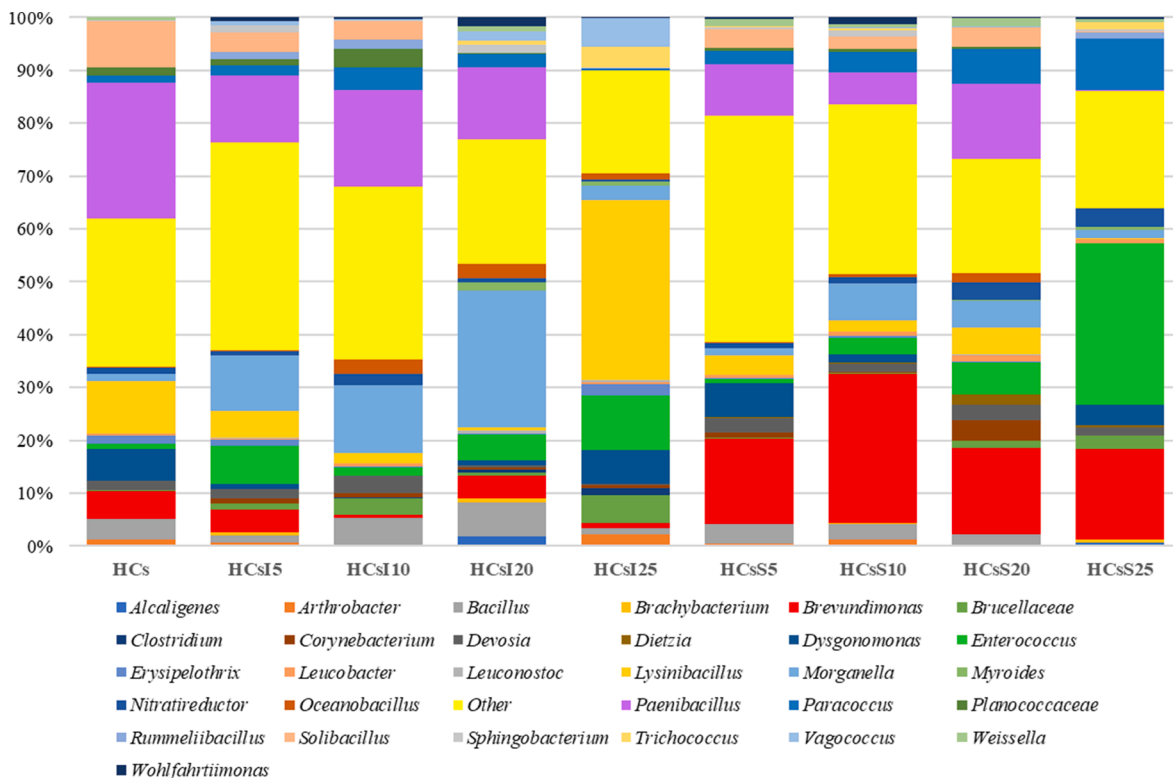


Fig. 2. Incidence of the major taxonomic groups detected by sequencing of larvae samples. HCs, larvae fed with 100% coffee silverskin; HCsI5, larvae fed with 95% Cs plus 5% *Isochrysis galbana*; HCsI10, larvae fed with 90% Cs plus 10% *I. galbana*; HCsI20, larvae fed with 80% Cs plus 20% *I. galbana*; HCsI25, larvae fed with 75% Cs plus 25% *I. galbana*; HCsS5, larvae fed with 95% Cs plus 5% *Schizochytrium limacinum*; HCsS10, larvae fed with 90% Cs plus 10% *S. limacinum*; HCsS20, larvae fed with 80% Cs plus 20% *S. limacinum*; HCsS25, larvae fed with 75% Cs plus 25% *S. limacinum*. Only OTUs with an incidence $\geq 1\%$ in at least one sample are shown. The OTUs with relative abundance below 1% are grouped together in “Other”. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

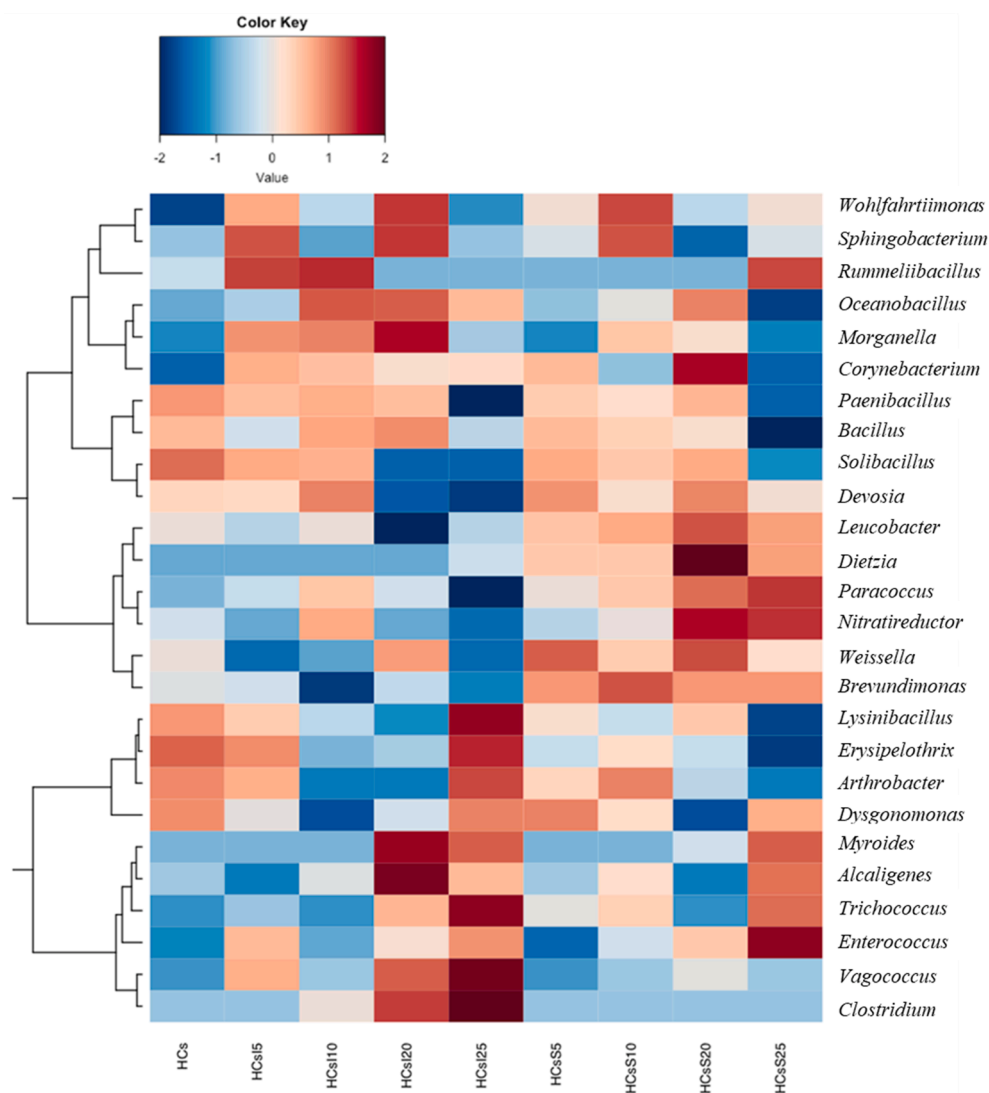


Fig. 3. Heat plot of the abundances of OTUs and samples (upper red bar). Rows are clustered by means of Ward linkage hierarchical clustering methods. The intensity of the colors represents the degree of correlation between the samples and OTUs with dark red positive association and dark blue negative association. HCs, larvae fed with 100% coffee silverskin; HCsI5, larvae fed with 95% Cs plus 5% *Ischrysis galbana*; HCsI10, larvae fed with 90% Cs plus 10% *I. galbana*; HCsI20, larvae fed with 80% Cs plus 20% *I. galbana*; HCsI25, larvae fed with 75% Cs plus 25% *I. galbana*; HCsS5, larvae fed with 95% Cs plus 5% *Schizochytrium limacinum*; HCsS10, larvae fed with 90% Cs plus 10% *S. limacinum*; HCsS20, larvae fed with 80% Cs plus 20% *S. limacinum*; HCsS25, larvae fed with 75% Cs plus 25% *S. limacinum*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are shown in Fig. 4. A high abundance of *Brevundimonas* (about 14, 12, 7 and 8% in FCsI5, FCsI10, FCsI20, and FCsI25, respectively) followed by *Alcaligenes* (5, 7, 4 and 12%) was seen in the frass from larvae fed *I. galbana*, whereas a predominance of *Brevundimonas* was observed in the frass from larvae fed *Schyzochytrium*-enriched diets and Cs (15, 24, 29, 27 and 18%, in FCsS5, FCsS10, FCsS20, and FCsS25, respectively). *Enterococcus* was prevalent in FCsI10 and FCsI20 with a 9 and 12% relative abundance, respectively, whereas *Trichococcus* was present at a 5% relative abundance in the frass from larvae fed Cs (FCs) and *Schyzochytrium*-enriched diets. Finally, *Devosia* (about 8%) was detected in FCs; moreover, *Myroides* was detected in FCsI25 with a relative abundance of about 10%.

4. Discussion

H. illucens can use a number of organic materials for its growth; however, not all substrates are able to assure the proper provision of the nutrients for the larval development (Wang & Shelomi, 2017). It is known that similar to other edible insects, the microbiological quality of *H. illucens* larvae strictly depends on the contamination of the rearing substrate and the rearing environment. It should be noted that *H. illucens* larvae are able to reduce the microbial load of the substrates containing Gram-negative bacteria of the Enterobacteriaceae family (Wang & Shelomi, 2017). Hence, the study of the microbial dynamics during the

rearing of this edible insect is crucial for a proper selection of the most suitable rearing substrate and the implementation of the proper preventive measures for microbiological risk management.

According to the microbiological analyses performed on Cs, high counts were detected for all microbial groups assessed, except Enterobacteriaceae. The data for total mesophilic aerobes, bacterial spores and presumptive lactic acid bacteria are in agreement with the results reported by Osimani et al. (2019) in the same rearing substrate. Cs is the tegument of green coffee beans and a byproduct of the roasting process. This substrate is a natural source of numerous bioactive compounds, such as chlorogenic acid, caffeic acid, melanoidins and dietary fiber, that can modulate the growth of microorganisms (Fernandez-Gomez et al., 2016; Truzzi, Giorgini, et al., 2020). The microbiological quality of Cs can vary depending on the roasting process and storage conditions before disposal. In the present study, viable counts were detected in the feed blends containing various levels of either *I. galbana* or *S. limacinum* and were generally comparable with the counts detected in the un-blended Cs.

As a general trend, larvae were characterized by higher counts than those detected in the feed blends. Loads of total mesophilic aerobes, bacterial spores, lactic acid bacteria and eumycetes were in agreement with the loads reported by Wynants et al. (2019) for *H. illucens* larvae reared on organic waste streams. In detail, Wynants et al. (2019) reported values up to 9.0 log cfu g⁻¹ for total mesophilic aerobes, whereas

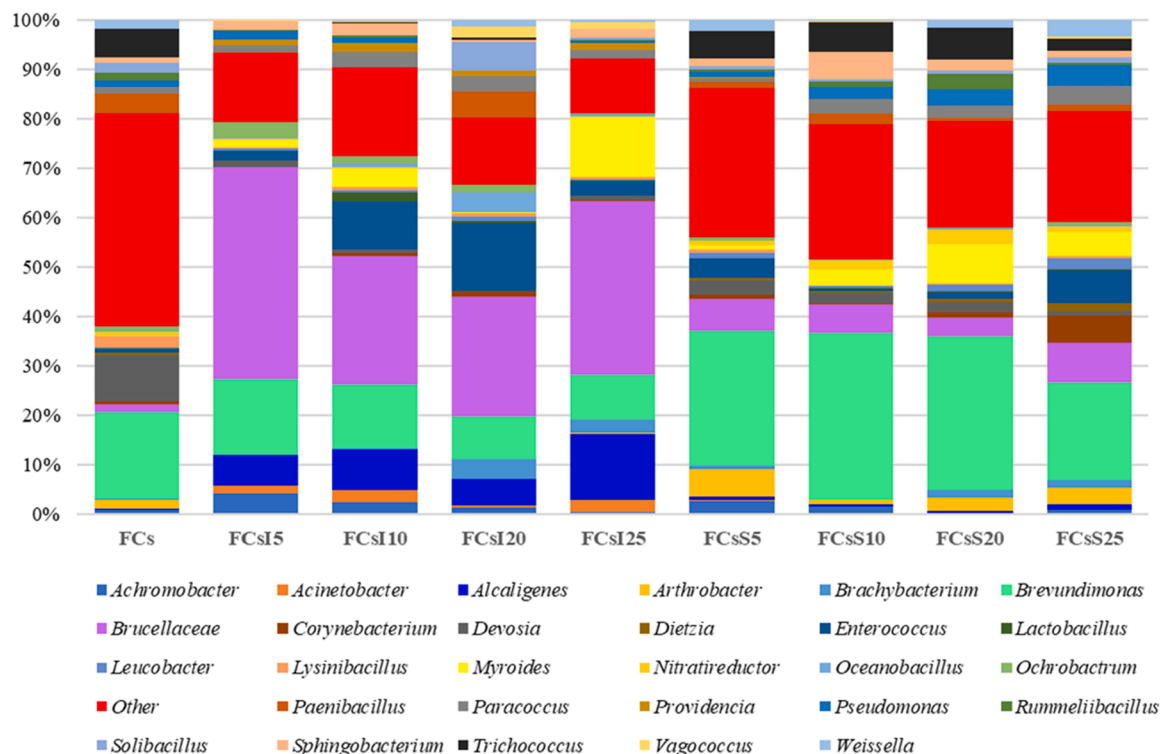


Fig. 4. Incidence of the major taxonomic groups detected by sequencing of frass samples. FCs, frass from larvae fed with 100% coffee silverskin; FCsI5, frass from larvae fed with 95% Cs plus 5% *Isochrysis galbana*; FCsI10, frass from larvae fed with 90% Cs plus 10% *I. galbana*; FCsI20, frass from larvae fed with 80% Cs plus 20% *I. galbana*; FCsI25, frass from larvae fed with 75% Cs plus 25% *I. galbana*; FCsS5, frass from larvae fed with 95% Cs plus 5% *Schizochytrium limacinum*; FCsS10, frass from larvae fed with 90% Cs plus 10% *S. limacinum*; FCsS20, frass from larvae fed with 80% Cs plus 20% *S. limacinum*; FCsS25, frass from larvae fed with 75% Cs plus 25% *S. limacinum*. Only OTUs with an incidence $\geq 1\%$ in at least one sample are shown. The OTUs with relative abundance below 1% are grouped together in “Other”. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

values up to $7.5 \log \text{cfu g}^{-1}$ were reported by the same authors for bacterial spores; in the case of lactic acid bacteria and eumycetes, values up to 8.5 and $6.8 \log \text{cfu g}^{-1}$ were reported by Wynants et al. (2019), respectively.

To the best of our knowledge, there is a paucity of the available data regarding microbial counts in the insect frass. Notwithstanding, the data obtained in the present study are in agreement with a previous study by Wynants et al. (2019) that indicated that in frass produced during the rearing of *H. illucens* larvae, detected counts of total mesophilic aerobes were up to $10.0 \log \text{cfu g}^{-1}$. Moreover, Wynants et al. (2019) reported bacterial spore counts up to $7.0 \log \text{cfu g}^{-1}$; in the case of lactic acid bacteria and eumycetes, the counts were up to 9.8 and $7.8 \log \text{cfu g}^{-1}$, respectively.

To the best of our knowledge, no previous reports on the counts of coagulase-positive cocci in the *H. illucens* rearing chain are available in the scientific literature, and thus, the data cannot be compared.

The results of the metataxonomic approach identified the major and minor taxa.

With regard to the rearing diets, lactic acid bacteria were massively present in the coffee byproduct, thus confirming the results of viable counting. Pothakos et al. (2020) have recently reported that *Lactococcus* and *Enterococcus* together with *Weissella*, *Leuconostoc* and *Lactobacillus* constitute the dominant lactic acid bacteria genera in the last phase of the coffee mucilage fermentation, thus explaining the occurrence of the latter three genera in the Cs used as a rearing substrate for *H. illucens* larvae. Lactic acid bacteria are known to be responsible for coffee fermentation and for the production of organic acids, esters and alcohols that directly influence the sensory properties of coffee (Martins et al., 2020).

With regard to the microbiota of the *H. illucens* larvae, a complex microbiota was detected and hence only the more relevant taxa will be

discussed. Interestingly, only a few bacterial taxa previously detected in the feed blends were found to be dominant OTUs in the larvae, thus suggesting that a stable resident gut microbiota is present in the larvae irrespective of their diet. In addition, a selective effect of microalgae inclusion in the rearing substrates on the composition of the larvae microbiota was observed. Regarding the latter aspect, Zheng, Chen & Cheong (2020) have recently reported that microalgae are a potential source of polysaccharides that can selectively enhance or reduce the viability of the microorganisms.

In detail, *Paenibacillus* was detected at variable abundances in the larvae, except for the larvae that were reared on the diet with the highest microalgae inclusion, irrespective of the type of algae. The genus *Paenibacillus* encompasses over 150 species of spore-forming and plant growth-promoting rhizobacteria (Eastman, Heinrichs & Yuan, 2014). In edible insects, *Paenibacillus* has been reported as an entomopathogen of the coconut rhinoceros beetle (Gahukar, 2020). The same genus has also been reported as a causative agent of a lethal intestinal infection of honeybee (*Apis mellifera*) (Morrissey et al., 2015). Currently, *Paenibacillus* has been detected in numerous edible insects, including giant water bugs, black ants, winged termites, rhino beetles, mole crickets and silkworm pupae (Osimani et al., 2018). Members of the *Paenibacillus* genus are known to inhibit other bacteria and even fungi due to their production of biologically active substances, such as antibiotics, bacteriocins and/or small active peptides (Singh, Ghodke & Chhatpar, 2009). Given the latter property, these microorganisms might have contributed to the modulation of the viable gut microbiota of the analyzed *H. illucens* larvae. In the present study, *Paenibacillus* was detected at the lowest relative abundance in the samples of larvae fed on the substrates with the highest substitution level of either *I. galbana* or *S. limacinum*, thus suggesting a negative effect of the algae on the occurrence of this bacterial genus.

With regard to the presence of *Lysinibacillus* in larvae fed 100% Cs, members of this genus can have pathogenic activity in mosquitoes, harlequin fly larvae, German cockroach, common cutworm and water scorpions (Berry, 2012). Moreover, *Lysinibacillus* has already been detected in a number of species of edible insects, thus confirming its adaptation to the gut of these organisms (Garofalo et al., 2019; Grabowski & Klein, 2017). In the present study, *Lysinibacillus* was detected at the highest relative abundance in larvae fed the substrate with the highest substitution level of *I. galbana*, whereas the lowest abundance was observed in larvae fed the highest substitution level of *S. limacinum*. It should be noted that the absence of *Lysinibacillus* in all substrates used for the rearing of the larvae suggests that occurrence of this genus in the gut of the *H. illucens* larvae can be ascribed to a direct contamination of the eggs from the mother to the offspring during egg-laying. Moreover, no effects of this microorganism on the viability of larvae were observed, thus suggesting the absence of any pathogenic activity of *Lysinibacillus* in this insect species.

The overall differences in the occurrence of *Lysinibacillus* and *Pae-nibacillus* in larvae fed various diets suggest an influence of the two tested algae on these two bacterial genera. These preliminary results suggest a need of further investigations on the inclusion of microalgae in the substrates for rearing of edible insects. To the best of our knowledge, no previous studies on the effect of algae on the microbiota of edible insects are available in the scientific literature and the data cannot be compared.

Moreover, an increase in the inclusion of algae resulted in a reduction in the relative abundance of *Solibacillus* detected in the larvae microbiota. *Solibacillus* belongs to the Planococcaceae family; this genus of spore-forming bacteria is generally of environmental origin (e.g., sediment of estuarine environment) (Markande, Acharya & Nerurkar, 2013). To the best of our knowledge, this is the first report of *Solibacillus* in edible insects. Interestingly, Wan, Zhao, Guo, Asraf, & Bai (2013) described a bioflocculating activity of *Solibacillus silvestris* towards microalgae, thus suggesting that a potential interaction between the two algae included in the *H. illucens* diets and *Solibacillus* should be investigated in the future.

In the case of *Brevundimonas*, its occurrence in insects has already been described in scorpions and in both larvae and adults of lepidoptera (Grabowski et al., 2017; Garofalo et al., 2019). Interestingly, *Brevundimonas* was detected as one of the major OTUs in the mucosa-associated bacteria of rainbow trout (*Oncorhynchus mykiss*) fed a *H. illucens*-enriched diet, thus confirming the strict association of this insect with *H. illucens* (Bruni, Pastorelli, Viti, Gasco & Parisi, 2018). In the present study, *Brevundimonas* was massively found in *H. illucens* fed *S. limacinum*, irrespective of the level of algae inclusion.

An increase in the inclusion of *I. galbana* was associated with an increase in the relative abundance of *Morganella* in the larvae, whereas no appreciable differences in the relative abundance of *Morganella* were detected in larvae fed *S. limacinum*-enriched diets. *Morganella* is a genus of the Enterobacteriaceae family, which includes Gram-negative rod-shaped aerobic and facultatively anaerobic bacterial species. This genus is usually isolated from freshwater, soil and intestinal tracts of animals (Oh et al., 2020). *Morganella* has already been detected in insects, including flies, spiders (Grabowski & Klein, 2017), and edible insects, such as fresh and processed mealworm larvae and *H. illucens* larvae (Garofalo et al., 2019; Liu, Yao, Chapman, Su & Wang, 2020).

Finally, the abundance of *Enterococcus* was increased concomitantly with an increase in the inclusion levels of *S. limacinum*. The presence of *Enterococcus* in insects is common and has been documented by numerous reports on its detection in mealworms, moths, ants, butterflies, flies and even *H. illucens* larvae (Garofalo et al., 2019; Grabowski & Klein, 2017; Osimani et al., 2019). As reported by Zheng et al. (2020), algae polysaccharides may stimulate the growth of enterococci, thus explaining an increase in the relative abundance of this microbial group in larvae fed diets containing the highest inclusion level of *S. limacinum*.

Schmitt & de Vries (2020) have recently reported that frass produced

during the rearing of *H. illucens* larvae can be used in a strained agricultural system. The environmental impact of application of this byproduct as a soil amendment is lower compared to the impact of existing products. However, the effectiveness of *H. illucens* frass varies depending on the diet used during the rearing of the insects and its physicochemical and microbiological features are thus variable (Schmitt & de Vries, 2020).

With regard to the latter aspect, insect frass can undoubtedly represent a rich culture medium for saprophytic microorganisms and a vehicle for transmission of the bacterial plant pathogens (Osimani et al., 2019). Moreover, the metabolic activity of the microorganisms in the frass can lead to the production of the molecules that can be perceived by other insects and influence their oviposition (Anbutu & Togashi, 2002).

In the present study, an enrichment in some of the species detected in the larvae was observed in the corresponding frass. To the best of our knowledge, only a few published reports have described the microbiota of frass, thus making comparison of the data difficult.

As a general trend *Alcaligenes* had the highest abundance in the frass from larvae fed Cs enriched with *I. galbana*. Species of the *Alcaligenes* genus have a potential detoxifying activity that still requires a clarification; *Alcaligenes* have been isolated from cerambycid beetle (Lemoine, Engl & Kaltenpoth, 2020).

A decrease in the relative abundance of the *Trichococcus* genus was detected in the frass from larvae fed increasing levels of *I. galbana*. *Trichococcus* belongs to the Carnobacteriaceae family and to the best of our knowledge, there are no previous reports in the scientific literature on the occurrence of this genus in the rearing chain of edible insects; thus, the data cannot be further compared.

With regard to the influence of microalgae on the microbial growth, Han, Pang, Wen, You, Huang & Kulikouskaya (2020) have recently reported that a sulfated polysaccharide produced by Rhodophyta algae can have a positive effect on the growth of Bacteroidetes and Proteobacteria and lead to a reduction in Firmicutes and Actinobacteria in the human feces. Moreover, Shang et al. (2017) reported that fucoidan (a sulfated polysaccharide) from the *Laminaria* and *Ascophyllum* algae can stimulate the growth of Bacteroides and selectively modulate the growth of other taxa present in the gut of mice. Finally, laminarin (a polysaccharide produced by brown algae from glucose) can negatively impact the growth of Firmicutes, while positively influencing the multiplication of Bacteroides (Nguyen et al., 2016).

With regard to the algae used in the present study, Jin et al. (2020) reported that in a simulated *in vitro* digestion and colonic fermentation, the supplementation of microalgae, including *Schizochytrium* spp., leads to a significantly increase in the abundance of *Bacteroides* and *Dialister* involved in propionate metabolism. Moreover, Lyons, Turnbull, Dawson, & Crumlishm (2017) have reported a notable increase in the relative abundances of *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Weissella* in the distal intestinal microbiome of rainbow trout fed a whole cell *S. limacinum* meal. With regard to *I. galbana*, Nuño, Villarruel-López, Puebla-Pérez, Romero-Velarde, Puebla-Mora & Ascencio (2013) observed an increase in the counts of intestinal lactic acid bacteria in experimental rats fed a diet including *Isochrysis* spp., thus suggesting a prebiotic effect of these microalgae. Moreover, members of the *Isochrysis* genus are known to produce fucoxanthin (Gullón, Gagaoua, Barba, Gullón, Zhang & Lorenzo, 2020), a marine carotenoid, which can significantly promote the growth of *Bifidobacterium lactis* (Hwang, Phan, Lu, Ngoc Hieu & Lin, 2016).

5. Conclusions

Numerous scientific studies reported that the rearing process together with the rearing substrates can positively or negatively influence the final microbiological quality of edible insects. Moreover, a fraction of microbiota of larvae and adults can be difficult to control, being directly transmitted from the mother to the offspring. A novel

approach to the latter issue can be based on the use of the feeding substrates that can selectively modulate the prevalence of potentially pathogenic microbial taxa in the edible insects. Based on the results of the present study, an effect of algae nutrient bioactive substances (e.g. polysaccharides, high-unsaturated fatty acids, taurine, carotenoids) on the relative abundance of some of the bacterial taxa detected in larvae may be hypothesized, thus opening new intriguing perspectives for the control of the entomopathogenic species and foodborne human pathogens potentially occurring in edible insects. Further studies are needed to support this hypothesis. Finally, new information on the microbial dynamics occurring in insect frass was also obtained.

CRedit authorship contribution statement

Andrea Osimani: Conceptualization, Writing - review & editing, Supervision. **Ilario Ferrocino:** Formal analysis. **Maria Rita Corvaglia:** Formal analysis. **Andrea Roncolini:** Formal analysis. **Vesna Milanović:** Formal analysis. **Cristiana Garofalo:** Formal analysis. **Lucia Aquilanti:** Writing - original draft. **Paola Riolo:** Writing - original draft. **Sara Ruschioni:** Formal analysis. **Elham Jamshidi:** Formal analysis. **Nunzio Isidoro:** Writing - original draft. **Matteo Zantoniello:** Formal analysis. **Luca Cocolin:** Writing - original draft. **Ike Olivotto:** Resources. **Francesca Clementi:** Writing - original draft, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Allegretti, G., Schmidt, V., & Talamini, E. (2017). Insect as feed: Species selection and their potential use in Brazilian poultry production. *World's Poultry Science Journal*, 73, 928–937. <https://doi.org/10.1017/s004393391700054x>.
- Anbutsu, H., & Togashi, K. (2002). Oviposition deterrence associated with larval frass of the Japanese pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae). *Journal of Insect Physiology*, 48, 459–465. [https://doi.org/10.1016/s0022-1910\(02\)00067-7](https://doi.org/10.1016/s0022-1910(02)00067-7).
- ANSES (French Agency for Food, Environmental and Occupational Health and Safety), 2015. Opinion on the use of insects as food and feed and the review of scientific knowledge on the health risks related to the consumption of insects. Available at: <https://www.anses.fr/en/documents/BIORISK2014sa0153EN.pdf>.
- Berry, C. (2012). The bacterium, *Lysinibacillus sphaericus*, as an insect pathogen. *Journal of Invertebrate Pathology*, 109, 1–10. <https://doi.org/10.1016/j.jip.2011.11.008>.
- Bruni, L., Pastorelli, R., Viti, C., Gasco, L., & Parisi, G. (2018). Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source. *Aquaculture*, 487, 56–63. <https://doi.org/10.1016/j.aquaculture.2018.01.006>.
- Bruno, D., Bonelli, M., De Filippis, F., Di Lelio, I., Tettamanti, G., Casartelli, M., ... Caccia, S. (2019). The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. *Applied and Environmental Microbiology*, 85(2), e01864–18. <https://doi.org/10.1128/aem.01864-18>.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335–336. <https://doi.org/10.1038/nmeth.f.303>. <https://doi.org/10.1038/nmeth.f.303>.
- Cuttrignelli, M. I., Messina, M., Tulli, F., Randazzo, B., Olivotto, I., Gasco, L., ... Bovera, F. (2018). Evaluation of an insect meal of the Black Soldier Fly (*Hermetia illucens*) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. *Research in Veterinary Science*, 117, 209–215. <https://doi.org/10.1016/j.rvsc.2017.12.020>.
- De Smet, J., Wynants, E., Cos, P., & Van Campenhout, L. (2018). Microbial community dynamics during rearing of black soldier fly larvae (*Hermetia illucens*) and impact on exploitation potential. *Applied and Environmental Microbiology*, 84(9), e02722–17. <https://doi.org/10.1128/aem.02722-17>.
- Eastman, A. W., Heinrichs, D. E., & Yuan, Z. C. (2014). Comparative and genetic analysis of the four sequenced *Paenibacillus polymyxa* genomes reveals a diverse metabolism and conservation of genes relevant to plant-growth promotion and competitiveness. *BMC Genomics*, 15, 851. <https://doi.org/10.1186/1471-2164-15-851>.
- EFSA Scientific Committee. Scientific Opinion on a risk profile related to production and consumption of insects as food and feed. (2015). EFSA Journal, 13(10), 4257. [10.2903/j.efsa.2015.4257](https://doi.org/10.2903/j.efsa.2015.4257) (60 pp.).
- FASFC (Belgian Scientific Committee of the Federal Agency for the Safety of the Food Chain), 2014. Food safety aspects of insects intended for human consumption. Common advice of the Belgian Scientific Committee of the Federal Agency for the Safety of the Food Chain (FASFC) and of the Superior Health Council (SHC). Available at: http://www.favv-afsc.fgov.be/scientificcommittee/advices/documents/ADVCE14-2014_ENG_DOSSIER2014-04.pdf.
- Fernandez-Gomez, B., Lezama, A., Amigo-Benavent, M., Ullate, M., Herrero, M., Ángeles Martín, M., ... del Castillo, M. D. (2016). Insights on the health benefits of the bioactive compounds of coffee silverskin extract. *Journal of Functional Foods*, 25, 197–207. <https://doi.org/10.1016/j.jff.2016.06.001>.
- Ferrocino, I., Bellio, A., Romano, A., Macori, G., Rantsiou, K., Decastelli, L., & Cocolin, L. (2017). RNA-based amplicon sequencing reveals the microbiota development during ripening of artisanal vs. industrial Lard d'Arnad. *Applied and Environmental Microbiology*, 83(16), e00983–17. <https://doi.org/10.1128/AEM.00983-17>.
- Gahukar, R. T. (2020). Edible insects collected from forests for family livelihood and wellness of rural communities: A review. *Global Food Security*, 25, 100348. <https://doi.org/10.1016/j.gfs.2020.100348>.
- Garofalo, C., Milanović, V., Cardinali, F., Aquilanti, L., Clementi, F., & Osimani, A. (2019). Current knowledge on the microbiota of edible insects intended for human consumption: A state-of-the-art review. *Food Research International*, 125, 108527. <https://doi.org/10.1016/j.foodres.2019.108527>.
- Gasco, L., Biancarosa, I., & Liland, N. S. (2020). From waste to feed: A review of recent knowledge on insects as producers of protein and fat for animal feeds. *Current Opinion in Green and Sustainable Chemistry*. <https://doi.org/10.1016/j.cogsc.2020.03.003>.
- Grabowski, N. T., & Klein, G. (2017). Bacteria encountered in raw insect, spider, scorpion, and centipede taxa including edible species, and their significance from the food hygiene point of view. *Trends in Food Science & Technology*, 63, 80–90. <https://doi.org/10.1016/j.tifs.2017.01.007>.
- Gullón, B., Gagaoua, M., Barba, F. J., Gullón, P., Zhang, W., & Lorenzo, J. M. (2020). Seaweeds as promising resource of bioactive compounds: Overview of novel extraction strategies and design of tailored meat products. *Trends in Food Science & Technology*, 100, 1–18. <https://doi.org/10.1016/j.tifs.2020.03.039>.
- Han, R., Pang, D., Wen, L., You, L., Huang, R., & Kulikouskaya, V. (2020). In vitro digestibility and prebiotic activities of a sulfated polysaccharide from *Gracilaria Lemaneiformis*. *Journal of Functional Foods*, 64, 103652. <https://doi.org/10.1016/j.jff.2019.103652>.
- Hwang, P. A., Phan, N. N., Lu, W. J., Ngoc Hieu, B. T., & Lin, Y. C. (2016). Low-molecular-weight fucoidan and high-stability fucoxanthin from brown seaweed exert prebiotics and anti-inflammatory activities in Caco-2 cells. *Food & Nutrition Research*, 60, 32033. <https://doi.org/10.3402/fnr.v60.32033>.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, 41(1), Article e1. <https://doi.org/10.1093/nar/gks808>.
- Lemoine, M. M., Engl, T., & Kaltenpoth, M. (2020). Microbial symbionts expanding or constraining abiotic niche space in insects. *Current Opinion in Insect Science*, 39, 14–20. <https://doi.org/10.1016/j.cois.2020.01.003>.
- Liu, C., Yao, H., Chapman, S. J., Su, J., & Wang, C. (2020). Changes in gut bacterial communities and the incidence of antibiotic resistance genes during degradation of antibiotics by black soldier fly larvae. *Environment International*, 142, 105834. <https://doi.org/10.1016/j.envint.2020.105834>.
- Lyons, P. P., Turnbull, J. F., Dawson, K. A., & Crumlish, M. (2017). Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, 48, 2438–2452. <https://doi.org/10.1111/are.13080>.
- Magoč, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27(21), 2957–2963. <https://doi.org/10.1093/bioinformatics/btr507>.
- Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology*, 197, 1–33. <https://doi.org/10.1016/j.anifeedsci.2014.07.008>.
- Markande, A. R., Acharya, S. R., & Nerurkar, A. S. (2013). Physicochemical characterization of a thermostable glycoprotein bioemulsifier from *Solibacillus silvestris* AM1. *Process Biochemistry*, 48, 1800–1808. <https://doi.org/10.1016/j.procbio.2013.08.017>.
- Martins, P. M. M., Nara Batista, N., da Cruz Pedrozo Miguel, M. G., Pavesi Simão, J. B., Ribeiro Soares, J., & Freitas Schwan, R. (2020). Coffee growing altitude influences the microbiota, chemical compounds and the quality of fermented coffees. *Food Research International*, 129, Article 108872. <https://doi.org/10.1016/j.foodres.2019.108872>.
- Morrissey, B. J., Helgason, T., Poppinga, L., Fünfhäus, A., Genersch, E., & Budge, G. E. (2015). Biogeography of *Paenibacillus larvae*, the causative agent of American foulbrood, using a new multilocus sequence typing scheme. *Environmental Microbiology*, 17, 1414–1424. <https://doi.org/10.1111/1462-2920.12625>.

- Mishra, P., Pandey, C. M., Singh, U., Gupta, A., Sahu, C., & Keshri, A. (2019). Descriptive statistics and normality tests for statistical data. *Annals of Cardiac Anaesthesia*, 22, 67–72. <https://doi.org/10.4103/aca.ACA.157.18>.
- Nguyen, S. G., Kim, J., Guevarra, R. B., Lee, J. H., Kim, E., Kim, S., & Unno, T. (2016). Laminarin favorably modulates gut microbiota in mice fed a high-fat diet. *Food and Function*, 7(10), 4193–4201. <https://doi.org/10.1039/c6fo00929h>.
- Nuño, K., Villarruel-López, A., Puebla-Pérez, A. M., Romero-Velarde, E., Puebla-Mora, A. G., & Ascencio, F. (2013). Effects of the marine microalgae *Isochrysis galbana* and *Nannochloropsis oculata* in diabetic rats. *Journal of Functional Foods*, 5, 106–115. <https://doi.org/10.1016/j.jff.2012.08.011>.
- NVWA (Netherlands Food and Consumer Product Safety Authority), 2014. Advisory report on the risks associated with the consumption of mass-reared insects. Available at: <http://www.nvwa.nl/actueel/risicobeoordelingen/bestand/2207475/consumentie-gekwekte-insecten-advies-buro>.
- Oh, W. T., Jun, J. W., Giri, S. S., Yun, S., Kim, H. J., Kim, S. G., ... Park, S. C. (2020). *Morganella psychrotolerans* as a possible opportunistic pathogen in rainbow trout (*Oncorhynchus mykiss*) fisheries. *Aquaculture*, 520, Article 735021. <https://doi.org/10.1016/j.aquaculture.2020.735021>.
- Osimani, A., & Aquilanti, L. (2021). Spore-forming bacteria in insect-based foods. *Current Opinion in Food Science*, 37, 112–117. <https://doi.org/10.1016/j.cofs.2020.10.011>.
- Osimani, A., Milanović, V., Cardinali, F., Garofalo, C., Clementi, F., Pasquini, M., ... Aquilanti, L. (2018). The bacterial biota of laboratory-reared edible mealworms (*Tenebrio molitor* L.): From feed to frass. *International Journal of Food Microbiology*, 272, 49–60. <https://doi.org/10.1016/j.ijfoodmicro.2018.03.001>.
- Osimani, A., Milanović, V., Garofalo, C., Cardinali, F., Roncolini, A., Sabbatini, R., ... Aquilanti, L. (2018). Revealing the microbiota of marketed edible insects through PCR-DGGE, metagenomic sequencing and real-time PCR. *International Journal of Food Microbiology*, 276, 54–62. <https://doi.org/10.1016/j.ijfoodmicro.2018.04.013>.
- Osimani, A., Milanović, V., Roncolini, A., Riolo, P., Ruschioni, S., Isidoro, N., ... Clementi, F. (2019). *Hermetia illucens* in diets for zebrafish (*Danio rerio*): A study of bacterial diversity by using PCR-DGGE and metagenomic sequencing. *PLoS ONE*, 14(12), e0225956. <https://doi.org/10.1371/journal.pone.0225956>.
- Pothakos, V., De Vuyst, L., Jiyuan Zhang, S., De Bruyn, F., Verce, M., Torres, J., ... Weckx, S. (2020). Temporal shotgun metagenomics of an Ecuadorian coffee fermentation process highlights the predominance of lactic acid bacteria. *Current Research in Biotechnology*, 2, 1–15. <https://doi.org/10.1016/j.crbiot.2020.02.001>.
- Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. Off. J. Euro. Union. Available online: <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015R2283&from=en>.
- Regulation (EU) 2017/893 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as regards the provisions on processed animal protein. Official Journal of the European Union, L 138/92-116. Available at: <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R0893&from=IT>.
- Schmitt, E., & de Vries, W. (2020). Potential benefits of using *Hermetia illucens* frass as a soil amendment on food production and for environmental impact reduction. *Current Opinion in Green and Sustainable Chemistry*. <https://doi.org/10.1016/j.cogsc.2020.03.005> (in press).
- Shang, Q., Song, G., Zhang, M., Shi, J., Xu, C., Hao, J., ... Yu, G. (2017). Dietary fucoidan improves metabolic syndrome in association with increased Akkermansia population in the gut microbiota of high-fat diet-fed mice. *Journal of Functional Foods*, 28, 138–146. <https://doi.org/10.1016/j.jff.2016.11.002>.
- Singh, A. K., Ghodke, I., & Chhatpar, H. S. (2009). Pesticide tolerance of *Paenibacillus* sp. D1 and its chitinase. *Journal of Environmental Management*, 91, 358–362. <https://doi.org/10.1016/j.jenvman.2009.09.001>.
- Truzzi, C., Annibaldi, A., Girolametti, F., Giovannini, L., Riolo, P., Ruschioni, S., ... Illuminati, S. (2020). A chemically safe way to produce insect biomass for possible application in feed and food production. *International Journal of Environmental Research and Public Health*, 17, 2121. <https://doi.org/10.3390/ijerph17062121>.
- Truzzi, C., Giorgini, E., Annibaldi, A., Antonucci, M., Illuminati, S., Scarponi, G., ... Olivotto, I. (2020). Fatty acids profile of black soldier fly (*Hermetia illucens*): Influence of feeding substrate based on coffee-waste silverskin enriched with microalgae. *Animal Feed Science and Technology*, 259, 114309. <https://doi.org/10.1016/j.anifeedsci.2019.114309>.
- Wan, C., Zhao, X. Q., Guo, S. L., Asraf, A. M., & Bai, F. W. (2013). Biofloculant production from *Solibacillus silvestris* W01 and its application in cost-effective harvest of marine microalga *Nannochloropsis oceanica* by flocculation. *Bioresource Technology*, 135, 207–212. <https://doi.org/10.1016/j.biortech.2012.10.004>.
- Wang, Y. S., & Shelomi, M. (2017). Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. *Foods*, 6, 91. <https://doi.org/10.3390/foods6100091>.
- Wynants, E., Frootinckx, L., Crauwels, S., Verreth, C., De Smet, J., Sandrock, C., ... Van Campenhout, L. (2019). Assessing the microbiota of black soldier fly larvae (*hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. *Microbial Ecology*, 77, 913–930. <https://doi.org/10.1007/s00248-018-1286-x>.
- Zarantoniello, M., Randazzo, B., Truzzi, C., Giorgini, E., Marcellucci, C., Vargas-Abúndez, J. A., ... Olivotto, I. (2019). A six-months study on Black Soldier Fly (*Hermetia illucens*) based diets in zebrafish. *Scientific Reports*, 9, 2045–2322. <https://doi.org/10.1038/s41598-019-45172-5>.
- Zarantoniello, M., Zimbelli, A., Randazzo, B., Delli Compagni, M., Truzzi, C., Antonucci, M., ... Olivotto, I. (2020). Black soldier fly (*Hermetia illucens*) reared on roasted coffee by-product and *Schizochytrium* sp. as a sustainable terrestrial ingredient for aquafeeds production. *Aquaculture*, 518, 734659. <https://doi.org/10.1016/j.aquaculture.2019.734659>.
- Zheng, L. X., Chen, X. Q., & Cheong, K. L. (2020). Current trends in marine algae polysaccharides: The digestive tract, microbial catabolism, and prebiotic potential. *International Journal of Biological Macromolecules*, 151, 344–354. <https://doi.org/10.1016/j.ijbiomac.2020.02.168>.