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The microbiota of marketed processed edible insects as revealed by high-throughput sequencing

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ABSTRACT

Entomophagy has been linked to nutritional, economic, social and ecological benefits. However, scientific studies on the potential safety risks in eating edible insects need to be carried out for legislators, markets and consumers. In this context, the microbiota of edible insects deserves to be deeply investigated.

The aim of this study was to elucidate the microbial species occurring in some processed marketed edible insects, namely powdered small crickets, whole dried small crickets (*Acheta domesticus*), whole dried locusts (*Locusta migratoria*), and whole dried mealworm larvae (*Tenebrio molitor*), through culture-dependent (classical microbiological analyses) and -independent methods (pyrosequencing). A great bacterial diversity and variation among insects was seen. Relatively low counts of total mesophilic aerobes, *Enterobacteriaceae*, lactic acid bacteria, *Clostridium perfringens* spores, yeasts and moulds in all of the studied insect batches were found. Furthermore, the presence of several gut-associated bacteria, some of which may act as opportunistic pathogens in humans, were found through pyrosequencing. Food spoilage bacteria were also identified, as well as *Spiroplasma* spp. in mealworm larvae, which has been found to be related to neurodegenerative diseases in animals and humans. Although viable pathogens such as *Salmonella* spp. and *Listeria monocytogenes* were not detected, the presence of *Listeria* spp., *Staphylococcus* spp., *Clostridium* spp. and *Bacillus* spp. (with low abundance) was also found through pyrosequencing. The results of this study contribute to the elucidation of the microbiota associated with edible insects and encourage further studies aimed to evaluate the influence of rearing and processing conditions on that microbiota.

1. Introduction

The worldwide intake of animal proteins has increased over the past few decades and is presumed to continue increasing through 2050 (van der Spiegel et al., 2013; van Huis et al., 2013; Makkar et al., 2014). However, meeting this demand by increasing the production of traditional livestock, such as swine and cattle, will exacerbate those sectors' known detrimental effects on the environment including high carbon emissions and increased use of land and water (Klunder et al., 2012; Makkar et al., 2014).

The need to replace livestock-derived proteins has driven the European feed and food markets toward innovative protein sources, among which are insects (van der Spiegel et al., 2013). It is estimated that insects constitute part of the traditional diets of at least 2 billion people, mainly in Asia, Africa and America. Currently, the industrial production of edible insects is well established in Thailand and other Asian countries. However, in most Western industrialized countries, consumer acceptance of edible insects remains limited by disgust and by the association of eating insects with primitive behavior (van Huis et al., 2013; Verbeke, 2015). Nevertheless, in Europe, especially in the Netherlands, the rearing of insects for human consumption is gradually becoming a reality (ANSES Opinion, 2014). Indeed, among the potential benefits of insect consumption (also called entomophagy), one may highlight that the majority of insects are rich in high-quality proteins, good lipids, vitamins, minerals (such as calcium, iron and zinc) and fiber due to the presence of chitin (Belluco et al., 2013; Rumpold and Schlüter, 2013; van Huis et al., 2013). Thanks to these nutritional

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benefits, increased entomophagy may also be a solution for undernourishment in developing countries, which is mainly linked to food poor in protein and/or energy (Klunder et al., 2012). Alongside the nutritional benefits, economic, social and ecological benefits have also been linked to entomophagy (Belluco et al., 2013; van Huis et al., 2013). Indeed, in comparison with traditional livestock, farmed insects multiply faster, are very efficient at converting feed into protein, require little space for breeding and cause less emissions of greenhouse gases and ammonia (Klunder et al., 2012; van Huis et al., 2013).

Although edible insects have always been a part of human diets, not all insects are safe to eat depending on taxonomy as well as rearing and processing procedures (Rumpold and Schlüter, 2013; van Huis et al., 2013). The main potential safety risks linked with insect consumption comprise a range of chemical and biological hazards including pesticides, heavy metals, steroids, benzoquinones, allergens, mycotoxins, bacterial toxins, parasites and microorganisms (van der Spiegel et al., 2013; Belluco et al., 2015; Milanović et al., 2016). The microbial agents that can potentially be transmitted via consumption of insects are mainly associated with the intrinsic flora of insects (intestinal tract and other anatomical compartments) or related to extrinsic sources, such as the environment and the rearing conditions (substrates and feed), handling, processing and preservation (ANSES Opinion, 2014). Until recently, European legislation has largely been silent concerning the use and the safety of edible insects for feed and food production (van der Spiegel et al., 2013; van Huis et al., 2013). Recently, the European Union introduced a new Regulation about the so-called novel foods, namely 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, that shall apply from 1 January 2018. This legislative act requests the reviewing, clarification and updating of the categories of food which constitute novel foods, including insects or their parts. Hence, in-depth studies on specified candidate insects aimed to define their safety for possible mass production are becoming even more important (van der Spiegel et al., 2013; ANSES Opinion, 2014).

Therefore, in this context the need emerges for an accurate and reliable identification of the microbiota of edible insects to evaluate the possible presence of pathogens, spoilage agents and beneficial microbes. To the author's knowledge, only a few papers to date have dealt with the microbiological aspects of fresh and processed edible insects, and most of them have relied solely on culture-dependent analyses and microbial identification by phenotypical methods (Mpuchane et al., 2000; Simpanya et al., 2000; Amadi et al., 2005; Banjo et al., 2006; Agabou and Alloui, 2010; Ali et al., 2010; Braide et al., 2011; Klunder et al., 2012; Opara et al., 2012; Hernández-Flores et al., 2015; Stoops et al., 2016).

Efficient and in-depth evaluation of the biodiversity in food is currently obtained by using high-throughput sequencing (HTS) approaches based on analyses of DNA or RNA directly extracted from the food matrices under study. Several next-generation high-throughput sequencing (NGS) techniques have been applied for the culture-independent study of food microbiota (Ercolini, 2013). To the author's knowledge, only one previous study exists on the analyses of the bacterial community of fresh grasshoppers and mealworm larvae samples for human consumption by pyrosequencing (Stoops et al., 2016).

Based on these premises, the aim of this study was to identify the microbial species occurring in samples of processed marketed edible insects, namely powdered small crickets, dried whole small crickets (*Acheta domesticus*), dried whole locusts (*Locusta migratoria*), and dried whole mealworm larvae (*Tenebrio molitor*), through classical microbiological analyses coupled with pyrosequencing.

2. Material and methods

2.1. Edible insect sampling

The edible insects, already marketed in the European Union, were purchased from a company located in the Netherlands. The following processed samples (boiled and dried, hence ready to be consumed) were analyzed: powdered small crickets, whole small crickets (*Acheta domesticus*), whole locusts (*Locusta migratoria*), and whole mealworm larvae (*Tenebrio molitor*) (Fig. 1). All of the samples were shipped in plastic bag packages weighing 500 g, by international express transport and stored at ambient temperature until analysis. For classical microbiological analyses, two batches of each insect sample under study were investigated: one batch was bought in March 2015 (batch 1) and one in July 2015 (batch 2) (Table 1). In analogy with Stoops et al. (2016), one batch (batch 1) of each edible insect was also subjected to pyrosequencing analyses.

No information is available on the rearing and hygiene conditions of processing, transport and storage applied to these edible insects before marketing.

2.2. Microbiological analyses

Twenty-five grams of each sample was aseptically crushed with mortar, diluted in 225 mL of sterile peptone water (bacteriological peptone 1 g L^{-1}, Oxoid, Basingstoke, UK) and homogenized in a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy). Ten-fold dilutions of homogenate were prepared in the same diluent and aliquots (1 mL) were inoculated by inclusion spreading on specific solid media for the enumeration of *Enterobacteriaceae*, counted in accordance with the ISO 21528−2:2004 standard method, and total mesophilic aerobes counted in Standard Plate Count (PCA) agar (Oxoid), with aerobic incubation at 32 °C for 48 h (Osimani et al., 2011). Aliquots (0.1 mL) of ten-fold dilutions of homogenate were inoculated by surface spreading on specific solid media for the enumeration of: lactic acid bacteria (LAB) on De Man Rogosa Sharpe (MRS) agar (Oxoid) incubated at 37 °C for 72 h under anaerobic conditions using the AnaeroGen 2.5 System (Oxoid) (Aquilanti et al., 2012). Yeasts and moulds on Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Difco, Sparks, MD, USA) with aerobic incubation at 25 °C for 5 days (Garofalo et al., 2015). For counting *Clostridium perfringens* spores,homogenates were treated in a water bath at 80 °C for 10 min and cooled in iced water. Aliquots (0.1 mL) of each dilution were spread on Tryptone Sulfite Neomycin (TSN) agar and incubated at 37 °C for 24 h under anaerobic conditions using the AnaeroGen 2.5 System (Oxoid).

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

All microbiological analyses were performed in duplicate. The results of the microbial counts were expressed as means of log colony-forming units (cfu) per gram of sample ± standard deviations.

2.3. DNA extraction from edible insect samples

The microbial DNA was extracted directly from the edible insect

Fig. 1. Edible insect samples subjected to microbiological analysis and pyrosequencing: a) powdered crickets; b) dried small crickets; c) dried locusts; d) dried mealworm larvae.

samples using a PowerFood™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). In detail, 1 mL of each insect homogenate (dilution 10^{-1}) was centrifuged to produce a pellet that was processed according to the kit manufacturer's instructions. The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, using a UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

2.4. Analysis of bacterial diversity by 16S rRNA gene pyrosequencing

The DNA extracted as described above was used to study the bacterial diversity by 16S rRNA amplicon pyrosequencing. In detail, the V1-V3 region of the 16S rRNA gene was analyzed using the primers and conditions previously described (Garofalo et al., 2015). After agarose gel electrophoresis, all PCR products were purified,

Table 1
Microbial counts (log cfu g^{-1}) of dried small crickets, powdered crickets, dried locusts, and dried mealworm larvae.

Microbiological parameter	Small crickets		Powdered crickets		Locusts		Mealworm larvae	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Enterobacteriaceae	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Total mesophilic aerobes	4.01 ± 0.05	4.50 ± 0.11	4.80 ± 0.06	3.91 ± 0.20	2.43 ± 0.12	2.01 ± 0.80	<2.00	<2.00
Lactic acid bacteria	4.10 ± 0.10	4.51 ± 0.05	<2.00	<2.00	2.11 ± 0.05	2.00 ± 0.01	<2.00	<2.00
Clostridium perfringens spores	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Yeasts	4.52 ± 0.22	5.10 ± 0.20	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Moulds	<2.00	<2.00	2.92 ± 0.01	3.10 ± 0.02	2.01 ± 0.11	2.10 ± 0.20	2.21 ± 0.20	2.30 ± 0.20
Salmonella spp.	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g

Means ± standard deviations of duplicate independent experiments are shown.
Cfu: colony-forming units.

quantified and processed as described by Garofalo et al. (2015). The bioinformatics and data analysis were performed as reported by Garofalo et al. (2015).

3. Results

3.1. Microbiological analysis

The results of the microbiological analysis of powdered small crickets, dried small crickets, dried locusts, and dried mealworm larvae are reported in Table 1. *Enterobacteriaceae* and *Clostridium perfringens* spores counts were <2.00 log cfu g^{-1} for all samples. Regarding total mesophilic aerobes, the highest counts were reported for powdered crickets (4.80 ± 0.06 log cfu g^{-1} in batch 1), whereas the lowest values (<2.00 log cfu g^{-1}) were recorded in both the batches of mealworm larvae. Counts of LAB were the highest for small crickets (4.51 ± 0.05 and 4.10 ± 0.10 log cfu g^{-1} in batches 2 and 1, respectively). Powdered crickets and mealworm larvae showed the lowest values (<2.00 log cfu g^{-1} in all of the batches), whereas locusts showed intermediate values ranging from 2.11 ± 0.05 to 2.00 ± 0.01 log cfu g^{-1}.

Yeast counts were the highest for small crickets (5.10 ± 0.20 and 4.52 ± 0.22 log cfu g^{-1} in batches 2 and 1, respectively) with respect to the other insect samples, which showed counts < 2.00 log cfu g^{-1}. Mould counts were the highest for powdered crickets (3.10 ± 0.02 and 2.92 ± 0.01 log cfu g^{-1} in batches 2 and 1, respectively) followed by mealworm larvae, locusts and small crickets, these latter with the lowest counts (<2.00 log cfu g^{-1}).

In small crickets, total mesophilic aerobes, LAB, and yeast counts were the highest in the samples taken in July (batch 2). Similarly, mould counts were the highest in July for powdered crickets, locusts and mealworm larvae.

The microbiological analyses of *L. monocytogenes* and *Salmonella* spp. revealed that these pathogenic bacteria were absent in 25 g of each insect sample analyzed.

3.2. Analysis of bacterial diversity by 16S rRNA gene pyrosequencing

A total of 15,537 reads passed the filters applied in the QIIME split_library.py script, with an average value of 3884 and an average length of 486 bp. The rarefaction analysis and the estimated sample coverage (Table 2) indicated that satisfactory coverage for all of the samples (ESC ≥ 99%) was achieved. Additionally, the richness of the samples varied from a minimum of 37 to a maximum of 157 OTUs. As shown in Fig. 2, small crickets and powdered crickets were dominated by three bacterial phyla ascribed to Proteobacteria (42.6% and 28.4% of all sequences, respectively), Firmicutes (34% and 54%, respectively), and Bacteroidetes (22.2% and 12.1%, respectively). Furthermore, Actinobacteria (0.3% and 3.3%, respectively), Tenericutes (0.7% and 1.6%, respectively) and Deferribacteres (0.2% only in small crickets) were also detected, although with low relative abundance. In contrast, locusts were almost completely dominated by Firmicutes (94.7%), while the remaining bacteria consisted of Proteobacteria (5.2%) and Actinobacteria (0.1%). In mealworm larvae, the bacterial population mainly belonged to Tenericutes (44.2%), Proteobacteria (39.22%), and Firmicutes (13.09%), whereas Fusobacteria (3.3%), Bacteroidetes (0.13%) and Actinobacteria (0.06%) were found only with low abundance.

Table 2
Number of sequences analyzed, observed diversity and estimated sample coverage (Good's coverage) for 16S rRNA amplicons analyzed.

Sample	Reads	OTUs	Good's coverage	Chao1	Shannon index
Small crickets	3190	157.0	99.00	178.13	5.66
Powdered crickets	3418	175.0	99.09	194.77	5.72
Locusts	4379	37.0	99.83	39.33	1.41
Mealworm larvae	4550	45.0	99.86	47.14	2.82

Focusing in within each phylum, the great bacterial diversity and variation among the samples under study was even more evident (Fig. 3). Among the dominant OTUs, *Enterobacteriaceae* occurred in all samples, although this clade was more abundant in mealworm larvae (26.2% *Enterobacter* spp. and 11.4% of other species belonging to *Enterobacteriaceae*) and small crickets (33.0% *Enterobacteriaceae*) than in powdered crickets (17.5% *Enterobacteriaceae*) and locusts (4.6% *Enterobacteriaceae*). Small crickets and powdered crickets were also characterized by the presence of *Pseudomonadaceae* (6.3% and 9.3%, respectively), *Ruminococcaceae* (13.4% and 18.1%, respectively), *Lachnospiraceae* (10.9% and 4.1%, respectively), *Bacteroides* (10.4% and 3.7%, respectively) and *Clostridium* spp. (2.3 and 4.8%, respectively). Furthermore, in small crickets, *Rikenellaceae* (4.9%) and *Dysgonomonas* spp. (2.9%) were detected, while in powdered crickets, *Lactococcus garviae* (14.0%), *Enterococcus haemoperoxidus* (4.8%), *Enterococcus* spp. (4.3%), *Corynebacterium variabile* (3.0%) and *Parabacteroides* spp. (2.9%) also occurred.

In addition to *Enterobacteriaceae*, locusts and dried mealworm larvae were found to be dominated by other bacterial groups. In detail, *Weissella* spp. was abundant (81.0%) in the locusts sample, and the remaining of the locusts microbiota mainly included other LAB such as *Pediococcus acidilactici* (7.8%) and *Enterococcus* spp. (2.7%). In the mealworm larvae, 44.1% of the bacterial population consisted of *Spiroplasma* spp. followed by *Enterococcus* spp. (5.4%), *Fusobacterium* spp. (3.4%) and *Enterococcus faecalis* (2.0%).

Several sub-dominant OTUs (below 2%) (Fig. 3, Table S1) were also found. Among these, *Listeria* spp. (in powdered crickets and mealworm larvae), *Staphylococcus* spp. (in all of the samples) and spore-forming bacteria such as *Clostridium* spp. and *Bacillus* spp. (in all of the edible insect samples but locusts) were detected.

4. Discussion

Currently, approximately 2000 species of insects are consumed worldwide, the most commonly consumed of which are Orthoptera (crickets, locusts and katydids), Hymenoptera (bees, wasps and ants), Coleoptera (weevils and longhorn beetles), caterpillars and Lepidoptera (butterflies and moths), termites and waterbugs (ANSES Opinion, 2014). They can be consumed at different stages of development such as eggs, larvae, pupae, or adults and can be included in the diet as a main dish, snack or ingredient for prepared dishes (Verkerk et al., 2007). Living and processed edible insects can be regarded as potential reservoirs and/or vectors of physical, chemical and biological agents, which may affect the health of humans and animals when consumed either directly or indirectly via livestock feed (ANSES Opinion, 2014).

In the present study, the microbial community of different marketed edible insects sold in powdered (crickets) or dried whole (mealworm larvae, adult crickets and locusts) form has been investigated by classical microbiological analysis coupled with pyrosequencing. Pyrosequencing is presently considered the most efficient culture-independent method for profiling microbial communities. Indeed, this technique allows thousands to billions of DNA fragments to be sequenced in a single run, and thanks to high-resolution optics, pyrosequencing is also very sensitive to low-abundance OTUs, thus leading to the detection of rare taxa. Therefore, an exhaustive and consistent investigation of the microbial diversity of a food ecosystem can be obtained (Dolci et al., 2015). Moreover, counting of total mesophilic aerobes, *Enterobacteriaceae*, *Clostridium perfringens* spores, LAB, yeasts and moulds

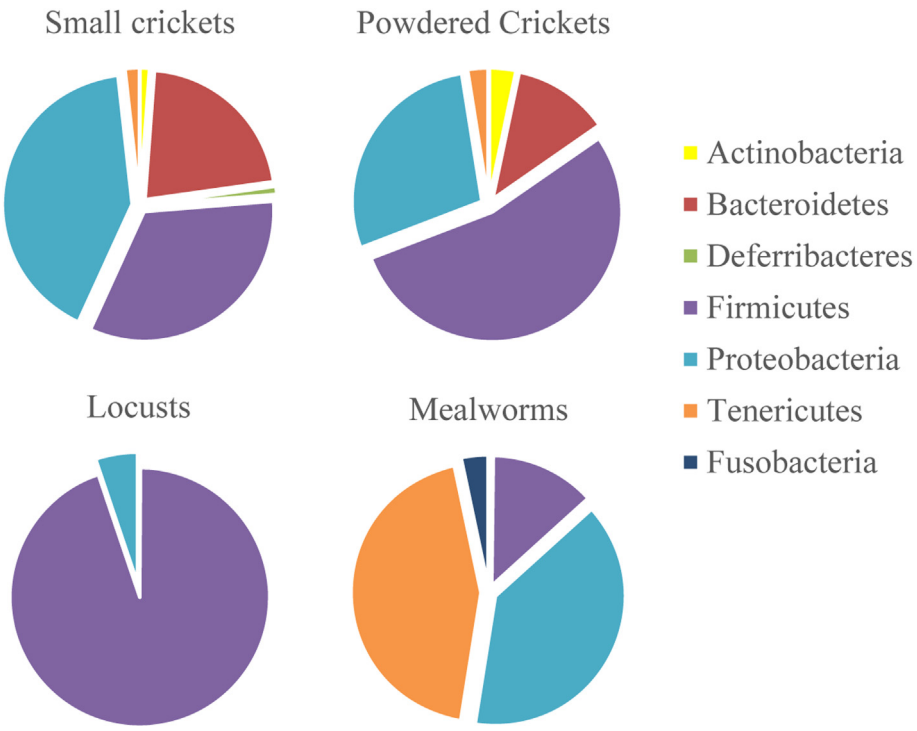

Fig. 2. Relative abundance (%) of phyla present in edible insect samples.

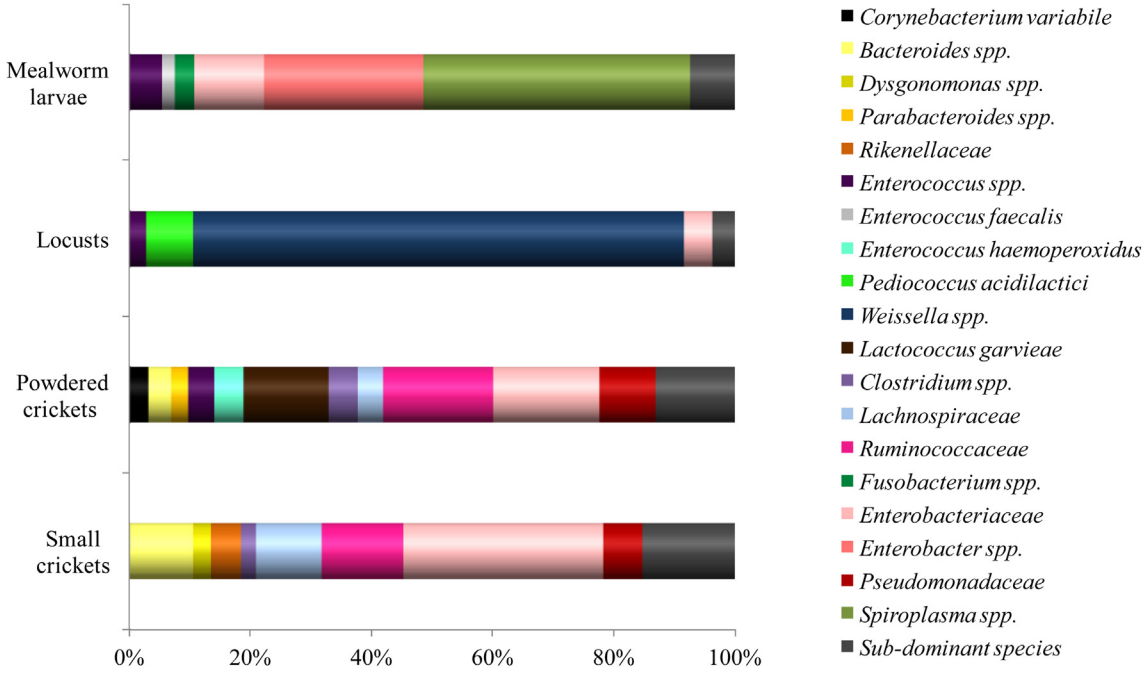

Fig. 3. Relative abundance (%) of the bacterial community in edible insect samples. The OTUs with relative abundance below 2% are grouped together in "sub-dominant OTUs".

used as hygiene and quality indicators was performed. The occurrence of *Salmonella* spp. and *L. monocytogenes* was also assessed. Indeed, pyrosequencing would not have been informative regarding the viability and load of these microorganisms because it was performed on DNA directly extracted from the edible insects under study and therefore derived either from live or uncultivable cells (i.e., cells in the Viable But Not Culturable state, dead and/or stressed/injured cells or cells unable to multiply on solid media due to unsuitable cultivation media). On the contrary, culture-dependent analyses would have limited the knowledge of the food microbiota under study, as > 99% of naturally occurring microorganisms are not cultivated using standard techniques (Cocolin et al., 2013).

Microbial contamination rates were generally higher in small crickets than in the other studied samples, while mealworm larvae showed the lowest microbial loads, which were <2.00 log cfu g^{-1}

for all microbial groups considered, but moulds. Small numerical differences were found among the microbial counts of the two batches of insects analyzed, although higher counts were seen for small crickets in the July sample. A shortage of data on the microbial load of edible insects is available in the scientific literature for the insect species analyzed in this study (Ali et al., 2010; Klunder et al., 2012; Stoops et al., 2016). It is worth noting that the dried whole insects under study were not subjected to pulverization before homogenization, indeed only a rough crushing was performed, hence the results of the counts may not completely reflect the entire gut microbiota of these insect samples. However, for all samples, the counts of all microbial groups considered were generally lower than those reported in the literature for fresh edible insects (Ali et al., 2010; Klunder et al., 2012; Stoops et al., 2016) thus indicating the importance of processing as heating as boiling and/or drying and/or frying in order to reduce the bacterial load as previously demonstrated and recommended by Klunder et al. (2012). Concerning yeasts, major contamination was found for small crickets, approximately 4.86 log cfu g^{-1} (mean of the two batches under study), whereas moulds were the highest in powdered crickets. Fungi are considered to be the main agents of food spoilage worldwide, and their possible sources are soils or leaves used by insects for feed (Simpanya et al., 2000).

Concerning the occurrence of viable cells of pathogenic bacteria, namely *L. monocytogenes* and *Salmonella* spp. in the samples under study, it is notable that despite their absence in all of the samples, pyrosequencing analyses revealed that powdered crickets and mealworm larvae contained a low abundance (below 2%) of *Listeria* spp., which was not possible to identify at the species level. Although no *Salmonella* spp. were identified by pyrosequencing, this pathogen could be included within *Enterobacteriaceae*, which were found at high abundance in small crickets and at lower abundance in powdered crickets, mealworm larvae and locusts. *Enterobacteriaceae* are Gram-negative, glucose-fermenting, oxidase-negative, and catalase-positive. Many of these bacteria are associated with human and animal feces. Consequently, these bacteria are frequently used to assess enteric contamination in foods and are considered as indicators of insufficient or unhygienic processing or inappropriate handling or storage conditions for the main livestock species (Barco et al., 2014). Furthermore, some species other than the *Salmonella* spp. within the *Enterobacteriaceae* family are also known as pathogens/opportunistic pathogens (Stoops et al., 2016). The ubiquity of *Enterobacteriaceae* in the studied samples may be ascribed to various reasons. Indeed, *Enterobacteriaceae* may originate from i) insect intestinal tracts, as the insects were not degutted; ii) rearing environment and conditions (the insects may act as vectors or intermediate hosts of bacteria); or iii) from unhygienic handling, processing and storage, as they were all processed through boiling and drying, and the small crickets were also ground into powder. Among *Enterobacteriaceae*, fecal and total coliforms were also previously detected in four different commercial species of insects (*Zoophobas morio*, *Tenebrio molitor*, *Galleria mellonella* and *Acheta domesticus*) (Giaccone, 2005) as well as in fresh grasshoppers together with *Escherichia coli* and *Salmonella* spp. (Ali et al., 2010). *E. coli* and *Klebsiella aerogenes* were also phenotypically identified from processed larvae of a lepidopteran, *Bunaea alcinoe*, and from fresh larvae (palm grubs) of *Rhynchophorus phoenicis* (African palm weevil) (Braide et al., 2011; Opara et al., 2012).

In the present study, other dominant OTUs were identified. In detail, small crickets and powdered crickets showed a similar bacterial pattern dominated by Firmicutes, Proteobacteria and Bacteroidetes. These phyla include the gut-associated bacteria of several insect species capable of many interactions with their hosts (Colman et al., 2012; Engel and Moran, 2013; Stoops et al., 2016). Within these phyla, small crickets and powdered crickets were found to be characterized by the presence of *Pseudomonadaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Bacteroides* spp. and *Clostridium* spp. The family *Pseudomonadaceae* includes strictly aerobic Gram-negative bacteria that are commonly found in soil or water and are pathogenic for insects and are well-known spoilage agents of different foods such as meat, cheeses and vegetables (Stellato et al., 2015; Stoops et al., 2016). Pseudomonads were also recently identified in edible fresh grasshoppers and mealworm larvae samples marketed in Belgium (Stoops et al., 2016). *Ruminococcaceae*, *Lachnospiraceae* and *Bacteroides* spp. are bacteria that are typical of the human and insect gut. In particular, the genera *Bacteroides* is the most represented within the human colonic microbiota (Shah et al., 2009; Engel and Moran, 2013; Cammarota et al., 2015). *Bacteroides* spp., together with *Parabacteroides* spp. (identified in powdered crickets) and *Fusobacterium* spp. (identified in mealworm larvae) are anaerobic Gram-negative bacilli that, in addition to the intestinal habitat, may also colonize the human oral cavity, the upper respiratory tract and the female genital tract (Shah et al., 2009; Boente et al., 2010; Cammarota et al., 2015). Species of the above-mentioned genera may act as symbiotic bacteria, with beneficial effects on the host metabolism and immune system, or as opportunistic pathogens causing human infections. They also represent an important medical challenge, as they have often developed resistance to the antibiotics commonly used in clinical practice (Shah et al., 2009; Boente et al., 2010). The bacterial population of small crickets also included *Rikenellaceae* and *Dysgonomonas* spp. In detail, members of the *Rikenellaceae* family were identified from a wide range of intestinal environments, such as goat rumen, termite gut, murine cecum and human colon, thus suggesting this bacterial group's extensive adaptation and positive impact on various intestinal ecosystems (Graf et al., 2006). The genus *Dysgonomonas* is represented by facultative anaerobes known as opportunistic human pathogens and frequently found in the gut of insects, such as larvae of *Rhynchophorus ferrugineus*, house flies and in *Drosophila* populations from different locations and diets (Tagliavia et al., 2014).

In powdered crickets, *C. variabile* and several LAB ascribed to *Lc. garviae*, *E. haemoperoxidus*, and *Enterococcus* spp. were found. The genus *Corynebacterium* belongs to the group of actinobacteria, which are located on the cuticles of insects and are considered mutualistic bacteria with a “healthcare” role for the hosts due to the production of antibiotics and to the metabolic versatility that may also have a role in insect nutrient utilization (Kaltenpoth, 2009). Specifically, the species *C. variabile* has been recently found within the cultivable bacterial community of the edible larvae of *Comadia redtenbacheri* Hammerschmidt (Hernández-Flores et al., 2015), although this species is typically associated with smear-ripened cheeses in which it plays a positive role during ripening (Schröder et al., 2011). Among LAB, *Lc. garviae* has been previously found to be associated with the digestive tract of the predatory ground beetle *Poecilus chalcites* (Lehman et al., 2009). *Lc. garviae* is a Gram-positive coccus, originally ascribed to the *Streptococcus* genus, and is considered as the main pathogen in aquaculture and sporadically in humans causing endocarditis, liver abscesses, spondylitis and osteomyelitis (Chao et al., 2013). The genus *Enterococcus* comprises species that are highly adapted to different habitats, including the digestive tract of humans, animals and insects, plants, soil, water and fermented foods. Among these species, *E. haemoperoxidus* has been recovered from water, while *E. faecalis* and *Enterococcus faecium* are the main known species leading to human infections. This latter effect is even more worrying due to the increasing prevalence of antibiotic-resistant enterococci since the 1970s and the possible involvement of insects as vectors/reservoirs of antibiotic-resistant enterococci in hospitals. *E. faecalis*

and *E. faecium* are also added in the preparation of fermented foods (cheeses, vegetables and sausages) to improve the flavor and overall quality of the products, although they have also been found to be associated with spoilage in cured meat products (Lebreton et al., 2014).

The locusts microbiota was found to be almost exclusively dominated by members of the Firmicutes; in particular, among the four major OTUs, three belonged to the LAB (*Weissella* spp., which was the most abundant OTU, *Enterococcus* spp. and *Pd. acidilactici*) and one to the *Enterobacteriaceae*. Species of *Weissella* occur in different environments such as skin, saliva, feces, and human and animal milk, as well as in several fermented foods (Garofalo et al., 2011; De Vuyst et al., 2014; Fusco et al., 2015; Osimani et al., 2015). Some species in this genus have probiotic potential; others have industrial application in the food sector thanks to their ability to produce exopolysaccharides (*Weissella cibaria* and *Weissella confusa*); yet others have been involved in human infections as opportunistic pathogens (Fusco et al., 2015). *Weissella paramesenteroides* and the novel species *Weissella diestrammenae* were recently isolated from insects and in particular from the gut microbiome of the European corn borer (*Ostrinia nubilalis*) and the camel cricket (*Diestrammena coreana*), respectively (Belda et al., 2011; Oh et al., 2013). Concerning *Pd. acidilactici*, to the author's knowledge this is the first report on the presence of this species in insects, although the *Pediococcus* genus was already detected in the gut microbiota of the tobacco hornworm (*Manduca sexta*) (van der Hoeven et al., 2008). Interestingly, a bacterial pattern very similar to that found for dried locusts was found in fresh grasshoppers analyzed by Stoops et al. (2016) who reported that LAB (*Weissella* spp., *Lactococcus* spp., *Enterococcus* spp.) and *Enterobacteriaceae* represented 88.5% of the bacterial sequences obtained. From this evidence, it is possible to speculate that taxonomy and/or the similar diet habits (locusts are also grass-feeders) give rise to a selective pressure of this specific microbiota. However, further study is necessary to confirm this hypothesis. Furthermore, according to Stoops et al. (2016), LAB and *Enterobacteriaceae* may also act to spoil edible insects during storage.

Concerning the bacterial community of mealworm larvae, it is notable that besides Proteobacteria and Firmicutes, the dominant phyla was Tenericutes, specifically represented by *Spiroplasma* spp. as the main OTU detected, followed by *Enterobacter* spp., *Enterobacteriaceae*, *Fusobacterium* spp. and *E. faecalis*. *Spiroplasma* spp. are small helical mycoplasmas that primarily live in plants and arthropods such as honeybees, wasps, beetles, flies, mosquitoes, butterflies, leafhoppers and leaf bugs (Henning et al., 2006; Zheng and Chen, 2014). Recently, *Spiroplasma* spp. have been isolated from field crickets (*Gryllus bimaculatus*), which are the main species of insect mass-reared in Taiwan (Nai et al., 2014). Some species of *Spiroplasma* are commensals, but others are pathogenic in insects (e.g., *Spiroplasma apis* and *Spiroplasma melliferum* in honey bees), plants (e.g., *Spiroplasma citri* and *Spiroplasma kunkelii*) and vertebrates. Indeed, *Spiroplasma* spp. was seen in crustaceans with tremor disease, and it was found to be associated with neurodegenerative diseases such as scrapie or transmissible spongiform encephalopathy (TSE) in humans and animals (Henning et al., 2006; Bastian et al., 2012; Nai et al., 2014; Zheng and Chen, 2014). *Spiroplasma* spp. are also able to form biofilms and are difficult to cultivate due to the peculiar growth requirements of the different species characterized by different biological features (Bastian et al., 2012). This latter aspect underlines the usefulness of culture-independent methods for the investigation of food microbiota, and in particular those of the edible insects.

Furthermore, in the present study, the genera *Clostridium*, *Bacillus*, and *Staphylococcus*, which can contain pathogenic species, were found not in all samples and only at low abundance.

Overall, the variations of the bacterial communities among the insects under study may be structured by i) insect diet, as the availability of specific substances may cause the selection of different species; ii) taxonomy, which is linked to pH values and the redox potential of the intestine compartments; and iii) rearing environment (Belda et al., 2011; Colman et al., 2012; Engel and Moran, 2013; Stoops et al., 2016). Furthermore, the processing, handling, transport and storage conditions may also have influenced the insects' microbial contamination, as already reported for the edible larvae of *Comadia redtenbacheri* Hammerschmidt (Hernández-Flores et al., 2015).

Finally, this study further demonstrates the usefulness of the combination of culture-dependent and -independent approaches for investigating the microbiota of such peculiar foods, as previously elucidated for more conventional food matrices (Garofalo et al., 2008, 2015; Greppi et al., 2015; Mariotti et al., 2014; Osimani et al., 2015; Zannini et al., 2009).

5. Conclusion

In this study, knowledge of the composition of microbiota occurring in powdered cricket, whole dried small crickets, whole dried locusts and whole dried mealworm larvae was obtained thanks to the combination of culture-dependent and -independent approaches. Great variety in the microbiota among the insects was seen. Despite the relatively low microbial counts in insect batches under study, the presence of several gut-associated bacteria, some of which may act as opportunistic pathogens for humans, was identified through pyrosequencing. Food spoilage bacteria were also found, as well as *Spiroplasma* spp. in mealworm larvae, which has been found to be related to neurodegenerative diseases in animals and humans. Although viable pathogens such as *Salmonella* spp. and *L. monocytogenes* were not detected, the presence of *Listeria* spp., *Staphylococcus* spp., *Clostridium* spp. and *Bacillus* spp. (with low abundance) was also found. These results must be considered within the context of the low number of edible insect samples analyzed. Therefore further studies are needed to improve our knowledge of microbiota thriving in insects with potential uses for food and feed production. More studies concerning the influence of the rearing conditions and processing on the edible insect-associated microbiota are also necessary.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.09.012.

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