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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