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Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (*Tenebrio molitor*)

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ABSTRACT

Specific processing steps after industrial rearing of insects for food and feed, being starvation and rinsing, are assumed to have an impact on their microbial quality. The aim of this study was to assess the effect on the microbiota of starvation (24 or 48 h, 10 or 30 °C) and rinsing (1 min using tap water) at the end of the rearing period of yellow mealworm larvae (*Tenebrio molitor*). Microbial numbers were determined using plate counts and the microbial community composition using metagenetic analyses. Total viable counts ranged from 7.7 to 8.4 log cfu/g for all treatments. Starvation did not evoke prominent shifts in the bacterial community, which was predominated by Proteobacteria and Firmicutes. No bacterial food pathogens were detected using metagenetics. Our data suggest that the processing steps under study do not contribute to a better microbial quality of fresh mealworm larvae.

Industrial relevance: As insects and insect-based foods are receiving more attention and are already being marketed in some European countries, more insects farms are being established. Rearing companies often optimise their practices by trial and error and no general hygiene codes are available. According to the Netherlands Food and Consumer Product Safety Authority (2014), mealworm larvae are generally starved and rinsed after rearing to empty their gut, but the impact of these practices has not been investigated so far. Hence, the necessity for rearers to incorporate these steps in their rearing procedures has not been demonstrated. The Belgian SHC (Superior Health Council) and FASFC (Federal Agency for the Safety of the Food Chain) have recommended in their advice (2014) to investigate these steps. In addition, as edible insects will be defined as novel foods as from 1 January 2018 according to the European Novel Food Regulation (EU) No. 2015/2283, more information is needed on their safety, which is related to production hygiene.

1. Introduction

In Western countries, the use of insects in feed and food is gaining increasing attention (Caparros Megido et al., 2014; Mlcek, Rop, Borkovcova, & Bednarova, 2014; Verbeke, 2015). Insects provide a qualitative source of nutrients such as proteins, fatty acids, and several vitamins and micronutrients (Rumpold & Schlüter, 2013; Sánchez-Muros, Barroso, & Manzano-Agugliaro, 2014). Furthermore, insect farming is generally characterized by a lower ecological footprint when compared to conventional livestock (Oonincx et al., 2010; Oonincx & De Boer, 2012; van Huis, 2013). In Europe - at least in Belgium and in the Netherlands - and also in African countries, new insect-rearing facilities are being set-up or existing facilities are being automated and scaled-up, in order to enlarge the availability of this alternative protein source.

Several studies on edible insects have shown that they contain high microbial numbers, with total viable counts ranging from 5.0 to 9.3 log cfu/g (Giaccone, 2005; Klunder, Wolkers-Rooijackers, Korpela, & Nout, 2012; Grabowski, Jansen, & Klein, 2014; Stoops et al., 2016; Vandeweyer, Crauwels, Lievens, & Van Campenhout, 2017; Vandeweyer, Lenaerts, Callens, & Van Campenhout, 2017). More specifically, edible insects can also contain large amounts of bacterial endospores (< 1.0 to 5.0 log cfu/g), Enterobacteriaceae (4.2 to 9.3 log cfu/g) and yeasts and moulds (3.5 to 7.2 log cfu/g) (Klunder

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et al., 2012; Stoops et al., 2016; Vandeweyer, Crauwels, et al., 2017; Vandeweyer, Lenaerts, et al., 2017), which potentially harbour food pathogens or produce mycotoxins. The rearing techniques, rearing environment, feeding substrate, hygiene measures and specific handling procedures, such as starvation and rinsing after harvest, are suggested to affect the microbiota of insects (Dillon, Webster, Weightman, & Charnley, 2010; Klunder et al., 2012; Engel & Moran, 2013; SHC & FASFC, 2014; EFSA Scientific Committee, 2015; Li, Xie, Dong, Wang, & Liu, 2016), but no specific information exists. According to a risk analysis of the NVWA (Netherlands Food and Consumer Product Safety Authority), mealworm larvae (*Tenebrio molitor*) are reared in industrial rearing companies at a temperature of 28 to 30 °C and a relative humidity of 60%. The feeding substrate generally consists of bran mixed with flour or ground chicken feed, supplemented with carrots, potatoes and water. After eight to ten weeks of rearing, the last larval stage of the mealworm is harvested by sieving. The larvae are then often starved for one or two days in order to empty their gut. Then they are rinsed with lukewarm to warm water and killed by freezing (NVWA, 2014, and personal communication with insect farmers). When they would be unnecessary, however, these procedures would imply a loss of time by adding extra steps to the rearing cycle, and starvation causes a weight loss in the larvae and hence a loss in produced biomass weight. Some rearing companies assume that the emptying of the gut and rinsing of the larvae enhance the microbial quality of the larvae. Indeed, it is known that the gut microbiota of insects can harbour a diversity of parasites, fungi and other microorganisms (SHC & FASFC, 2014). Rumpold et al. (2014) observed that the overall microbial load of the mealworm larvae was generally higher (approximately one log cycle) than the surface contamination, which was suggested to be due to the gut microbiota. However, although applied by several companies, the impact of these practices on the microbial quality of insects as a feed and food matrix has never been investigated. More information is needed for insect farmers in order to optimise rearing practices and also to support the evaluation of insects as Novel Food as they will receive the Novel Food status as from 1 January 2018, according to the renewed European Novel Food Regulation (EU) No. 2015/2283. Research on the effect of starvation of insects for consumption on their food safety was also recommended in an advisory report by the Belgian Superior Health Council (SHC) and Federal Agency for the Safety of the Food Chain (FASFC) (2014).

The goal of this study was to examine whether two specific industrial practices performed at the end of the rearing cycle of mealworm larvae, *i.e.* starvation and rinsing, have an impact on the microbiota of freshly harvested larvae. In a first experiment, starvation was investigated under different conditions with respect to duration, temperature and contact with faeces. Both culture-dependent plate counts as well as Next Generation Sequencing based community profiling (based on the Illumina Miseq platform) were used to evaluate the microbiota. In a second experiment, the effect of rinsing on the microbial load of both starved and non-starved larvae was assessed by means of plate counts.

2. Materials and methods

2.1. Experimental design

Final instar mealworm larvae were obtained from an industrial rearing company in Belgium. The larvae were kept for maximum 24 h in the feeding substrate, which consisted of wheat bran supplemented with carrot pieces as supplied by the company, in a disinfected plastic container (39.5 × 34 × 19.1 cm) until use. Starvation was performed under four different conditions. In particular, larvae were starved either at 10 °C or at 30 °C, representing the two temperatures that are commonly used in industry (NVWA, 2014). For both temperatures, starvation was carried out for larvae in contact with their faeces (as is the case in industrial rearing) as well as for larvae that could not take up their faeces (to examine whether a more stringent way of starvation would make a difference). For each condition, a control group of non-starved larvae was included. The experiment was performed on three different batches for each of the four conditions: 30 °C with faecal contact (batches 1.1 to 1.3), 30 °C without faecal contact (batches 2.1 to 2.3), 10 °C with faecal contact (batches 3.1 to 3.3) and 10 °C without faecal contact (batches 4.1 to 4.3).

In a second series of experiments, the effect of rinsing was studied for both non-starved and starved larvae. Microbial counts were determined and compared to those of a non-rinsed control group. The microbial load of the tap water before rinsing and of the residual rinsing water was also determined. These experiments were performed with three batches of larvae.

2.2. Starvation

For each batch, three 30-g samples of larvae were sieved out of the substrate and analysed (counts and metagenetics, see below). Then, 800 g of larvae were sieved out of the substrate. Four hundred grams of larvae was placed back in the substrate as control group and kept in a first container (see Section 2.1), while the remaining 400 g was transferred into a second, empty container for starvation. That container was, depending on the batch, either or not equipped with a sieve consisting of a plastic mosquito net (mesh size 1 mm). The sieve allowed the faeces to fall through during starvation, while the larvae were kept on the sieve. When faecal contact (and thus possible consumption of the faeces) was allowed, the larvae were placed directly, without sieve, into the container. Subsequently, both the control and starvation group were placed, depending on the batch, in an incubator (Heratherm, Thermo Scientific, Waltham, Massachusetts, USA) with set point at 30 °C and ranging between 28 and 32 °C, or in a refrigerator (DynaCool, Miele, Gütersloh, Germany) with set point at 10 °C and ranging between 8 and 12 °C. From each group, three replicate 30-g samples of larvae were taken after 24 and 48 h for analysis.

2.3. Rinsing

Each batch of mealworm larvae was divided into a control group of non-starved larvae and larvae that were starved for 48 h at room temperature and without faecal contact as described above. Subsequently, both groups of larvae were subjected to a rinsing procedure: 30-g aliquots of larvae were transferred into a sterile 250-ml flask containing 100 ml of tap water and shaken for 1 min at 200 rpm on a laboratory shaker (HS501 Digital, IKA Labortechnik, Staufen, Germany). Then, the larvae were drained over a disinfected sieve and the rinsing water was collected. Microbial counts of the non-rinsed larvae, the rinsed larvae and the tap water before and after rinsing were determined. For each batch, samples were analysed in two- or threefold, resulting in a total of eight replicates per condition for all batches.

2.4. Classical microbiological analyses

Each larvae sample was kept at 3 °C for approximately 1 h for sedation, after which it was pulverised prior to analysis as described by Stoops et al. (2016). Water samples from the rinsing experiment were kept at 3 °C until analysis. Plate counts were performed according to the ISO standards for microbial analyses of food as compiled by Dijk et al. (2015), except for yeasts and moulds which were determined according to Dijk et al. (2007). Total viable aerobic counts were determined on Plate Count Agar (PCA, Biokar Diagnostics, Beauvais, France) and incubated at 30 °C for 72 h. Enterobacteriaceae were determined on Violet Red Bile Glucose medium (VRBG, Biokar Diagnostics) after incubation at 37 °C for 24 h. Aerobic bacterial endospores were determined by giving the 10^{-1} dilution a heat-shock condition (10 min at 80 °C), followed by a ten-fold serial dilution, plating onto PCA and incubation at 37 °C for 48 h. Yeasts and moulds were determined on