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PII: S1466-8564(16)30741-X
DOI: doi: 10.1016/j.ifset.2017.06.004
Reference: INNFOO 1772

To appear in: *Innovative Food Science and Emerging Technologies*

Received date: 25 November 2016
Revised date: 1 June 2017
Accepted date: 7 June 2017

Please cite this article as: E. Wynants, S. Crauwels, B. Lievens, S. Luca, J. Claes, A. Borremans, L. Bruyninckx, L. Van Campenhout , Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (Tenebrio molitor), *Innovative Food Science and Emerging Technologies* (2017), doi: 10.1016/j.ifset.2017.06.004

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.

KEYWORDS

Mealworm larvae; rearing; starvation; rinsing; microbial numbers; next generation sequencing

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, 2013). 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 et al., 2012; Stoops et al., 2016; Vandeweyer et al., 2017a; Vandeweyer et al., 2017b), 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) N° 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 x 34 x 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 (batch 1.1 to 1.3), 30 °C without faecal contact (batch 2.1 to 2.3), 10 °C with faecal contact (batch 3.1 to 3.3) and 10 °C without faecal contact (batch 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 were placed back in the substrate as control group and kept in a first container (see 2.1), while the remaining 400 g were 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, Massachusets, 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 one hour 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 Oxytetracycline Glucose Agar (OGA, Biokar Diagnostics) supplemented with oxytetracycline (90.91 mg/l, Biokar Diagnostics) incubated at 25 °C for five days. Psychrotrophic aerobic counts were determined by plating onto PCA and incubating at 6.5 °C for ten days.

2.5. Metagenetic analyses

In order to study possible changes in the bacterial community composition, 25 g of the pulverised larvae samples taken at 0 h and at 48 h (see 2.4) were subjected to high-throughput 16S ribosomal RNA gene sequencing using the MiSeq Illumina platform. DNA of each sample was extracted in duplicate using the Powersoil DNA Isolation Kit (0.25 g sample/extraction, MO BIO Laboratories, Carlsbad, California, USA), and subsequently diluted 10 times to avoid inhibitory concentrations of potential DNA inhibitors co-extracted

with the DNA. Next, samples were subjected to PCR amplification of the V4 region of the 16S rRNA gene using barcode-tagged versions (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013; dual indexing strategy) of the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011) (Table S1, Supporting Information). PCR reactions were performed in duplicate in a 20 µl reaction volume, containing 150 µM of each dNTP, 0.5 µM of each primer, one unit of Titanium Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France), 1x Titanium Taq PCR buffer and 5 ng genomic DNA (as measured using a Nanodrop instrument (Thermo Scientific Nanodrop Products Inc., Wilmington, USA)). The PCR amplification protocol consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 60 °C for 45 s and primer extension at 72 °C for 45 s, and a final extension of 10 min at 72 °C. After amplification, duplicate PCR products were combined and resolved using agarose gel electrophoresis. The amplicons within the expected size range were excised and extracted/purified from the gel using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Purified dsDNA amplicons were then quantified with the Qubit fluorometer with the high-sensitivity reagent kit according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). Subsequently, all samples were pooled in equimolar concentrations, and the library was diluted to 2 nM. Finally, the library was sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) using 2 x 250 bp chemistry at the Center of Medical Genetics Antwerp (University of Antwerp, Antwerp, Belgium) according to the principle outlined in Kozich et al. (2013).

Resulting sequences were received in the format of a de-multiplexed FASTQ file. Paired-end reads were merged using USEARCH (v.8.1) (Edgar, 2013) to form consensus sequences originating from each sample with a maximum number of 10 mismatches allowed in the

overlap region. Subsequently, reads with a total expected error threshold above 1.0 for all the bases in the read were discarded. Next, remaining sequences with a minimum abundance of two, were grouped into species-level operational taxonomic units (OTUs) based on a 3 % sequence dissimilarity cut-off and discarding chimeric sequences using the UPARSE greedy algorithm implemented in USEARCH (Edgar, 2013). Global singletons (*i.e.* OTUs representing only a single unique sequence in the entire dataset) were removed after UPARSE clustering in order to minimize the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud, Busschaert, Ruyters, Jacquemyn, & Lievens, 2014). Due to uneven sequencing depth the number of sequences was rarefied to 9,000 sequences per sample. Two DNA-extracts that rendered too few sequences were omitted from further analysis, leaving one DNA-extract for those samples (batch 1.3, non-starved (control) larvae (48 h) and batch 4.2, starved larvae (48 h)). Next, OTUs were assigned taxonomic identities using the "classify.seqs" command in Mothur (v. 1.36.1) (Schloss et al., 2009) against the Silva taxonomy database (Quast et al., 2013) and taxonomic assignments were considered reliable when ≥ 0.80 score value was found. DNA-sequences originating from chloroplasts or mitochondria were eliminated with Mothurs "remove lineage" command. Additionally, OTU representative sequences (selected by UPARSE) were subjected to a BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) search against GenBank (Benson et al., 2013), excluding uncultured/environmental entries. Nonmetric multidimensional scaling (NMDS), Chao1 and Shannon-Wiener diversity indices calculations were performed on the microbial communities of the samples using R-packages (R Development Core Team, 2013) Vegan (v.2.41) and Phyloseq (v. 1.19.0). Two DNA extracts that rendered a coverage, based on Chao1, of below 50 % were omitted from the dataset, leaving one replicate DNA extract for those samples (batch 1.1 non-starved (control) larvae (0 h) and batch 4.2 non-starved (control) larvae (48 h)).

2.6. Statistical analyses

Statistical analyses were performed using SPSS (IBM SPSS Statistics version 23, New York, USA). For starvation, data were compared per condition and per microbial count by one-way ANOVA. Hence, statistical differences could be detected not only between the control and the starvation group at 24 and 48 h, but also between the time intervals. In addition, one-way ANOVA was performed on the Chao1 and Shannon-Wiener diversity indices of each condition after 0 and 48 h. One-way ANOVA was also performed for each microbial count of the rinsing experiment, comparing larvae subjected to starvation (48 hours), rinsing (1 min), both steps or neither of them (control). For all analyses, multiple comparisons were performed using Tukey's *post hoc* test, while considering a 0.05 significance level.

3. RESULTS AND DISCUSSION

3.1 Starvation

3.1.1 Classical microbiological analyses

In the first part of this study, the effect of starvation on the microbial numbers of mealworm larvae was examined. Tables 1 and 2 show the microbial counts for each condition, averaged over all batches investigated. The initial total viable counts (7.9 to 8.4 log cfu/g; Tables 1 and 2), the initial Enterobacteriaceae counts (6.9 to 7.6 log cfu/g; Tables 1 and 2) and the initial psychrotrophic counts (6.7 to 7.0 log cfu/g; Tables 1 and 2) of the larvae examined in this study were comparable to numbers found for fresh mealworm larvae in literature (Klunder et al., 2012; Stoops et al., 2016; Vandeweyer et al., 2017a; Vandeweyer et al., 2017b). The average endospore counts (1.5 to 2.0 log cfu/g; Tables 1 and 2), however, were generally lower compared to those reported in literature, which generally range from 1.7 to 5.0 log cfu/g (Klunder et al., 2012; Stoops et al., 2016; Vandeweyer et al.,

2017a; Vandeweyer et al., 2017b). The average initial counts for yeasts and moulds (5.6 to 6.5 log cfu/g; Tables 1 and 2) were comparable to results obtained by Stoops et al. (2016) and Vandeweyer et al. (2017a). Additionally, the count was comparable to results obtained by Vandeweyer et al. (2017b) for those batches that were obtained from the same rearing company as in our research (4.8 – 5.0 log cfu/g), whereas the counts obtained from another rearing company in that study were lower (ranging from 3.5 to 3.8 log cfu/g).

Several insect-rearing companies believe that the emptying of the gut content of mealworm larvae, as stated by the NVWA (2014), may have a positive impact on the microbial quality and reduce microbial numbers. Furthermore, preliminary experiments showed that mealworm faeces excreted after 24 and 48 h of starvation contain very high microbial numbers, with a total viable aerobic count ranging from 8.8 to 11.4 log cfu/g (data not shown). Our data, however, do not show considerable changes in microbial numbers of mealworm larvae during the starvation period for any of the conditions investigated (Tables 1 and 2). After both 24 and 48 h, no statistical differences were observed between starved and non-starved larvae for any of the counts in any of the conditions (all p-values > 0.05). Some statistically significant differences could be detected between numbers at different moments within a condition. For example, at 30 °C and with faecal contact (Table 1), the psychrotrophic aerobic count of the larvae was statistically lower ($p = 0.046$) after 48 h starvation (6.1 log cfu/g) than at that start of the experiment (6.7 log cfu/g). At the same temperature but without faecal contact (Table 1), a significant increase ($p = 0.031$) in yeasts and moulds was observed between 24 and 48 h starvation. Those results, though having statistical significance, are not considered to indicate notable changes from a microbiological point of view. Only numbers differing by one or more log cycles would be of microbial significance in this context. However, in whatever way the larvae were starved, difference between starved larvae and control and differences between time intervals were always below one log cycle. The results strongly indicate that starvation

of mealworm larvae does not reduce their microbial load as often assumed, neither when they are starved at rearing temperature nor chilled, and neither with nor without the ability to consume their faeces. The opposite hypothesis, being that the gut microbiota can multiply intensively during starvation, in the absence of a plug flow of substrate through the gut, can be rejected as well.

It should be noted that the starvation period in our study only lasted for 48 h, which was selected based on practices in rearing companies. This may indicate that 48 h may not be enough to eradicate certain pathogens, or by extension any microorganism that may be present in the larval gut. Starvation for a period that exceeds 48 h is, however, not practiced by rearing companies, as the larvae would either dehydrate or start pupation.

Importantly, it has to be noted that the effect of starvation on the chemical quality (*i.e.* with respect to chemical contaminants such as pesticide residues, heavy metals, mycotoxins, veterinary substances, …) remains to be established as well. Starvation may be useful to eliminate chemical contaminants, but this has not been demonstrated so far. It is known, for aquatic insects, that the gut content can contribute significantly to the total body load of chemical pollutants (Cain, Luoma, & Axtmann, 1995). Furthermore, faeces of mealworm larvae that were fed with a deoxynivalenol (DON)-contaminated substrate were found to contain the mycotoxin (van Broekhoven, 2014 in EFSA scientific committee, 2015). Therefore, the question remains from a chemical point of view whether starvation should be incorporated as a necessary procedure into guidelines for insect-rearing companies.

3.1.2 Metagenetic analyses

High-throughput 16S rRNA gene sequencing was used to unravel possible changes in the microbial community composition upon starvation. Relative OTU abundances and diversity indices were averaged over two DNA extracts per sample. The average coverage per sample, based on Chao1, ranged from 68.18 % to 95.32 %, suggesting that the most abundant

community members were covered in our study. The average number of recovered OTUs ranged from 21 to 57 (average 36.7 ± 9.2 (SD)) per sample. Table 3 shows the diversity indices, averaged over all batches, that were obtained per condition for non-starved and starved larvae after 0 and 48 h. The main phyla present were Proteobacteria and Firmicutes, followed by Tenericutes (67.86 %, 24.67 % and 5.67 % of all sequences, respectively). The most abundant OTUs, represented by more than 5 % of the sequences in any sample (Figures 1 and 2), all belonged to those phyla. The Bacteroidetes and Actinobacteria were low in abundance (0.76 % and 0.74 %, respectively). This is in contrast to Stoops et al. (2016), who reported higher abundances for the latter groups (26.9 % for Actinobacteria and 2.9 % for Bacteroidetes), but did not report the presence of Tenericutes in fresh mealworm larvae. On the other hand, Garofalo et al. (2017) reported a large abundance of Tenericutes (44.2 %), Proteobacteria (39.22 %) and Firmicutes (13.09 %), and a low abundance of Bacteroidetes (0.13 %) and Actinobacteria (0.06 %) in dried mealworm larvae. The reason for these differences between studies may be the difference in rearing company or rearing techniques from which the larvae were obtained. The feeding substrate and the rearing environment are believed to determine the microbial community inside the insect gut (SHC & FASFC, 2014; Li et al., 2016).

Some of the most abundant OTUs (*i.e.* OTUs 2, 10 and 12) could not be identified reliably to the genus level (score value < 0.80) (Table S2, Supporting Information). Therefore, these OTUs were further refined to taxonomic ranks by a BLAST search against the GenBank nucleotide database (Table S3, Supporting Information). In most samples, Enterobacteriaceae (OTUs 2, 6 and 10), a member of the Gammaproteobacteria (OTU 1), a number of lactic acid bacteria (OTUs 4, 5 and 7), and a *Spiroplasma* member (OTU 3) represented more than 80% of all sequences (Figures 1 and 2). These findings correspond to the large amount of Enterobacteriaceae obtained in the plate counts, as well as to the large amount of lactic acid

bacteria that has been found for mealworm larvae in other studies (Stoops et al., 2016; Vandeweyer et al., 2017a; Vandeweyer et al., 2017b). Notably, these microbes may contain possible spoilage organisms (Sperber & Doyle, 2009), but they are easily reduced in numbers by a heat treatment before consumption (Vandeweyer et al., 2017b). Some samples contained a considerable relative abundance of the genus *Pseudomonas* (OTU 11 and 12), which also contains important spoilage organisms (Sperber & Doyle, 2009). The genus *Spiroplasma* is known to harbour insect pathogens. It is, however, thought to not affect mealworm larvae, since the genus has been detected in the mealworm gut in several previous studies (Jung et al., 2014; Wang & Zhang, 2015; Garofalo et al., 2017). The composition of the most abundant OTUs in our samples largely differs from that reported by Stoops et al. (2016), who found fresh mealworm larvae to contain mostly Enterobacteriaceae species, *Haemophilus*, *Lactobacillus*, *Pseudomonas*, *Propionibacterium*, *Staphylococcus* and *Streptococcus*. Other studies report large numbers of Enterobacteriaceae and *Spiroplasma* for dried mealworm larvae (Garofalo et al., 2017) and the gut of fresh mealworm larvae (Jung et al., 2014).

Some genera were identified among the most abundant taxa that may contain food pathogens, such as *Cronobacter* and *Staphylococcus* (Figures 1 and 2). To date, no specific hygiene measures are considered in insect-rearing companies, and the feeding substrate is often provided with bare hands, which may be a source of contamination (Rediers, Claes, Kinnerk, Peeters, & Willems, 2008). Furthermore, in some batches, *Listeria*, *Clostridium* and/or *Bacillus* were identified, albeit in relatively low abundances (0.23 %, 0.32 % and 0.05 % of all sequences, respectively). However, to our knowledge, neither *Listeria monocytogenes*, *Clostridum perfringens*/*botulinum* or *Bacillus cereus* have yet been detected in mealworm larvae. Our results, however, indicate the possible presence of spore-forming food pathogens. Endospores generally survive heat treatments applied so far for insects, such as blanching and boiling (Klunder et al., 2012; Vandeweyer et al., 2017b). Therefore, further

studies should be conducted to characterize the risks related to the occurrence of spore-forming pathogens in or on edible insects such as mealworm larvae. Finally, the genera *Shigella/Escherichia* and/or *Vibrio* were identified (0.18 % and < 0.01 % of all sequences, respectively) in some batches.

Studies have shown that dietary changes in some insect species, such as crickets, cockroaches and fruit flies, affect the composition of the gut microbiota (Kane & Breznak, 1991; Domingo, Kaufman, Klug, & Tiedje, 1998; Broderick, Raffa, Goodman, & Handelsman, 2004; Broderick & Lemaitre, 2012). Furthermore, Dillon & Dillon (2004) suggested that an insect that is constantly fed is likely to possess a different gut bacterial community as compared to a starved insect due to the food transit in the gut of the former. This was confirmed in a study by Dillon *et al.* (2010), where the microbial diversity in the gut of desert locusts (*Schistocerca gregaria*) increased after a 5-day starvation period. It should be noted, however, that the gut microbial community composition differs between insect species (Yun et al., 2014; Stoops et al., 2016; Garofalo et al., 2017). It is clear, when considering the relative OTU abundances in our study, that no consistent changes in the microbial community composition occurred during starvation (Figure 1 and 2). Statistical analysis showed no difference ($p > 0.05$) in the Chao1 index between non-starved and starved larvae after 0 and 48 h, indicating that starvation does not affect the bacterial species richness of the larvae. However, the Shannon-Wiener diversity indices, which also take into account species evenness, were significantly higher ($p = 0.020$) for non-starved larvae after 48 h as compared to starved larvae that were kept at 30 °C without faecal contact. Nevertheless, in general it seems variation between samples was more prominent than a shift in the community composition due to starvation. Furthermore, NMDS (Figure 3) showed no visual clustering of samples, neither of the four conditions, nor of samples of non-starved versus starved larvae,

confirming that starvation under whatsoever conditions does not influence the bacterial community composition.

3.2 Rinsing

The second part of the study focussed on the effect of rinsing on both non-starved and starved larvae. The initial counts of non-starved, non-rinsed larvae (Table 4) were comparable to those observed in the starvation experiment (Tables 1 and 2). Additionally, no significant differences were observed between non-rinsed and rinsed larvae, for any type of larvae (starved or not) and for any microbial count. Those findings correspond very well with results obtained in preliminary experiments in which sterile demineralised water was used for rinsing (Wynants, Bruyninckx, & Van Campenhout, 2016). This indicates that neither starvation, neither rinsing and neither the combination of both procedures affected the microbial load of the larvae. Decontamination using water is a technique that is often industrially applied for products such as fresh fruit, vegetables and meat. However, in these cases, the water is often enriched with disinfecting chemicals or it is heated (e.g. > 74 °C in the case of meat carcasses) (Beuchat, 1998; Huffman, 2002). For lesser mealworm beetles, Crippen & Sheffield (2006) tested multiple chemical washes as external disinfectants, including combinations of 70-95 % EtOH, NaOCl, H_2O_2 and H_2O. They found that only a 95 % EtOH condition followed by a 20 % H_2O_2 wash resulted in total external disinfection of all beetles. Given the results of those authors, a low disinfection efficiency of water without the addition of disinfecting agents can be speculated. However, the total viable aerobic counts of the rinsing water increased with 4.9 to 5.5 log cfu/ml during rinsing, indicating that the procedure removes a considerable amount of microorganisms from the larvae. Rumpold et al. (2014) showed indirect plasma condition to be effective for surface decontamination, as the technology could not eradicate the total microbiota. It illustrates the large share of gut microorganisms in the total microbiota. From our study it can be concluded that rinsing of

larvae, without the addition of chemicals or without the use of higher water temperatures, does not reduce the microbial numbers on larvae. Nevertheless, mealworm rearers will likely hold on to the rinsing practice, as it yields clean larvae free from any substrate or exuviae. The advice is, as for any food product that is rinsed, to use clean water with a low microbial load in order not to contaminate the larvae during this step.

4. CONCLUSIONS

Starvation and rinsing of mealworm larvae are procedures commonly included at the end of the rearing process of mealworm larvae for human consumption. These practices are often assumed to enhance the microbial quality of the edible insects; however, the results of this study show no differences in microbial numbers between larvae that were starved, rinsed or were subjected to a combination of both treatments. Furthermore, a starvation period of 48 h does not bring about a systematic shift in the composition of the bacterial community. Further research on the impact of other rearing practices, hygiene measures and the substrate on the microbiota of edible insects is necessary in order to provide additional guidelines for the emerging insect-rearing industry to ensure food safety of their end products.

ACKNOWLEDGEMENTS

Funding: This work was supported by the Belgian Federal Public Service Health, Food Chain Safety and Environment (project EDINCO, RT 15/9).

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TABLE 1 Microbial counts for non-starved (control) and starved mealworm larvae after 0, 24 and 48 h at 30 °C and with or without faecal contact during starvation

Microbial counts (log cfu/g)		With faecal contact			Without faecal contact		
		0 h	24 h	48 h	0 h	24 h	48 h
Total viable aerobic count	Control	7.9 ± 0.2[a]	8.0 ± 0.3[a]	8.0 ± 0.3[a]	7.9 ± 0.2[a]	8.0 ± 0.3[a]	8.0 ± 0.2[a]
	Starvation		7.9 ± 0.4[a]	7.9 ± 0.5[a]		7.8 ± 0.4[a]	7.8 ± 0.3[a]
Enterobacteriaceae	Control	7.0 ± 0.3[a]	7.0 ± 0.3[a]	6.8 ± 0.7[a]	6.9 ± 0.3[a]	6.9 ± 0.4[a]	7.0 ± 0.3[a]
	Starvation		7.1 ± 0.3[a]	7.0 ± 0.5[a]		7.1 ± 0.5[a]	7.1 ± 0.3[a]
Aerobic bacterial endospores	Control	1.5 ± 0.6[a]	1.5 ± 0.3[a]	1.2 ± 0.3[a]	1.9 ± 0.2[a]	1.9 ± 0.3[a]	2.0 ± 0.3[a]
	Starvation		1.4 ± 0.7[a]	1.2 ± 0.2[a]		1.7 ± 0.3[a]	1.6 ± 0.4[a]
Psychrotrophic aerobic count	Control	6.7 ± 0.4[a]	6.4 ± 0.6[ab]	6.5 ± 0.5[ab]	7.0 ± 0.3[a]	6.8 ± 0.2[a]	6.9 ± 0.3[a]
	Starvation		6.5 ± 0.4[ab]	6.1 ± 0.3[b]		6.8 ± 0.2[a]	7.1 ± 0.3[a]
Yeasts and moulds	Control	5.6 ± 0.8[a]	5.4 ± 0.6[a]	5.6 ± 0.8[a]	6.2 ± 0.4[ab]	6.3 ± 0.2[ab]	6.2 ± 0.3[ab]
	Starvation		5.3 ± 1.1[a]	5.8 ± 0.5[a]		5.7 ± 0.4[a]	6.4 ± 1.0[b]

Data are the mean of two to three replicates from each of three different batches ± standard deviation.

[a, b] Means for one microbial count within one condition (with or without faecal contact) with the same superscript are not significantly different ($p > 0.05$).

TABLE 2 Microbial counts for non-starved (control) and starved mealworm larvae after 0, 24 and 48 h at 10 °C and with or without faecal contact during starvation

Microbial counts (log cfu/g)		With faecal contact			Without faecal contact		
		0 h	24 h	48 h	0 h	24 h	48 h
Total viable aerobic count	Control	8.4 ± 0.4[a]	8.2 ± 0.2[a]	8.4 ± 0.5[a]	8.0 ± 0.2[a]	7.8 ± 0.2[a]	7.9 ± 0.3[a]
	Starvation		8.2 ± 0.2[a]	8.3 ± 0.4[a]		7.7 ± 0.1[a]	7.7 ± 0.3[a]
Enterobacteriaceae	Control	7.6 ± 0.4[a]	7.5 ± 0.3[a]	7.9 ± 0.6[a]	7.2 ± 0.2[a]	7.0 ± 0.3[a]	7.1 ± 0.4[a]
	Starvation		7.5 ± 0.4[a]	7.7 ± 0.5[a]		6.9 ± 0.2[a]	7.0 ± 0.6[a]
Aerobic bacterial endospores	Control	2.0 ± 0.8[a]	2.2 ± 1.0[a]	1.7 ± 0.5[a]	1.5 ± 0.4[a]	1.7 ± 0.4[a]	1.5 ± 0.4[a]
	Starvation		1.8 ± 0.5[a]	1.6 ± 0.3[a]		1.4 ± 0.5[a]	1.8 ± 0.9[a]
Psychrotrophic aerobic count	Control	7.0 ± 0.3[a]	7.3 ± 0.2[ab]	7.6 ± 0.3[b]	6.8 ± 0.4[a]	7.0 ± 0.5[a]	7.1 ± 0.7[a]
	Starvation		7.1 ± 0.3[a]	7.5 ± 0.6[ab]		6.8 ± 0.3[a]	7.0 ± 0.6[a]
Yeasts and moulds	Control	6.5 ± 0.8[a]	6.1 ± 0.5[a]	5.8 ± 0.5[a]	6.1 ± 1.0[a]	5.6 ± 0.9[a]	5.7 ± 0.4[a]
	Starvation		6.1 ± 0.6[a]	5.9 ± 0.6[a]		5.7 ± 0.9[a]	5.3 ± 0.9[a]

Data are the mean of two to three replicates from each of three different batches ± standard deviation.

[a, b] Means for one microbial count within one condition (with or without faecal contact) with the same superscript are not significantly different ($p > 0.05$).

TABLE 3 Microbial community diversity indices for samples of non-starved and starved larvae after 0 and 48 h incubation under different conditions. Values are the mean ± standard deviation of two analyses performed on two DNA extracts from the same sample from each of three different batches (n = 2 x 3).

Condition		Observed richness	Chao1[1]	Coverage (%)[2]	Shannon-Wiener[3]
30°C, faecal contact	0h	35.33 ± 4.37	47.91 ± 8.05[a]	74.92 ± 8.95	1.48 ± 0.35[a]
	Control (48 h)	36.00 ± 8.76	41.19 ± 8.29[a]	87.57 ± 4.44	1.60 ± 0.29[a]
	Starvation (48 h)	28.33 ± 6.43	35.10 ± 9.49[a]	82.10 ± 6.41	1.36 ± 0.26[a]
30°C, no faecal contact	0h	37.00 ± 2.60	43.67 ± 2.98[a]	86.07 ± 2.71	2.03 ± 0.02[ab]
	Control (48 h)	35.33 ± 8.50	42.67 ± 6.68[a]	81.56 ± 6.09	2.20 ± 0.21[a]
	Starvation (48 h)	29.67 ± 3.82	33.90 ± 5.69[a]	88.12 ± 7.14	1.74 ± 0.16[b]
10°C, faecal contact	0h	42.67 ± 5.80	57.04 ± 16.29[a]	77.63 ± 14.29	2.02 ± 0.24[a]
	Control (48 h)	36.83 ± 7.85	44.76 ± 10.22[a]	82.95 ± 3.55	2.01 ± 0.10[a]
	Starvation (48 h)	36.83 ± 4.65	46..25 ± 1.47[a]	81.26 ± 10.43	1.92 ± 0.07[a]
10°C, no faecal contact	0h	46.00 ± 9.73	51.63 ± 12.45[a]	89.68 ± 4.52	2.18 ± 0.23[a]
	Control (48 h)	36.50 ± 4.33	46.78 ± 3.73[a]	80.62 ± 4.70	1.82 ± 0.34[a]
	Starvation (48 h)	42.17 ± 9.41	48.30 ± 12.72[a]	89.60 ± 5.07	2.11 ± 0.18[a]

[1]Chao1 richness estimator: an estimantion of the total number of OTUs present in the community. A higher number indicates a higher richness (Chao, 1984).

[2]Observed richness/Chao1 estimate * 100

[3]Shannon-Wiener index: a community diversity index that combines the number of OTUs present and their distribution (Shannon, 1948).

[a,b] Means for the Chao1 or Shannon-Wiener diversity indices within one condition with the same superscript are not significantly different ($p > 0.05$).

TABLE 4 Microbial counts of mealworm larvae that were (1) non-starved and non-rinsed, (2) starved and non-rinsed, (3) non-starved and rinsed, and (4) starved and rinsed. Additionally, microbial counts of the tap water before rinsing and of the residual water after rinsing are shown.

		Microbial counts (log cfu/g)			
Parameter	Type of larvae	Non-rinsed larvae	Rinsed larvae	Tap water	Residual water
Total viable aerobic count	Non-starved	8.0 ± 0.2[a]	7.9 ± 0.2[a]	1.5 ± 0.4	6.0 ± 0.7
	Starved	7.8 ± 0.4[a]	8.0 ± 0.3[a]	1.6 ± 0.6	5.5 ± 0.6
Enterobacteriaceae	Non-starved	7.0 ± 0.2[a]	7.1 ± 0.2[a]	<0.0 ± 0.0	4.0 ± 0.6
	Starved	7.0 ± 0.4[a]	7.2 ± 0.5[a]	<0.0 ± 0.0	4.2 ± 0.9
Aerobic bacterial endospores	Non-starved	1.7 ± 0.3[a]	1.6 ± 0.3[a]	0.0 ± 0.1	0.7 ± 0.3
	Starved	1.7 ± 0.5[a]	1.7 ± 0.4[a]	<0.0 ± 0.0	0.5 ± 0.3

Data are the mean values of two to three replicates originating from each of three different batches ± standard deviation.

[a] Means for the larvae for one parameter with the same superscript are not significantly different ($p > 0.05$).

LIST OF FIGURE LEGENDS

Figure 1 Relative abundance (%) of the most abundant bacterial operational taxonomic units (OTUs) in non-starved (control) and starved mealworm larvae after 0 and 48 h incubation at 30 °C with (A) or without (B) faecal contact. OTUs with a maximum abundance below 5 % in all samples were grouped in "Other OTUs". Identifications were performed using the SILVA reference database and taxonomic assignments were considered reliable when a score value ≥ 0.80 was found. When OTUs could not be reliably identified to the genus level, OTUs were further refined by a BLAST analysis against the GenBank nucleotide database (uncultured/environmental sample sequences excluded).

Figure 2 Relative abundance (%) of the most abundant bacterial operational taxonomic units (OTUs) in non-starved (control) and starved mealworm larvae after 0 and 48 h incubation at 10 °C with (A) or without (B) faecal contact. OTUs with a maximum abundance below 5 % in all samples were grouped in "Other OTUs". Identifications were performed using the SILVA reference database and taxonomic assignments were considered reliable when a score value ≥ 0.80 was found. When OTUs could not be reliably identified to the genus level, OTUs were further refined by a BLAST analysis against the GenBank nucleotide database (uncultured/environmental sample sequences excluded).

Figure 3 Nonmetric multidimensional scaling (NMDS) ordination (stress value = 0.24) representing the bacterial community composition of mealworm larvae after 0 h (circles), non-starved larvae (control) after 48 h (squares) and starved larvae after 48 h (triangles). Different colours represent different treatments: incubation at 30 °C with faecal contact (blue) or without faecal contact (green), or at 10 °C with faecal contact (yellow) or without faecal contact (red).

Figure 1

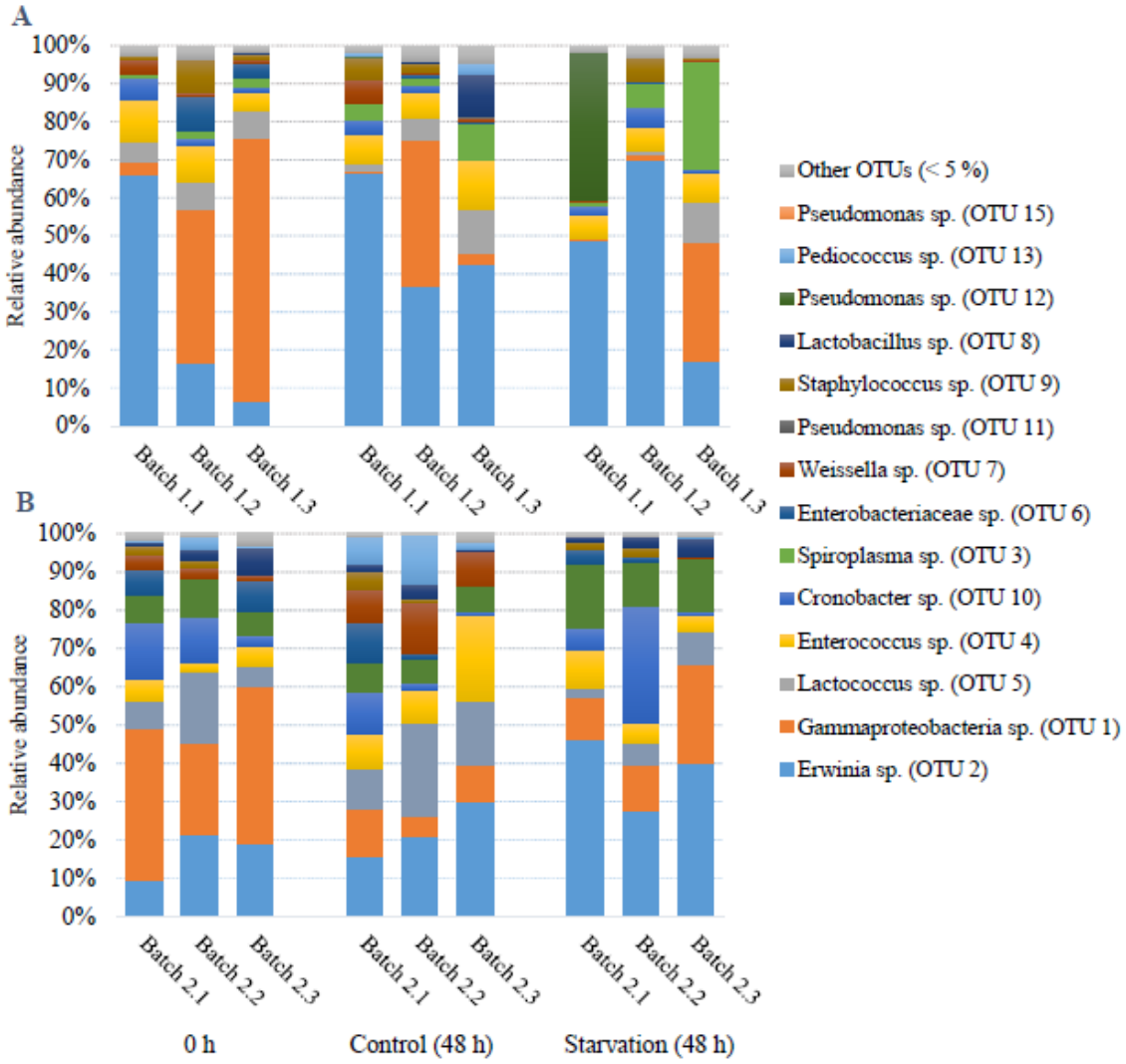


Figure 2

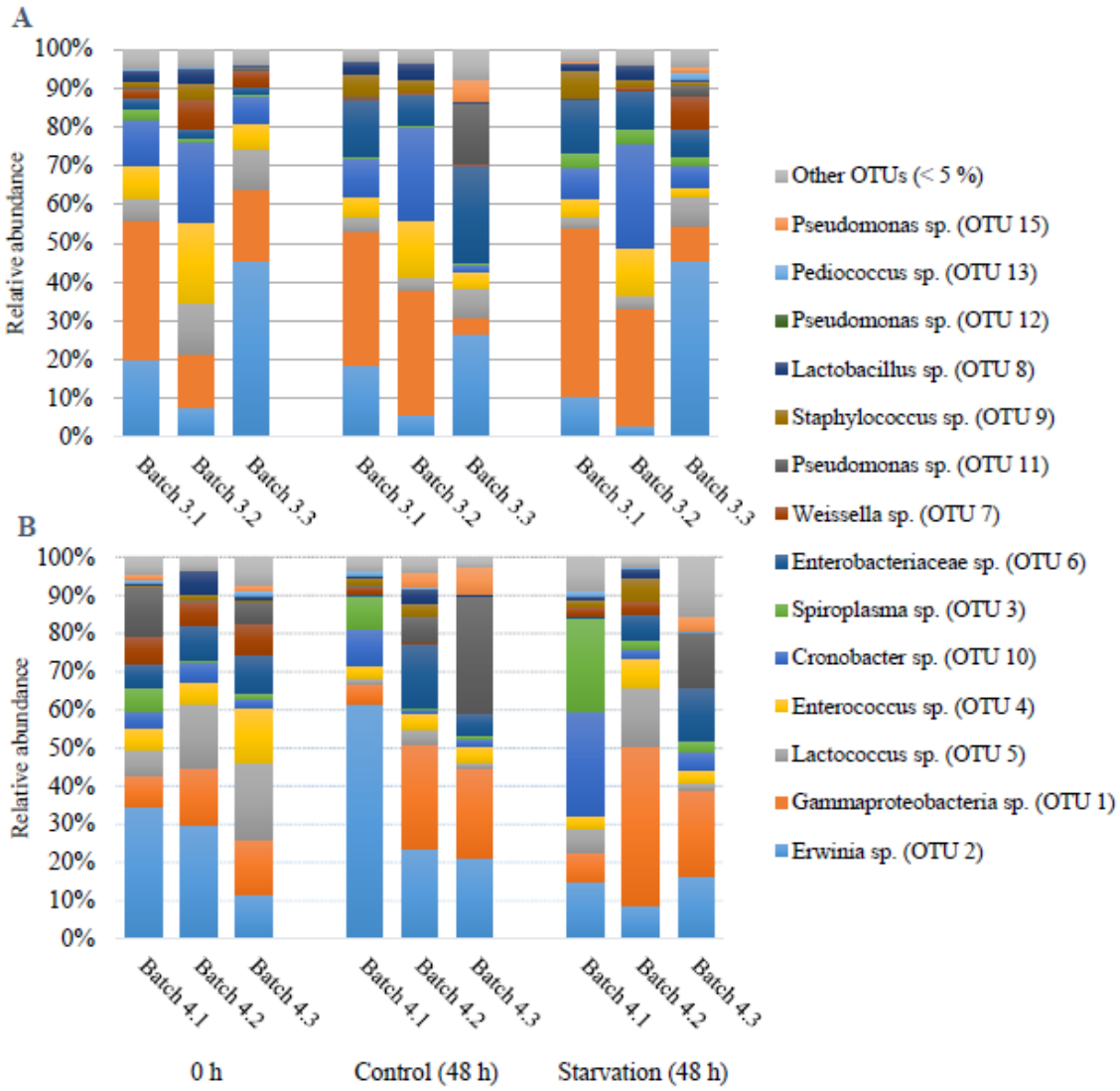
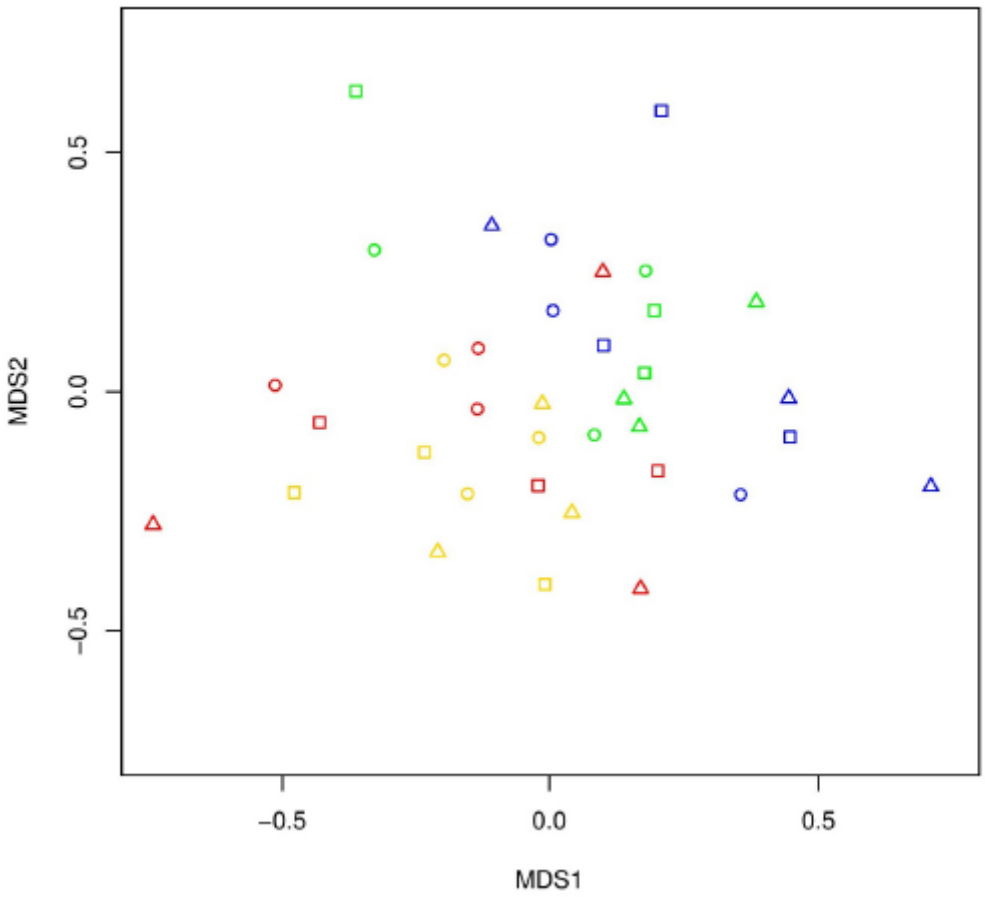


Figure 3



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) N° 2015/2283, more information is needed on their safety, which is related to production hygiene.

Highlights

- Mealworm larvae were starved (24/48 h, 10/30 °C) and rinsed (1 min, tap water)
- Total viable counts ranged from 7.7 to 8.4 log cfu/g for all treatments
- Neither starvation nor rinsing of mealworms after rearing reduced microbial numbers
- The bacterial community was dominated by the phyla Proteobacteria and Firmicutes
- Starvation did not consistently change the bacterial community composition