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Microbial communities and food safety aspects of crickets (*Acheta domesticus*) reared under controlled conditions

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RESEARCH ARTICLE

Abstract

In an approach combining microbiological culture methods with high-throughput sequencing, this study investigated the microbial communities (bacteria, moulds and yeasts) in Swedish-produced edible crickets (*Acheta domesticus*) reared in a controlled environment. The effects of different feeds on microbial loads and populations in crickets were also studied. The crickets used were third-generation offspring from wild-caught individuals from Sweden, which are adapted to grow in a laboratory environment. The efficiency of rinsing to decrease microbial load was evaluated not obtaining a significant decrease of plating counts for total aerobic counts (TAC) and Enterobacteriaceae. Crickets were divided into three batches and fed different diets (control feed, early-cut red clover hay (ECH), late-cut fresh red clover (LCF)) for 62 days. Bacterial numbers (TAC and Enterobacteriaceae) on whole raw crickets ranged between 7 and 8 log cfu/g. Pre-rinsing in water did not reduce these levels (P=0.19). All batches tested negative for the food-borne bacteria *Salmonella*, *Listeria monocytogenes*, *Bacillus cereus* and *Clostridium perfringens*. The mean mould count for crickets fed control feed was 2.8 log cfu/g, while the values for crickets fed ECH and LCF were 4.2 and 4.5 log cfu/g, respectively. The dominant bacterial communities were Proteobacteria, Bacteroidetes and Firmicutes, with Firmicutes and Proteobacteria dominating in crickets fed control feed, Firmicutes dominating in crickets fed LCF and Proteobacteria dominating in crickets fed ECH. *Aspergillus flavus,* a fungus that is capable of producing mycotoxins, was detected in control feed and ECH reared crickets. More work is needed to identify specific food-borne pathogens in edible crickets and establish possible bacterial quality reference values, as an important step in developing microbial quality and safety parameters to ensure consumer safety.

Keywords: insects, food safety, novel food, culture-independent methods, microbial load

1. Introduction

Entomophagy is a common practice in large parts of the world, such as Africa, Asia, Oceania, and South America, with more than 2,100 insect species being consumed worldwide (Jongema, 2017). Despite the large number of insect species consumed, only a limited number of species are cultivated for food production on a large scale. In European countries, insect consumption has not been traditional practice, especially the consumption of whole insects (Hartmann *et al.*, 2015). However, this is expected to change as demand for edible insects increase in Western markets. Compared to traditional livestock, insects appear to be a more ethical and sustainable source of animal protein for many consumers. Insect production can help to meet the increasing demand for animal protein for a growing human population (FAO, 2009). Moreover, insects have attractive nutritional profiles, representing a potential source of essential amino acids and proteins of animal origin and other vitamins and minerals (Rumpold

and Schlüter, 2013). Compared with traditional livestock, insects may have a smaller ecological footprint, their production requires smaller amounts of food and water and less space, and they have better biomass conversion rates. Consequently, insects are an environmentally friendly nutrient source (FAO, 2013; Oonincx and De Boer, 2012; Oonincx *et al.*, 2010). Among reared insects, the house cricket (*Acheta domesticus*) is viewed as one of the most promising species due to its nutritional profile and efficient feed conversion rate (Rumpold and Schlüter, 2013; Van Huis, 2013).

The Novel Foods Regulation within the European Union (EU 2015/2283; http://data.europa.eu/eli/reg/2015/2283/oj) considers insects and insect-derived products to be novel foodstuffs. Hence insects are covered by the EU's novel food legal framework. Owing to their rather recent introduction to Western markets, there are currently no specific hygiene guidelines or microbiological criteria for edible insects in EU legislation (EC 2073/2005; http://data.europa.eu/eli/reg/2005/2073/oj). Data on microbial loads in raw edible or processed crickets have been published in recent years (EFSA, 2015; Grabowski and Klein, 2017a,7b; Vandeweyer *et al.*, 2017a). A recent risk profiling of the microbial and chemical hazards arising from consumption of house crickets showed that data on microbial hazards are still scarce, despite the increasing number of studies being published, and that more focused studies are needed to define the species-specific microbial populations (Fernandez-Cassi *et al.*, 2018, 2019). Microbial load and community composition seem to be influenced by rearing conditions, the feed used and processing treatments applied to edible insects (Vandeweyer *et al.*, 2018). Unfortunately, very few of the metagenomic studies available provide information about feed and environmental conditions during the rearing stage of commercial insects. These insects are often imported from outside the EU, with unknown feeding regime, transport, packaging or production conditions, which may play an important role in microbial loads and also influence microbial populations. Very few studies have explored the presence of fungi in edible crickets and those conducted to date have focused on marketed insects whose rearing conditions are poorly described (Fernandez-Cassi *et al.*, 2019). Fungal growth is heavily influenced by environmental factors such as temperature, humidity and light and conditioned by other factors such as stocking density. Lack of information on the possible presence of mycotoxins and mycotoxin-producing fungi in edible crickets has been identified as an important data gap (Garofalo *et al.*, 2019).

The present study investigated the microbial communities (including yeast, moulds and bacteria) associated with Swedish-produced edible house crickets by combining microbiological culturing methods with high-throughput sequencing (HTS). In addition, to weight the contribution of external microbiota to the total bacterial load, a rinsing step prior plating has been evaluated. The crickets were kept under environmentally controlled rearing conditions, which minimised the possibility of external contamination during processing, handling and storage and allowed better characterisation of the indigenous microbial hazards present in crickets during the rearing stage. The knowledge of microbial communities present in reared crickets under environmentally controlled conditions is important for developing future hazard analysis critical control points systems specific for crickets.

2. Materials and methods

Sample collection and preparation

The crickets used in the study were third-generation offspring of individuals captured from Swedish fields and adapted to grow in a farming/laboratory environment at Swedish University of Agricultural Sciences facilities. They were fed exclusively with local products from Sweden, in order to produce a sustainable, environmental-friendly, low carbon footprint food for human consumption. The present study was part of a larger project aiming to develop a novel protein- and mineral-rich Swedish foodstuff based on *A. domesticus*.

The crickets were kept under controlled conditions with 12 hour lighting regime, temperature 31±1 °C and relative humidity 50-60%, during January-May 2018. The crickets were kept in plastic boxes (W21×D17×H15 cm) fitted with stainless steel mesh on the side for ventilation. One-day-old cricket nymphs were divided into three groups and allocated to different diets. Each group contained six replicates per feed, with 60 crickets per replicate. Thus, around 1,080 crickets in total were included in the study. As a water source, 10-ml plastic vials with cotton at the opening were placed in all boxes and changed every 15 days. Feed was available *ad libitum* and new feed was provided and feed refusals removed every five days. The three groups of crickets were fed: (1) control feed (pelleted feed mixture, prepared from commercial wheat flour (30.8%), oat bran (29.6%), wheat bran (22.4%), rapeseed meal (Expro 00SF, 15.0%), $CaCO_3$ (1.8%) and vitamin-mineral premix (0.04%); (2) early-cut red clover hay (ECH); or (3) late-cut fresh red clover (LCF). Previous testing (data not shown) demonstrated that these feeds have potential for use in commercial cricket rearing.

All replicate batches in each of the three feed groups were collected 62 days after hatching, when the first crickets had reached adulthood. The insects from each batch were placed in separate plastic jars, killed by freezing (-18 °C) and stored at -18 °C until further analysis. The crickets were thawed at room temperature for approximately 20 minutes before preparation. Each replicate within the three different

feed regimes tested (control, ECH and LCF) contained between 15 and 24 individuals. Crickets fed ECH and LCF were much smaller than crickets fed control feed, and hence all replicates from the ECH and LCF groups were pooled for analysis as bulk samples, with a total sample weight of 0.5 and 0.6 g for ECH and LCF, respectively. Control feed crickets had a mean weight of 3.4 g (range 1.6-4.9 g) and the six different replicates were analysed individually.

Evaluation of rinsing in water prior to plating

To assess the effect of rinsing on the microbial load of the crickets, a study was conducted using total aerobic counts (TAC) and Enterobacteriaceae as microbial indicators. Crickets reared under the same environmental conditions and on the same diet (control feed) were divided into two groups (rinsed and non-rinsed) with four replicates per group. Whole non-thermally treated crickets were killed as described above. The rinsing pre-treatment consisted of brief immersion with constant agitation for 1 minute in 150 ml of potable water in a sterile stomacher bag. The crickets were then poured into a strainer and allowed to dry for 10 minutes before being transferred to a fresh sterile stomacher bag. The second group was directly transferred to the stomacher bag without being exposed to immersion. Each sample was crushed with a mortar for 30 s before homogenisation with buffered peptone water (BPW) (1:9) in a stomacher (easyMIX Lab Blender, AESChemunex, Weber Scientific, Hamilton, NJ, USA) for 2 minutes. Ten-fold dilutions of homogenate were prepared using BPW and aliquots of 1 ml or 0.1 ml were used for plating. At all stages, sterile sampling equipment and aseptic techniques were applied.

Enumeration of viable and culturable bacteria

Enumeration of TAC, Enterobacteriaceae, *Bacillus cereus* and *Clostridium perfringens* was performed according to Nordic Committee on Food Analysis (NMKL) methods No. 86 (NMKL, 2013), No. 144 (NMKL, 2005), No. 67 (NMKL, 2010a) and No. 95 (NMKL, 2009), respectively. A qualitative method for all *Salmonella* serovars based on NMKL method No. 187 (NMKL, 2016) was used. To evaluate the presence of *Listeria monocytogenes*, a qualitative method based on NMKL No. 136 (NMKL, 2010b) was followed. However, due to the limited size and weight of the crickets, samples of less than 5 g were used to perform the Salmonella and Listeria spp. analyses, rather than the 25-g samples stated in the methods.

Enumeration of moulds and yeasts from crickets

Yeasts and moulds were enumerated using an in-house method. In brief, 0.1 ml aliquots of suitable homogenate dilutions were plated on Sabouraud dextrose agar with chloramphenicol (National Veterinary Institute) and incubated at 25±1 °C for 5-7 days. Preliminary identification was performed based on macro- and micromorphology, using a microscope (Samson *et al.*, 2010). Representative mould from samples of crickets and cricket feeds were plated on malt extract agar (MEA) at 25 °C for 7 days prior to DNA extraction following the Cenis method (Cenis, 1992). Selected genes for each isolate were amplified using DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Vilnius, Lithuania) and 2 µl template DNA as follows: the *CaM* gene region in *Aspergillus* spp. with primers cmd5/cmd6 (Hong *et al.*, 2006); translation elongation factor 1α in *Fusarium* spp. with primers EF1/EF2 (O'Donnell *et al.*, 1998); β-tubulin gene in *Penicillium* subgenus *Penicillium* spp. with primers bt2a/bt2b (Glass and Donaldson, 1995); rDNA internal transcribed spacer in all species with primers ITS1F/ITS4 (White *et al.,* 1990). Amplicons were sequenced by Macrogen Corporation (Amsterdam, the Netherlands). Isolates were identified by comparing the curated sequences with the GenBank database and Westerdijk Fungal Biodiversity Institute databases (http://www.cbs.knaw.nl), using Blast search (Altschul *et al.*, 1990a). Specific media and microscopy were used for further confirmation of fungal species isolated. Media and incubation times were in brief: *Penicillium* at 25 °C for 7 days on MEA, creatine sucrose agar, yeast extract sucrose agar and Czapek yeast extract agar (CYA) (also at 30 °C) (Pitt and Hocking, 2009); *Mucor circinelloides* on MEA at 37 °C for 3 days; presumptive *Aspergillus sydowii* on CYA at 25 °C for 7 days; and presumptive *Aspergillus flavus* on *A. flavus* and *Aspergillus parasiticus* agar at 25 °C for 7 days and on coconut cream agar (CCA) (Pitt and Hocking, 2009) at 30 °C for 5 days, with fluorescence of the reverse of the plate examined under 365 nm UV light to screen for strains capable of aflatoxin production (Dyer and McCammon, 1994). *A. parasiticus* SLV481, a strain from the internal fungal collection at the Swedish National Food Agency, was used as a toxigenic control and fluoresced bluish white under long UV light.

Analysis of bacterial communities using 16s rRNA gene amplicon sequencing

DNA extraction was performed on the 10-fold serial dilution used for culture-dependent analysis. In brief, a 1 ml sample of homogenate was centrifuged at 13,000×*g* for 2 minutes to produce a bacterial pellet. Supernatant was discarded and DNA was extracted from the bacterial pellet using the DNeasy PowerFood Microbial Kit from QIAGEN (Cat No./ID: 21000-100, Hilden, Germany) following the manufacturer's instructions, with inclusion of an incubation step at 65 °C for 10 minutes to ensure appropriate lysis of Gram-positive bacteria. Extracted DNA was quantified using the Qubit dsDNA BR Assay Kit (cat no. Q32850; Invitrogen, Waltham, MA, USA) with a Qubit DNA 2.0 fluorometer (Invitrogen). Extracted DNA was kept at -80 °C before being used to study bacterial communities

by following an amplicon sequencing approach targeting the V3-V4 region of the 16S rRNA gene (466 bp). Libraries were generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina Inc. (San Diego, CA, USA) and sequenced on an Illumina MiSeq 2×250 bp paired-end run obtaining 100,000 tags per sample. PCR amplification, library preparation and sequencing were performed by Novogene (Beijing, China).

After classifying the paired-end reads according to their unique barcode, primers and barcodes were removed. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). Merged paired-end reads were quality filtered following QIIME (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality control recommendations (Bokulich *et al.*, 2013; Caporaso *et al.*, 2010). Quality tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences and remove them (Edgar *et al.*, 2011). Data obtained from the MiSeq run are summarised in Supplementary Table 1. Effective tags that passed the quality criteria cut-off were further analysed using Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) (Edgar, 2013). To define non-redundant operational taxonomic units (OTUs), a similarity criterion of ≥97% was applied. Representative sequences for each OTU were screened for further annotation using Mothur software against the SSUrRNA database in SILVA (http://www.arb-silva.de/) for species annotation at each taxonomic rank (Threshold: 0.8-1) (Quast *et al.*, 2013; Wang *et al.*, 2007). Obtained OTU's were used to create a species abundance heatmap using the RDP and Blast (ITS) software and plotted by in-house script developed by Novogene. In an attempt at further taxonomic classification, OTUs were blasted against the NCBI GenBank complete database, excluding unclassified and environmental entries, using BLAST (Altschul *et al.*, 1990b) and Geneious software (11.1.5) (https://www.geneious.com) with an e-value threshold of 10^{-5}.

Statistical analyses

To assess the statistical significance of the rinsing treatment prior to plating, an unpaired t-test was conducted ($P<0.05$). Statistical analyses were performed using the software package tidyverse developed in R (R Core Team, 2013), using the log values of TAC and Enterobacteriaceae bacterial counts.

Table 1. Effect of pre-rinsing with water on microbial loads in reared crickets (*Acheta domesticus*).

	Treatment									
	Rinsed					Not rinsed				
Replicate	1	2	3	4	Mean (±sd)	5	6	7	8	Mean (±sd)
Total aerobic counts (log cfu/g)	10.8	8.6	7.5	8.1	8.8±1.4	7.9	7.7	7.4	8.6	7.3±0.8
Enterobacteriaceae (log cfu/g)	7.3	8.4	6.7	6.9	7.3±0.8	7.1	7.1	6.7	7.1	7.0±0.2

Table 2. Microbial counts (log cfu/g) in unprocessed reared crickets (*Acheta domesticus*).[1]

Crickets samples	Total aerobic counts	Enterobacteriaceae	Moulds	Yeast
Control 1	6.8	6.6	3	–
Control 2	7.7	7.5	2.6	–
Control 3	7.3	7.3	2.5	–
Control 4	7.1	6.8	–	5.2
Control 5	8.3	8.5	3.2	–
Control 6	7.6	7.4	2.8	–
Mean (±sd)	7.5 (±0.5)	7.3 (±0.6)	2.8 (±0.3)	5.2
Early-cut red clover hay	8.0	7.6	4.2	–
Late-cut fresh red clover	8.3	7.7	4.5	–

[1] – = negative findings or under the detection limit.

3. Results

Effect of rinsing in water on total aerobic counts and Enterobacteriaceae

The exact log cfu/g values obtained for TAC and Enterobacteriaceae are presented in Table 1. The mean value for TAC in rinsed crickets was 8.8 log cfu/g and in non-rinsed crickets 7.9 log cfu/g. The mean Enterobacteriaceae count in rinsed crickets was 7.3 log cfu/g and in non-rinsed crickets 7.0 log cfu/g. Use of a rinsing treatment prior to plating did not appear to reduce the microbial loads of TAC and Enterobacteriaceae compared with non-rinsed plated crickets (P=0.19).

Microbiological plating counts

Plating counts for commonly analysed foodborne bacteria, determined based on the non-rising method, are presented in Table 2. Crickets fed the control diet had mean counts of 7.5 log cfu/g for TAC. Crickets fed the ECH and LCF diets had TAC counts of 8.0 log cfu/g and 8.3 log cfu/g, respectively. The mean count of Enterobacteriaceae was 7.3 log cfu/g for control diet crickets, while crickets fed ECH and LCF showed counts of 7.6 and 7.7 log cfu/g, respectively. None of the samples analysed tested positive for the foodborne pathogens *B. cereus*, *C. perfringens*, *Salmonella* or *L. monocytogenes.*

Moulds and yeasts isolated in reared crickets and their feed

The mean count of moulds was 2.8 log cfu/g. Yeast were only detected in crickets fed the control feed, at a count of 5.2 log cfu/g. Fungi identified in the crickets or in their feed are listed in Table 3. Crickets fed the control diet hosted colonies of *M. circinelloides*, which seemed to be present also in crickets fed LCF. *A. flavus,* a fungal species capable of producing mycotoxins, was detected in the animals fed under control and ECH regimes, with counts ranging from 4.0 to 5.0 log cfu/g. Crickets fed ECH showed a high diversity of moulds, with four different fungal species being detected, including *A. sydowii, Cladosporium sphaerospermum* and *Penicillium brevicompactum* species in counts of 4.0 log cfu/g. Regarding the fungi species detected in feed used to rear the crickets, *M. circinelloides* was the only mould species that could be taxonomically identified on the feed used to rear control crickets and was present in a low concentration, 1.0 log cfu/g. Similarly, crickets fed ECH had a low concentration of *M. circinelloides* but also different populations of other moulds, including *Cladosporium* spp. (3.0 log cfu/g) and *Botrytis cinerea* (2.3 log cfu/g). Analysis of the results for LCF feed revealed greater diversity in fungi communities compared with control feed and ECH (Table 3). The dominant fungal species belonged to the genus *Neoascochyta*, with the species *Neoascochyta exitialis* and *Neosetophoma samarorum* being quantified at 3.0 log cfu/g. Fungi from the *Fusarium* genus, identified as *Fusarium avenaceum*, were quantified at 2.7 log cfu/g in dried feed. Other fungi, such as *Alternaria tenuissima* and *Heterosporicola chenopodii*, were detected at lower counts.

Microbial communities in reared crickets

The MiSeq analysis generated 602 different OTUs after application of the Uparse software, as summarised in Supplementary Figure S1. Crickets fed control feed presented an average of 402 different OTUs, while ECH and LCF crickets had 369 and 396 OTUs, respectively. Bacterial communities were dominated by three major phyla, Proteobacteria, Bacteroidetes and Firmicutes (Figure 1). Control cricket replicates presented variable proportions of these three phyla, with Firmicutes and Proteobacteria dominating. LCF crickets were dominated by Firmicutes

Table 3. Moulds identified and concentrations in reared crickets (*Acheta domesticus*) and their feed.[1]

Type of feed	Crickets (log cfu/g)	Feed analysis (log cfu/g)
Control feed	*Aspergillus. flavus*∞ (4.7-5.0) *Mucor circinelloides* (4.3-6.1)	*Mucor circinelloides* (1.0)
Early-cut red clover hay	*Aspergillus flavus*∞ (4.0) *Aspergillus sydowii* (4.0) *Cladosporium sphaerospermum* (4.0) *Penicillium brevicompactum* (4.0)	*Botrytis cinerea* (2.3) *Cladosporium cladosporioides* species *complex* (3.0) *Mucor circinelloides** (2.0)
Late-cut fresh red clover	*Mucor circinelloides** (5.2-5.5)	*Alternaria tenuissima** (2.0) *Fusarium avenaceum* (2.7) *Heterospora chenopodii* (1.6) *Neoascochyta exitialis* (3.0) *Neosetophoma samarorum* (3.0)

[1] * = opportunistic pathogen; ∞ = mycotoxin production.

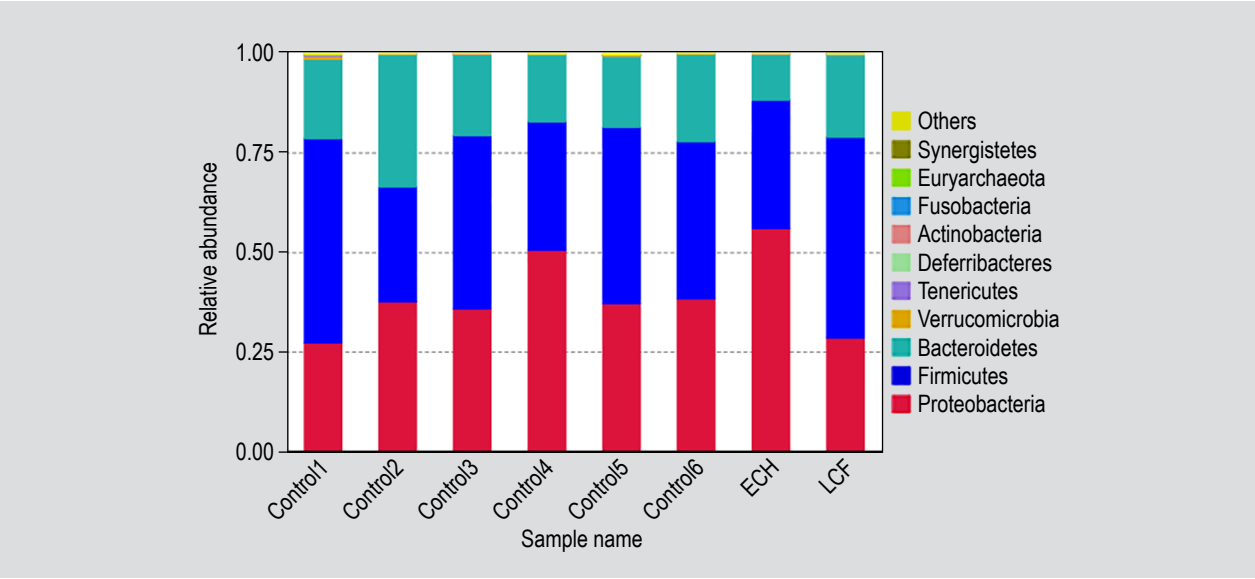

Figure 1. Relative abundance (%) of the top 10 phyla in reared crickets (*Acheta domesticus*) (ECH = early-cut red clover hay; LCF = late-cut fresh red clover).

and ECH crickets by Proteobacteria. Bacteroidetes were present in all samples, but in minor proportions compared with Firmicutes and Proteobacteria. Among the total OTUs generated, 306 OTUs were common to all three cricket groups, irrespective of feed regime. A small number of OTUs were only present in LCF (6 OTUs) and ECH (8 OTUs) (Supplementary Figure S1). In contrast, higher numbers of exclusive OTUs were detected in control feed crickets but not LCF and ECH crickets (211 and 225 OTUs, respectively). The abundance of the 35 dominant genera in all samples is presented in the species abundance heatmap in Figure 2. A complete list of the taxonomic classification of the OTUs generated is presented in Supplementary Data S1. Following this methodology, 580 OTUs were taxonomically classified. The remaining 22 OTUs could not be taxonomically assigned. Several lactic acid bacteria from the genera *Lactobacillus* spp., *Streptococcus* spp., Lactococcus spp. and *Micrococcus* spp. were detected by HTS. Important genera from a food safety perspective detected using the HTS approach are listed in Table 4. Those identified included putative members of the *Streptococcus*, *Bacillus* and *Escherichia-Shigella* genera. The *Streptococcus* genus was highly abundant in crickets fed ECH, *Bacillus* was highly abundant in control feed crickets and *Escherichia-Shigella* was frequently detected in control and LCF feed crickets. *Wolbachia endosymbiont* (OTU 1) was one of the most abundant OTUs detected in all samples, irrespective of the feed used. Reads related to *Klebsiella* spp. (OTU 3) were detected in all samples but were especially abundant in crickets fed ECH. Sequences related to *Methanobrevibacter smithii* (OTU 241) were detected only on one of the replicates under control feed regime.

4. Discussion

The crickets used in this study were third-generation offspring from wild specimens of *A. domesticus* captured in Sweden, which were domesticated and reared to assess their potential suitability as a novel food for human consumption. The use of endogenous species in controlled environments is important for several reasons: (1) Any escape of insects from rearing facilities would have a minimal ecological impact on local ecosystems and biodiversity; (2) the crickets can be sourced locally and sustainably; and (3) the reared crickets do not carry any pathogen with negative effects on farming (i.e. cricket densovirus). This study represents a first attempt to rear crickets using local Swedish resources. In this context, information regarding the microbial food safety of the crickets produced was needed.

The TAC counts detected were as high as those reported by Klunder *et al.* (2012), with an average value of 7.5 log cfu/g in control samples. The TAC values obtained for both LCH and ECH crickets were slightly higher compared to control samples and were similar to values reported by Caparros Megido *et al.* (2017). The feed regime used do not seem to have a big impact on TAC plating counts. However, as observed by the use of specific plating media and HTS, the feed used may influence the microbial communities present. Enterobacteriaceae were present in average counts of 7.4 log cfu/g in control feed crickets, i.e. within the same log range as TAC counts, suggesting that a large proportion of viable bacteria in edible crickets belong to this bacterial family. Slightly higher average Enterobacteriaceae levels were reported by Vandeweyer *et al.* (2017a) (7.7 log cfu/g) compared to detected values in the present study for control crickets (7.4 log cfu/g). Similar values were recorded for

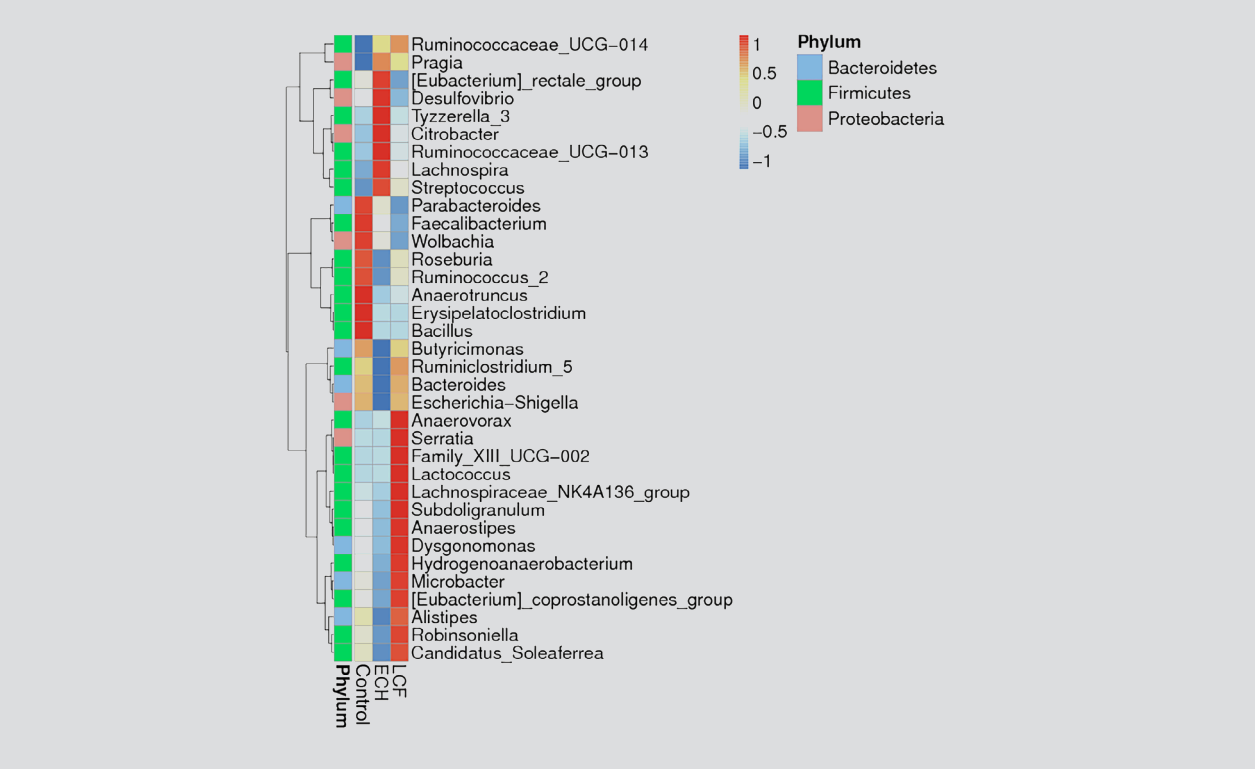

Figure 2. Abundance distribution of the dominant 35 genera among all batches of reared crickets (*Acheta dometicus*) in a species abundance heatmap. Sample name is presented on X-axis whereas the genus is presented in Y-axis. The value of 'Z' is standardised treatment of species relative abundance and is represented by a colour scale. The value of 'Z' of the sample in specific classification represents the distance (between the relative abundance of the sample and the mean relative abundance of all samples in this classification) divided by standard deviation of all samples in this classification. 'Z' is negative when the relative abundance is below the mean, and vice versa.

Table 4. Pathogenic genera detected in reared crickets (*Acheta domesticus*) by high throughput sequencing. Taxonomic classification was obtained by blasting the operational taxonomic units (OTUs) against the NCBI GenBank complete database, excluding unclassified and environmental entries, with an e-value threshold of 10^{-5}.

Genus/species	OTU	Sequence length	% pairwise identity	Query coverage	Previously reported in crickets (Y/N) (reference)
Klebsiella sp.	3	429	100%	100%	Y (Ulrich *et al.*, 1981)
Escherichia coli/Shigella flexneri	43	429	100%	100%	N
Enterococcus faecium	64	429	100%	100%	Y (Fasolato *et al.*, 2018; Garofalo *et al.*, 2017)
Clostridium perfringens	103	404	100%	100%	Y (Garofalo *et al.*, 2017; Osimani *et al.*, 2017)
Haemophilus parainfluenzae	100	429	100%	100%	N
E. coli/Salmonella enterica	329	429	100%	100%	N
Serratia sp.	429	429	100%	100%	N
Aeromonas hydrophila	554	429	99.50%	100%	N
Bacteroides fragilis	242	424	100%	100%	Y (Garofalo *et al.*, 2017)
Streptococcus anginosus	338	429	100%	100%	N
Neisseria sp.	409	429	100%	100%	N
Acinetobacter lwoffii	369	430	100%	100%	Y (Vandeweyer *et al.*, 2018)[1]
Enterobacter sp.	125	429	100%	100%	Y
Citrobacter sp.	366	429	98.80%	100%	Y (Osimani *et al.*, 2017)
Bacillus sp.	559	429	100%	100%	Y (Garofalo *et al.*, 2017; Osimani *et al.*, 2017)

[1] Reported in *Gryllodes sigillatus*.

LCH and ECH crickets, reinforcing this hypothesis. This observation is also supported by the HTS results, with a high number of OTUs taxonomically related to this group. The high microbial loads reported for raw edible crickets is of relevance from a consumer perspective if whole crickets are consumed, including their gut content which is usually not removed, and are in line with other non-processed raw natural foods consumed as a whole. Overall, however, these high plating counts highlight the importance of thermal processing of edible crickets prior to consumption to ensure consumer safety (Fernandez-Cassi *et al.*, 2019). In order to increase the shelf-life of reared crickets and avoid rapid spoilage, an additional processing treatment after thermal processing, such as drying, acidification or addition of nitrifying salts, should be explored.

Crickets fed control feed had lower average mould counts (2.8 log cfu/g) than crickets fed ECH and LCF (4 log cfu/g). Both these values are lower than those reported in other studies on whole crickets (Caparros Megido *et al.*, 2017; Vandeweyer *et al.*, 2017a, 2018). The higher mould counts in crickets fed the LCF feed could be related to the higher moisture content (86%) compared with the pelleted control feed (4%). However, the moisture content of the ECH feed was only 6%, suggesting the moulds present on those crickets may have originated from the drying and processing step or from the fields where the clover was harvested. Yeasts were only detected in one control sample.

The following mould species were identified in the crickets: *A. flavus*, *A. sydowii*, *C. sphaerospermum*, *M. circinelloides* and *P. brevicompactum*. To our knowledge, *A. sydowii*, *C. sphaerospermum* and *P. brevicompactum* have not been reported previously in edible crickets. From a food safety perspective, the identification of *A. flavus* is an interesting finding. These fungi are considered a main source of aflatoxins, a group of well-known carcinogenic metabolites (Bennett and Klich, 2003). However, based on their growth on CCA, the *A. flavus* isolated from the control and ECH feeds were non-toxigenic strains or strains with low potential to produce mycotoxins. *A. sydowii* and *C. sphaerospermum* are not mycotoxigenic moulds and their inability to grow at 37 °C makes unlikely their role as opportunistic human pathogens. *M. circinelloides*, an opportunistic pathogen with extremely rapid growth belonging to the genus *Zygomycetes* (Pitt and Hocking, 2009), was identified both in the feed and in the reared crickets. *M. circinelloides* has been isolated from soil and vegetables and has been also reported in reared crickets (Grabowski and Klein, 2017c), which suggests a dietary transmission route when environmental conditions are favourable (Pitt and Hocking, 2009).

Feed is considered one of the main potential sources of moulds entering the food chain, as observed here for *M. circinelloides*. However, according to plating results the isolated moulds in cricket feed did not match those identified on crickets, which might indicate different sources. The absence in crickets' feed of *A. flavus* and the other moulds detected on crickets could be explained by their low concentration (under the limit of determination of the technique), the low input sample tested (10 g) or non-uniform distribution of moulds in the feeds. Moulds were possibly already present in the feed at low concentrations and were able to grow due to favourable environmental conditions in the experiment, i.e. the presence of 10-ml plastic vials of water in the cricket boxes, which may have created a localised high-humidity area suitable for their germination. In addition, insects are known to play an important role in aflatoxin contamination in the field and in storage conditions, so proliferation in edible insects is feasible. Mycotoxin-producing fungi have been reported previously on edible crickets or their rearing environment (Vandeweyer *et al.*, 2018). *Cladosporium* spp. and *B. cinerea* can be isolated from a wide variety of fresh, dried and processed foods. Overall, these results suggest a need for better monitoring of rearing conditions, including the fungi present on feeds, to prevent growth of *Aspergillus* spp. and mycotoxin production in edible crickets.

Two different protocols were used to assess the effects of rinsing and non-rinsing crickets prior to microbial plate counting. Based on microbial loads obtained, this pre-treatment had a negligible effect on the plating counts, suggesting that gut microbial load was more important than external microbiota for the total plating counts. Similar findings have been reported for other insects with a more intimate contact with the ground such as mealworms (Rumpold *et al.*, 2014; Wynants *et al.*, 2017). Therefore, as inclusion of a rinsing step is time-consuming, adds difficulty in standardisation and does not significantly reduce the microbial loads, it was omitted in other stages of this study. However, the inclusion of a rinsing treatment with a high water-flow rate and for a long time, has the potential to decrease the bacterial and fungi loads that colonise the outer surface of crickets, reducing the risk of sporadic contamination by microbial pathogens during the rearing or during food handling. According to Vandeweyer *et al.* (2017a,b, 2018) *Bacteroides* spp., *Parabacteroides* spp., *Erwinia* spp. and *Fusobacterium* spp. are typical members of the gut bacterial communities of fresh house crickets or tropical house crickets. In the present study, *Parabacteroides* spp. and *Bacteroides* spp. were among the dominant microbial genus in crickets, but not *Erwinia* spp. and *Fusobacterium* spp. This difference might be explained by differences in dietary exposure or different microbiota in the Swedish-adapted *A. domesticus*.

One of the most abundant OTUs, *W. endosymbiont*, has been reported previously as a common species infecting crickets and producing several forms of sex aberrations in arthropods (Kageyama *et al.*, 2012; Marshall, 2007).

Presence of this genus might thus have a negative impact in cricket farming facilities, resulting in significant economic losses. Interestingly, a low number of reads taxonomically close to *M. smithii*, a methane-producing archaeon present in the intestine of humans and other animals, including insects, were detected in one control sample. Important food-borne pathogens such as *B. cereus*, *C. perfringens*, *Salmonella* and *L. monocytogenes* were not detected using culture-based methodologies. Despite the increasing number of studies examining food-borne bacterial pathogens in edible crickets and other insects, *Salmonella* and *L. monocytogenes* are rarely detected by culture-based methods, as reviewed elsewhere (Fernandez-Cassi *et al.*, 2019; Garofalo *et al.*, 2019). This suggests absence of common food-borne pathogens in the microbial communities of reared crickets. Therefore, their sporadic presence might be due to poor hygienic practices during rearing and/or cross-contamination. However, genera that include pathogenic bacteria, such as *Clostridium* spp., *Bacillus* spp., *Escherichia/Shigella* spp. and *Salmonella*, were detected here using non-culture-based methodologies. For some bacteria such as *Shigella* spp. and *Salmonella*, the study of 16S rRNA is limited and additional testing for specific DNA markers within these particular genera is needed to confirm their detection and rule out the presence of *Escherichia coli*. Hence, it is important to remember that, despite the higher sensitivity of molecular methods, culture-based methods are still the gold standard from a food safety perspective and that microbial results derived from HTS do not provide information regarding the viability of detected food-borne pathogens.

The high Enterobacteriaceae counts observed in plating are explained by the amount of reads taxonomically assigned to *Klebsiella* spp., *Citrobacter* spp. and *Enterobacter* spp. in the culture-independent methods. These Gram-negative aerobic bacteria are ubiquitous and can be detected in many environments, including the gastrointestinal tract of humans. Presence of members of these genera in *A. domesticus* has been reported previously (Osimani *et al.*, 2017; Ulrich *et al.*, 1981). *C. perfringens* (OTU 103) and *Bacillus* spp. are two important sporulating bacteria, highly resistant to heat treatment and frequently involved in food-poisoning outbreaks causing diarrhoea or vomiting (Bottone, 2010; Kiu and Hall, 2018). These genera have been detected previously in edible crickets and associated processed products by both culture-dependent and independent methods, suggesting a role as part of the microbiome in the gastrointestinal tract of crickets (Fasolato *et al.*, 2018; Osimani *et al.*, 2017, 2018). In the present study, several OTUs were taxonomically assigned to these two sporulating genera representing low homology or coverage (lower than 95%). This indicates the presence of non-characterised sporulated bacteria closely related to these two genera in the cricket microbiome, which merits further study from a food safety perspective. *Acinetobacter* spp. were detected by HTS in both control and LCF crickets. *Acinetobacter* spp. have been described in a wide variety of environments and are considered one of the most important nosocomial opportunistic pathogens and well-known multi-drug resistant bacteria (Atrouni *et al.*, 2016). Vandeweyer *et al.* (2018) reported their presence in crickets reared on carrots. It seems feasible that the vegetal fraction of the feed is the source of this bacterial species. *Enterococcus faecium*, identified in all cricket samples, has been identified in previous studies and is suggested to be an spoilage microorganism during storage (Garofalo *et al.*, 2017). Reads belonging to the genus *Aeromonas* were also detected in the present study. *Aeromonas* spp. are known foodborne and waterborne opportunistic pathogens that cause gastroenteritis and bacteraemia (Figueras and Beaz-Hidalgo, 2015). Furthermore, sequences related to important bacterial genera such as *Neisseria* spp. and *Streptococcus anginosus* species were detected.

No conclusions can be drawn as regards the effect of different feeds on the microbial community in crickets, as only one batch of crickets fed LCF or ECH could be analysed in the present study.

Despite being fed with the same production feed batch and exposed simultaneously to the same laboratory-controlled conditions (temperature, humidity and light conditions), the different control sample replicates showed different OTU profiles that did not cluster in principal component analysis (PCA) plots (data not shown). This suggests diverse microbial composition within each reared batch. The presence of different microbial communities within different batches at the same facility has been suggested by others (Vandeweyer *et al.*, 2017b). Despite the lack of replicates for microbial communities' analysis for ECH and LCF feed crickets, a similar variability within batches may be expected. More studies assessing the microbial communities in edible insects are needed in order to better characterise the microbial populations at species-specific level and gain a better perspective on the risks associated with consumption of edible insects.

5. Conclusions

High microbial loads of TAC and Enterobacteriaceae were detected in edible crickets, indicating a high risk of rapid spoilage. Thus, thermal treatments, the use of new technological approaches (i.e. high-pressure processing) and additional post-processing treatments (acidification, addition of food preservatives, the use of modified atmosphere packaging, etc.) should be applied to extend cricket shelf-life. All cricket batches in this study tested negative for common food-borne bacteria using standard culture methods. Use of a rinsing procedure before plating did not decrease the microbial loads, suggesting a minimal impact of external microbiota on the total counts, at least

at the conditions applied under the current study. Due to their high microbial loads and in order to prevent any possible food safety issues, edible crickets should be thermally treated at harvest, immediately after the rearing phase is over, to extend their shelf-life. The implementation of these treatments (thermal treatments, application of post-processing measures, etc.) are especially important as regards sporulating bacteria, which seem to be part of the cricket microbiome according to our HTS results. Despite negative plating results, sequences related or closely related to the 16S rRNA of *Bacillus* and *Clostridium* species were detected, raising concerns about sporulating bacteria in crickets. More efforts should be made to characterise these sporulating species and their putative food risks. However, HTS results do not provide information on the viability of detected species. Low mycotoxigenic strains of *A. flavus* were identified in reared crickets, indicating a risk of possible presence of mycotoxins in edible crickets. Some bacteria and fungi species detected in the present study have not been described previously in *A. domesticus*. There is a need for new methods to characterise microbial species in crickets that overcome the limited resolution of 16S rRNA sequencing or the limited level of fungi identification. More efforts are needed to identify specific food-borne pathogens in edible crickets and define possible bacterial quality reference values. This is important for establishing microbial quality and safety parameters to ensure consumer safety.

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Conflict of interest

The authors have no conflict of interest to declare.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2019.0048.

Table S1. Summary of sequencing statistics from the 16-S RNA V3-V4 gene from reared crickets.

Figure S1. Venn diagram of microbial communities detected in edible crickets fed three different diets (control; early-cut red clover hay (ECH); late-cut fresh red clover (LCF)).

Supplementary Data S1. List of the taxonomic classification of generated OTUs.

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