International Journal of Food Microbiology 261 (2017) 11–18

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies

D. Vandeweyer[a,b], S. Crauwels[b,c], B. Lievens[b,c], L. Van Campenhout[a,b,*]

[a] KU Leuven, Department of Microbial and Molecular Systems (M^2S), Lab4Food, Campus Geel, B-2440 Geel, Belgium
[b] KU Leuven, Leuven Food Science and Nutrition Research Centre (LFoRCe), B-3001 Leuven, Belgium
[c] KU Leuven, Department of Microbial and Molecular Systems (M^2S), Laboratory for Process Microbial Ecology and Bioinspirational Management (PME & BIM), Campus De Nayer, B-2860 Sint-Katelijne-Waver, Belgium

ARTICLE INFO

Keywords:
Acheta domesticus
Bacterial community composition
Edible insects
Gryllodes sigillatus
Illumina MiSeq sequencing
Tenebrio molitor

ABSTRACT

Despite the continuing development of new insect-derived food products, microbial research on edible insects and insect-based foods is still very limited. The goal of this study was to increase the knowledge on the microbial quality of edible insects by comparing the bacterial community composition of mealworms (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from several production cycles and rearing companies. Remarkable differences in the bacterial community composition were found between different mealworm rearing companies and mealworm production cycles from the same company. In comparison with mealworms, the bacterial community composition of the investigated crickets was more similar among different companies, and was highly similar between both cricket species investigated. Mealworm communities were dominated by *Spiroplasma* and *Erwinia* species, while crickets were abundantly colonised by (*Para*)*bacteroides* species. With respect to food safety, only a few operational taxonomic units could be associated with potential human pathogens such as *Cronobacter* or spoilage bacteria such as *Pseudomonas*. In summary, our results implicate that at least for cricket rearing, production cycles of constant and good quality in terms of bacterial composition can be obtained by different rearing companies. For mealworms however, more variation in terms of microbial quality occurs between companies.

1. Introduction

Although consumer acceptance of edible insects and insect-derived foods is still limited (Caparros Megido et al., 2014; House, 2016; Lensvelt and Steenbekkers, 2014; Verbeke, 2015; Yen, 2009), insect-based products are increasingly being investigated (Tan et al., 2017) as well as developed (Cadesky, 2017; Stoops et al., 2017) and insects are getting progressively more attention as food source in Western countries (Mlcek et al., 2014). While insect products are entering the market - despite the Novel Food status of insects and their derived products as from 2018 (Regulation 2015/2283) - the microbial quality of the insects is still not fully revealed. Some studies have already assessed the microbial quality of fresh edible insects (Klunder et al., 2012; Stoops et al., 2016; Vandeweyer et al., 2017a) and/or insect-derived products (Caparros Megido et al., 2017; Garofalo et al., 2017; Grabowski and Klein, 2016; Stoops et al., 2017). However, except for Vandeweyer et al. (2017a), these studies did not compare different production cycles and rearing companies. Furthermore, most studies only used culture-dependent methods for microbial analysis, leading to an observed microbial diversity which may be incomplete and/or biased (Justé et al., 2008). Garofalo et al. (2017) and Stoops et al. (2016) recently investigated the bacterial community composition of respectively processed and fresh edible insects using culture-independent 454 pyrosequencing of partial 16S ribosomal RNA (rRNA) genes. These studies revealed that some potential food pathogen and spoilage genera can be present, which could not be proved on this taxonomic level by general culture-dependent counts alone. Both the edible insect sector and the legislative authorities (ANSES, 2015; EFSA Scientific Committee, 2015; SHC and FASFC, 2014) are highly interested in additional microbiological (and other) data from different sources. The data are also useful for insect rearing and processing companies to gain further insight into insects as a food matrix and to complete the Novel Food dossiers they are currently preparing.

The purpose of this study was to assess and compare the bacterial communities of fresh mealworms (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different production cycles, produced at industrial rearing companies in Belgium and The Netherlands. In both countries, crickets and mealworms are produced

* Corresponding author at: Lab4Food, KU Leuven, Campus Geel, Kleinhoefstraat 4, B-2440 Geel, Belgium.
E-mail address: leen.vancampenhout@kuleuven.be (L. Van Campenhout).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.08.018
Received 20 June 2017; Received in revised form 28 July 2017; Accepted 22 August 2017
Available online 24 August 2017

Table 1
Samples investigated in this study.[a]

Sample ID	Rearing company	Production cycle	Sampling month (2015)	Insect type	Species	Rearing purpose (human/pet food)
MW 1.1	1	1	March	Mealworm	*T. molitor*[b]	Human food
MW 1.2	1	2	May	Mealworm	*T. molitor*	Human food
MW 1.3	1	3	September	Mealworm	*T. molitor*	Human food
MW 2.1	2	1	March	Mealworm	*T. molitor*	Human food
MW 2.2	2	2	June	Mealworm	*T. molitor*	Human food
MW 2.3	2	3	October	Mealworm	*T. molitor*	Human food
MW 3.1	3	1	May	Mealworm	*T. molitor*	Pet food
MW 3.2	3	2	July	Mealworm	*T. molitor*	Pet food
MW 3.3	3	3	November	Mealworm	*T. molitor*	Pet food
CR 1.2	5	2	June	Cricket	*A. domesticus*[c]	Human food
CR 1.3	5	3	September	Cricket	*A. domesticus*	Human food
CR 2.1	6	1	April	Cricket	*A. domesticus*	Human food
CR 2.2	6	2	July	Cricket	*A. domesticus*	Human food
CR 2.3	6	3	October	Cricket	*A. domesticus*	Human food
CR 3.1	7	1	August	Cricket	*G. sigillatus*[d]	Human food
CR 3.2	7	2	October	Cricket	*G. sigillatus*	Human food
CR 3.3	7	3	December	Cricket	*G. sigillatus*	Human food

[a] Table adjusted from Vandeweyer et al. (2017a).
[b] T.: *Tenebrio*.
[c] A.: *Acheta*.
[d] G.: *Gryllodes*.

intensively for human consumption, but fresh crickets have never been investigated with next-generation sequencing techniques before and fresh mealworms only once on a small scale in a preliminary study (Stoops et al., 2016). In addition to the intrinsic properties and the traditional culture-dependent microbial counts previously determined and described in Vandeweyer et al. (2017a), this study reports on the metagenetic data obtained for the samples collected in the aforementioned study.

2. Material and methods

2.1. Sample preparation and DNA extraction

Concurrent with the culture-dependent analyses performed in Vandeweyer et al. (2017a), DNA extractions were executed on samples collected in that study (Table 1). A 30 g subsample of living insects from each production cycle was pulverised as described earlier (Stoops et al., 2016; Vandeweyer et al., 2017a) and used to execute two extractions using 2 g starting material (manufacturer's protocol, Power Soil DNA Elution Accessory Kit, Mo Bio laboratories, Carlsbad, CA, USA). DNA samples were stored at − 80 °C until further use.

2.2. Metagenetic analysis

To perform the metagenetic analysis, a tenfold dilution of each DNA extract was amplified in twofold by PCR targeting the V4 region of the 16S rRNA gene using sample-specific barcode-labelled versions of primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) (Caporaso et al., 2011; dual-index sequencing strategy, Kozich et al., 2013; Table S1, Supporting Information). Each 20 µl PCR reaction contained 1 × Titanium Taq PCR buffer, 150 µM of each dNTP, 0.5 µM of each primer, 1 × Titanium Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France) and 1 µl 10-times diluted DNA. The reaction was initiated by denaturation at 95 °C for 120 s, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 45 s. Replicate amplification products were combined and loaded on an agarose gel. Next, visible bands of the expected size were excised and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). After quantification of all purified DNA amplicons (Qubit High Sensitivity Fluorometer kit, Invitrogen, Carlsbad, CA, USA), DNA samples were equimolarily combined into a library and purified once again (Agencourt AMPure XP kit, Beckman Coulter Life Sciences, Indianapolis, IN, USA). The library was diluted to 2 nM and sequenced at the Centre of Medical Genetics Antwerp (University of Antwerp, Antwerp, Belgium), using an Illumina MiSeq sequencer with v2 500 cycle reagent kit (Illumina, San Diego, CA, USA).

Resulting sequences were received as a de-multiplexed FASTQ file (data deposited in a Sequence Read Archive; BioProject accession PRJNA390238). Paired-end reads were merged using USEARCH (v. 8.1) to form consensus sequences (Edgar, 2013) with no more than 10 mismatches allowed in the overlap region. Subsequently, sequences were truncated at the 250th base. Shorter reads or reads with a total expected error threshold above 1.0 for all the bases were discarded. Due to uneven sequencing depth, the number of sequences was rarefied to 54,000 sequences per sample. Remaining sequences with a minimum abundance of two were grouped into operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the UPARSE greedy algorithm in USEARCH, during which chimeric sequences were also removed (Edgar, 2013). Global singletons (i.e. OTUs represented by only a single sequence in the entire dataset) were not taken into account to minimize the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud et al., 2014). Subsequently, 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 v1.23 (Quast et al., 2013). With Mothur's “remove.lineage” command, OTUs originating from chloroplasts or mitochondria were deleted.

The taxonomic origin of each OTU was determined with the SINTAX algorithm implemented in USEARCH, (Edgar, 2016) based on the Silva Living Tree Project v123 (LTP v123) database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.80. Additionally, OTU representative sequences (selected by UPARSE) were subjected to a BLAST (Altschul et al., 1990) search against GenBank (Benson et al., 2013), excluding uncultured/environmental entries. Principally, assignments were based on SINTAX results, but BLAST results were used when SINTAX assignment was inconclusive or produced assignment scores below 0.80. Finally, nonmetric multidimensional scaling (NMDS) and 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).