Food Microbiology 53 (2016) 122–127

Contents lists available at ScienceDirect

Food Microbiology

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

Short communication

Microbial community assessment of mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria migratorioides*) sold for human consumption

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ARTICLE INFO

Article history:
Received 8 May 2015
Received in revised form
19 August 2015
Accepted 16 September 2015
Available online 21 September 2015

Keywords:
Edible insects
Mealworm larvae
Grasshoppers
Next-generation sequencing
Culture-dependent analyses

ABSTRACT

In Western countries, the popularity of edible insects as an alternative animal protein source is increasing. Nevertheless, there is a lack of profound insight into the microbial safety and shelf life of living insects sold for human consumption. The purpose of this study was to characterise the microflora of fresh edible mealworm larvae and grasshoppers in a quantitative and qualitative way. Therefore, culture-dependent analyses (the total viable aerobic count, Enterobacteriaceae, lactic acid bacteria, yeasts and moulds, and bacterial endospores) and next-generation sequencing (454amplicon pyrosequencing) were performed. High microbial counts were obtained for both insect species. Different insect batches resulted in quite similar microbial numbers, except for bacterial endospores. However, the bacterial community composition differed between both insect species. The most abundant operational taxonomic unit in mealworm larvae was *Propionibacterium*. Also members of the genera *Haemophilus*, *Staphylococcus* and *Clostridium* were found. Grasshoppers were mainly dominated by *Weissella*, *Lactococcus* and *Yersinia*/*Rahnella*. Overall, a variety of potential spoilage bacteria and food pathogens were characterised. The results of this study suggest that a processing step with a microbiocidal effect is required to avoid or minimize risks involved with the consumption of edible insects.

1. Introduction

Current animal protein sources will not be sufficient for the increasing human population and alternative sources need to be found (Foley et al., 2011). Several studies suggest that the consumption of edible insects, i.e. entomophagy, can be a valuable alternative to conventional animal proteins (Megido et al., 2014; Oonincx and de Boer, 2012; Yen, 2009). Entomophagy is well-appreciated in many regions in Asia, Africa, Australia and America (MacEvilly, 2000). In contrast, in Western countries the use of insects as human food is still very scare (van Huis, 2013; Yen, 2009). Nevertheless, also in these regions the popularity of edible insects as a new, interesting food matrix is increasing (Mlček et al., 2014). Many studies have reported on the nutrient composition of various insect species (e.g. recently reviewed in Rumpold and Schlüter, 2013; van Huis et al., 2013). Overall, insects provide a valuable source of proteins, lipids, carbohydrates, and certain vitamins and minerals (Mlček et al., 2014). Moreover, rearing of insects involves a smaller environmental footprint compared to traditional animal husbandry (Yen, 2009), making edible insects a promising alternative to animal protein products.

In contrast to what is known on the nutritional properties of edible insects, their food safety and shelf life are hardly investigated to date (van der Spiegel et al., 2013). Only a few studies have reported actual microbial numbers present in/on edible insects. Often these numbers are averages across several insects species and therefore very general (Giaccone, 2005; Grabowski et al., 2014; Klunder et al., 2012; Rumpold et al., 2014). Moreover, in these studies only traditional culture-dependent methods were used for the characterisation of the microflora. It is known that the

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http://dx.doi.org/10.1016/j.fm.2015.09.010
0740-0020/

cultivable microbes in a certain matrix or ecological niche represent only a small fraction of the total microbial community (Olofsson et al., 2007; Justé et al., 2008). Some microorganisms, including food pathogens, are uncultivable because the cultivation conditions are not suitable or they have entered a Viable But Not Culturable (VBNC) state (Amann et al., 1995; Oliver, 2010; Olofsson et al., 2007). As a consequence, the current (limited) view on the microbial community structure of edible insects, and thus their microbial quality, may be incomplete and/or biased. Next-generation sequencing technologies, such as 454 amplicon pyrosequencing have been used to study microbial communities in diverse environments, among which various food products (e.g. De Filippis et al., 2013; Solieri et al., 2013; Stoops et al., 2015; Xiao et al., 2013), surprisingly they have not yet been applied to investigate the microbial quality of fresh edible insects.

The aim of this study was to assess the microbial quality of fresh mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria migratorioides*) which are currently present in the Belgian market and specifically intended for human consumption. The objective was to characterise the microbial community structure both in a quantitative and qualitative way. To this end, culture-dependent (classical plate counts) and culture-independent (454 amplicon pyrosequencing) methods were used.

2. Material and methods

2.1. Sample preparation

Living mealworm larvae (*T. molitor*) and grasshoppers (*L. migratoria migratorioides*, fifth instar larvae) were obtained from an eco-shop selling insects for human consumption (Antwerp, Belgium). No information could be obtained on the rearing process or on specific hygiene measures taken for marketing in the food chain. After purchase, insects were kept at 4 °C until further utilisation (24 h after purchase). For determination of microbial loads by plate counts, three batches of mealworm larvae (one batch obtained begin August, one end August and one in September 2014) and two batches of grasshoppers (August and September 2014) were investigated. It is not known whether the different batches originated from different rearing cycles of the insects. One additional batch of each insect species (February 2014) was subjected to culture-independent 454 pyrosequencing.

Fresh living insects (30 g) were transferred aseptically into a sterile beaker. The insects were anesthetized by incubating them in 100% food grade nitrogen (Praxair, Schoten, Antwerp) for 20 min. Then they were pulverised using a home type mixer (Bosch CNHR 25, speed 12, 2 min), resulting in a homogenous mixture for further microbial analysis.

2.2. Plate counts

Plate counts were determined according to the ISO standards for microbial analyses of food as compiled by Dijk et al. (2007). Five gram of insect mixture was transferred aseptically into a sterile stomacher bag and 45 ml of sterile peptone physiological salt solution (0.85% (w/v) NaCl, 0.1% (w/v) peptone, Biokar Diagnostics, Beauvais, France) was added. The mixture was homogenised for 60 s in a stomacher (Bagmixer® 400 W, Interscience, St Nom, France). A ten-fold serial dilution series was plated on different media using the pour plate method. Total viable counts were determined on Plate Count Agar (PCA, Biokar Diagnostic) incubated at 30 °C for 3 days, Enterobacteriaceae on Violet Red Bile Glucose medium (VRBG, Biokar Diagnostic) incubated at 37 °C for 24 h, lactic acid bacteria (LAB) on de Man Rogosa Sharpe medium (MRS, Biokar Diagnostic) incubated at 30 °C for 3 days, yeasts and moulds on Oxytetracycline Glucose Agar (OGA, Biokar Diagnostic) supplemented with oxytetracycline (50 mg/550 ml OGA, Biokar Diagnostic) incubated at 25 °C for 5 days. Bacterial endospores were determined by giving the 10^{-1} dilution a heat shock treatment (10 min at 80 °C), followed by serial dilution, plating onto PCA and incubation at 37 °C for 24 h. All microbial counts were expressed as log cfu/g. For each insect batch, three samples were analysed (three biological replicates). Obtained data were statistically analysed using SPSS (IBM© SPSS Statistics ver. 22, New York, USA). One-way ANOVA was performed to determine significant differences in microbial counts between different batches of mealworm larvae and grasshoppers. Multiple comparison was performed by Tukey's *post hoc* test. For all statistical analyses, a significance level of 0.05 was considered.

2.3. 454 ampliconpyrosequencing

To assess the bacterial community structure using culture-independent 454 amplicon pyrosequencing, DNA extraction, PCR amplification, library preparation and pyrosequencing were performed as described previously (Stoops et al., 2015). Briefly, DNA extractions were performed on two separate 0.5 g subsamples (biological replicates) per insect mixture using the phenol chloroform DNA extraction method (Lievens et al., 2003). Subsequently, an amplicon library was created using the primers 577F and 926R (Rosenzweig et al., 2012) containing the 454 GS-FLX adapters and barcodes for sample identification. PCR amplification was performed in duplo (technical replicates). After resolving the amplicons by agarose gel electrophoresis, target amplicons were excised and extracted/purified from the gel using the QIAquick® gel extraction kit (Qiagen, Hilden, Germany). Purified dsDNA amplicons were then quantified using the Qubit fluorometer with the high-sensitivity DNA reagent kit (Invitrogen, Carlsbad, CA, USA) and combined at equimolar concentrations (1.00×10^9 molecules/μl) of each amplicon. The quality of the resulting library was assessed using an Agilent Bioanalyser 2100 with high-sensitivity chip (Agilent Technologies, Waldbronn, Germany). Finally, the library was sequenced using the GS-FLX instrument with Titanium Lib L chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Obtained sequences were assigned to the appropriate sample based on both barcode and primer sequences, allowing zero discrepancies, and processed as described previously (Stoops et al., 2015) using Mothur version 1.32.1 (Schloss et al., 2009). Sequences which passed all quality control procedures were combined per sample, rarefied to the lowest number of sequences per sample (950 sequences), and grouped into operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off. OTUs were assigned taxonomic identities to the highest taxonomic rank possible (in general genus level) based on BLAST (Altschul et al., 1990) results of the OTU representative sequences (selected by Mothur) using the GenBank nucleotide (nt) database (Benson et al., 2008), including uncultured/environmental entries.

3. Results

3.1. Plate counts

Microbial counts of fresh edible mealworm larvae and grasshoppers are presented in Table 1. Total aerobic viable counts of mealworm larvae were 8.3 (average) ± 0.1 (standard deviation), 7.7 ± 0.3 and 8.0 ± 0.1 log cfu/g for batch 1, 2 and 3 respectively. Similar counts were obtained for grasshoppers (7.8 ± 0.1 log cfu/g for batch 1 and 8.6 ± 0.4 log cfu/g for batch 2). Counts of LAB and Enterobacteriaceae ranged between 7.0 and 8.5 log cfu/g and