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House crickets (Othroptera: Gryllidae: *Acheta domesticus*) reared in small-scale laboratory conditions harbour limited viral flora

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SHORT COMMUNICATION

Abstract

Insects, such as crickets, are being used as a viable food source in many regions of the world, given their nutritional value for human and animal consumption. This study investigated the viral communities present in European house crickets and whether feed influences the composition of the crickets' virome. The crickets were reared under environmentally controlled conditions and fed fresh red clover (fresh), red clover haylage (haylage), red clover hay (hay) or control feed. The viral metagenomic analysis of six replicates from each feed treatment showed that only a few reads were classified as viruses, mainly assigned to phages and insect-related viruses. A significant difference (P<0.001) was observed between the different treatments in regard to the number of viral reads. The highest number of viral reads was identified in the fresh treatment (2,568 reads), whereas the control treatment had the fewest viral reads (90). Phages were identified in all the treatments; however, they were clearly dominant in the fresh and hay feed treatments. A limited number of insect and plant viral reads from *Xinmoviridae*, *Polydnaviridae, Metaviridae,* unclassified and 'other' viruses were also found in all the feed treatments. The results from this study may indicate that the feed for the crickets determines the richness of the viral flora of crickets, but overall, very few viral reads were identified, making it hard to draw any conclusion regarding the impact of the feed on viral richness.

Keywords: insects, feeds, red clover, viruses, metagenomics

1. Introduction

Insects are part of the traditional diets of an estimated 2 billion people worldwide and offer excellent future food and feed sources for environmental, nutritional and economic reasons (DeFoliart, 1999; Van Huis, 2013). More than 2,111 insect species are being used as food worldwide; among these, crickets, grasshoppers and locusts are some of the most consumed insects (Jongema, 2017). There are several different species of cricket consumed worldwide, and the most common species include *Brachytrupes membranaceus*, *Gryllus similis*, *Gryllus bimaculatus*, *Gryllotalpa orientalis* and *Acheta domesticus* (Ayieko *et al.*, 2016; Halloran *et al.*, 2016a,b; Hanboonsong, 2013; Homann *et al.*, 2017). The rich protein, vitamin, and mineral contents of crickets make them a possible nutritional source for both humans and animals (Rumpold and Schlüter, 2013; Van Huis, 2013). Different studies have examined the use of crickets as feed for different animals, such as poultry, fish and lizards (Jeong *et al.*, 2021; Paoletti, 2005; Taufek *et al.*, 2018; Wang *et al.*, 2005). Previous reports have shown the nutritional value of house crickets (*A. domesticus*), which have high protein and mineral contents (Barker, 1997; Magara *et al.*, 2021; Rumpold and Schlüter, 2013). The need for alternative nutritional food sources is important, as there has been increased global demand for proteins for the growing human population (FAO, 2009).

Recent viral metagenomic studies show that insects are major reservoirs for viruses with a high diversity, and some viruses are likely to be ancestors of those that infect vertebrates (Li *et al.*, 2015; Shi *et al.*, 2016b). Some viral families taxonomically share viruses that can infect insects as well as humans, e.g. *Parvoviridae*, *Poxviridae*,

Picornaviridae, *Orthomyxoviridae* and *Reoviridae*. The majority of the viruses in insects are specific to their host, and some of these insect-specific viruses are a major concern, as they can lead to economic losses in the insect farming industry. The European house cricket *A. domesticus* was shown to be susceptible to different viruses, which affect mass-reared crickets (Eilenberg *et al.*, 2015). The devastating decimation of crickets in commercial rearing facilities by *A. domesticus* densovirus is common and an example of a virus that can have a severe impact on the cricket rearing industry (Szelei *et al.*, 2011). Similarly, cricket paralysis virus, a dicistrovirus, has decimated the cricket population in farms (Maciel-Vergara and Ros, 2017). In addition, there are a number of other viruses known to infect and cause disease in crickets, such as *A. domesticus* mini ambidensovirus (Pham *et al.*, 2013b), *A. domesticus* volvovirus (Pham *et al.*, 2013a), invertebrate iridescent virus type 6 (Kleespies *et al.*, 1999) and *G. bimaculatus* nuduvirus (Wang and Jehle, 2009).

Studies have also indicated that the microbiome composition can be influenced by rearing conditions, the feed used and processing treatments used for edible insects (Vandeweyer *et al.*, 2018). In Sweden, commercially reared crickets are used as feed for pet animals but not for humans. As part of an ongoing project investigating the potential of rearing crickets for future food and feed, the aim of the present study was to investigate the viral flora of small-scale laboratory-reared crickets using a combination of high-throughput sequencing and bioinformatics and to further evaluate the impact of different feed treatments on viral flora in these crickets.

2. Materials and methods

Animals and experimental design

Fourth- and fifth-generation offspring from wild house crickets (*A. domesticus*) were used in the study. The animals were kept at the Swedish University of Agricultural Sciences facilities under climate-controlled conditions (31±1 °C, 50-60% relative humidity and a 12 h lighting regimen) in transparent plastic boxes (21×17×15 cm; W×D×H) with a hole (10×4 cm) covered by a stainless-steel mesh for ventilation. Plastic straws (L≈5 cm) and tubes made of black polyethylene medium density pipes (∅ 25 mm) were placed in the boxes to provide hiding places and allow the crickets to express their natural hiding behaviour (Vaga *et al.*, 2020a).

The metagenomics of crickets with four feed treatments were evaluated: three red clover-only diets (*Phleum pratense*, cv. Yngve) and one control treatment including oats and wheat bran, wheat and heat-treated rapeseed meal (Vaga *et al.*, 2020a). Red clover was harvested from fields at the Röbäcksdalen research station, Umeå, Sweden (63°35´N, 20°45´E) and was stored as fresh (frozen at -18 °C), haylage (airtight) or dried hay. Sixty one-day-old nymphs per replicate were fed for 45 days with each feed treatment. Feed was provided from plastic petri dishes during the first 30 days and thereafter from feeders (3 mm mesh galvanised steel net). Feed denials were removed, and new feed was offered every 5 days. Water (deionised and filtered) was provided in plastic vials (10 cm, ∅ 14 mm) with cotton at the opening and was changed every 15 days. Salt blocks (Albert Kerbl BmbH, Buchbach, Germany) were freely available for all crickets to account for sodium deficiency in the plant-based diets (Vaga *et al.*, 2020b). Two to six crickets per replicate (Table S1) were collected after feeding for 45 days, euthanised by freezing and processed further for metagenomic studies.

Extraction of nucleic acids

Each cricket was rinsed in 70% alcohol and twice with double distilled water. Individual crickets were then mechanically homogenised with 1 ml of TRIzol LS reagent (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 10296028) and a Precellys CK14 lysing kit containing 1.4 mm ceramic beads (Bertin Corp., Rockville, MD, USA, cat. no. P000933-LYSK0A) using a Precellys Evolution tissue homogeniser (Bertin Corp., cat. no. P000062-PEVO0-A) for 30 cycles/sec for 4 min. The supernatant was collected after centrifugation at 13,000×*g* for 10 min at 4 °C. Total RNA was extracted using modified TRIzol and a column method (GeneJet RNA Purification kit, Thermo Fisher Scientific, cat. no. K0732), and the RNA was eluted in 35 µl of nuclease-free water. After extracting RNA from the aqueous phase of the TRIzol homogenate, the remaining slurry containing the interphase/organic phase was saved at -80 °C for DNA extraction. Next, 5 µl of RNA from each tube was combined into a single pool according to the replicate and treatment, and the RNA was treated with DNase from the RNAse-free DNase set (Qiagen, Venlo, the Netherlands, cat. no. 79253) and purified with the RNeasy MinElute Cleanup kit (Qiagen, cat. no. 74204). Ribosomal RNA was depleted using the RiboZero kit (Ribo-Zero plus rRNA depletion kit, Illumina, San Diego, CA, USA, cat. no. 20040526) according to the manufacturer's protocol, and the RNA was again concentrated using the RNeasy MinElute Cleanup kit. For DNA extraction, 500 µl of back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris) was added to the interphase/organic phase slurry. These samples were mixed by inverting the tubes and then centrifuged at 12,000×*g* for 30 min at room temperature. The upper aqueous phase was collected, mixed with 400 µl of isopropanol and then incubated at room temperature for 5 min. The tubes were then centrifuged for 15 min at 4 °C. After removing the supernatant, the pellets were washed with 70% ethanol and centrifuged as before. DNA pellets were then dissolved in 25 µl of nuclease-free water.

cDNA labelling, amplification and sequencing for viral metagenomics

For RNA, cDNA synthesis and amplification were performed using the Ovation RNA-seqV2 system (Tecan Genomics Inc., Redwood City, CA, USA, cat. no. M01206) as per the manufacturer's protocol. For DNA, the ends were labelled and amplified as described by Reyes and Kim (1991). The final amplified products were submitted to the SNP&SEQ Technology platform in Uppsala, Sweden for library preparation and sequencing. High-throughput sequencing was performed with the Ion-Torrent 5S XL sequencing platform using an Ion 318™ chip (v2) and 400 bp read length chemistry. The sequencing data are accessible through NCBI's Sequence Read Archive under the Bioproject accession number PRJNA742181.

Sequence processing and taxonomy assignment

The sequences produced from the Ion-Torrent platform were quality checked by filtering reads with low quality scores (Q<20), removing exact duplicate reads and trimming the ends of the reads with PRINSEQ (Prinseq-lite-0.20.4) (Schmieder and Edwards, 2011). Good-quality reads were subjected to Diamond BLASTx (version 0.9.22; Max Planck Institute for Biology, Tübingen, Germany) querying against the NCBI protein sequence (nr) database (Buchfink *et al.*, 2015), and the output files were analysed by MEGAN Community Edition (version 6.18.0, University of Tübingen, Tübingen, Germany, and mapping file -megan-map-Jul2019.db.zip) (Huson *et al.*, 2016). Reads that were classified as viruses were further assembled to generate longer sequences using the SPAdes assembler and CodonCode Aligner 6.02 (CodonCode Corporation, Centerville, MA, USA), with default settings.

Statistical analysis

Generalised linear models were used. Viruses and phages were modelled by a generalised linear model assuming a Poisson distribution, i.e. a Poisson regression. These two variables showed overdispersion, i.e. a higher variation than assumed in a Poisson distribution. Therefore, a separate dispersion parameter defined as the deviance divided by the degrees of freedom was added to the model using the statement 'dscale' in the GENMOD procedure. All analyses were conducted in SAS software, Version 9.4 (SAS Institute, Cary, NC. USA), and the data are presented as LSmeans ± SE. The dataset was normalised before analysis.

3. Results and discussion

Crickets with 4 different feed treatments (six replicates/treatment) and two to six crickets per replicate were included in the viral metagenomic analysis (Table S1). The metagenomic workflow generated 1-2.3 million reads per library, of which 91-95% were of good quality, with a mean read length ranging from 261-295 nucleotides. The datasets were normalised to the dataset with the lowest number of reads (1,013,423 reads), and Diamond, a fast sequence aligner, was used to assign taxa for each read. A majority of the reads (70-80%) were not assigned to any taxon, and these reads could, for example, be noncoding host sequences and highly divergent microbial sequences. The remaining reads were classified as eukaryotes, bacteria, archaea and viruses (Table S2). The eukaryotic reads (40-90%) mainly belonged to insect hosts such as crickets, including the Japanese burrowing cricket (*Velarifictorus micado*), the black cricket (*Teleogryllus commodus*) and the house cricket. In addition, reads matching plants, fungi and other arthropod insects were found among the eukaryotic reads. Some of the reads may have originated from the feed of the crickets. The bacterial reads (10-60%) corresponded to different Proteobacteria, Spirochetes, Bacteroidetes and other phyla. The number of reads classified as viruses (up to 0.06%) and archaea (0.05%) were relatively low in each replicate when compared to the other taxa (Table S2).

The BLASTx analysis identified reads related to 19 different viral families and unclassified viruses among all the feed treatments, and the number of viral reads varied among the feeds, with most viral reads (2,568) in the fresh group and the least in the control treatment (90) (Table S2). The viral reads were mainly classified into different viral phage families, although a few viral reads were classified into nonphage families, such as *Xinmoviridae*, *Polydnaviridae*, *Metaviridae*, and unclassified viruses. The majority of these viral reads are rather divergent from previously sequenced viruses, showing a protein identity of only 30-65% to known viruses. A significant difference (P<0.001) was observed between the different feed treatments in regard to the number of viral reads. The distribution of individual viral families per feed treatment is displayed in Figure 1. Table S4 shows the exact number of reads belonging to the different viral families from each treatment and replicate.

In the crickets with the fresh feed, the majority of the viral reads were classified as phages, such as *Myoviridae*, *Podoviridae* and *Siphoviridae*, which was similar for all replicates. Viral reads belonging to *Xinmoviridae*, which consist of insect viruses, were identified in four replicates. These reads show 50-65% identity at the protein level to the RNA-dependent RNA polymerase of Hubei chuvirus-like virus 3. Reads in the unclassified virus group showed close similarity to Hubei orthoptera virus 5, a virus previously identified in insects through a metagenomic study (Shi *et al.*, 2016a). Three reads related to insect viruses (*Cotesia sesamiae mombasa* bracovirus (CsMBV)) belonging to the *Polydnaviridae* family were also identified in two replicates (Table S3). The haylage treatment showed a different pattern, with limited phages (5-9 reads), except in one replicate (55 reads). Additionally, in this treatment,

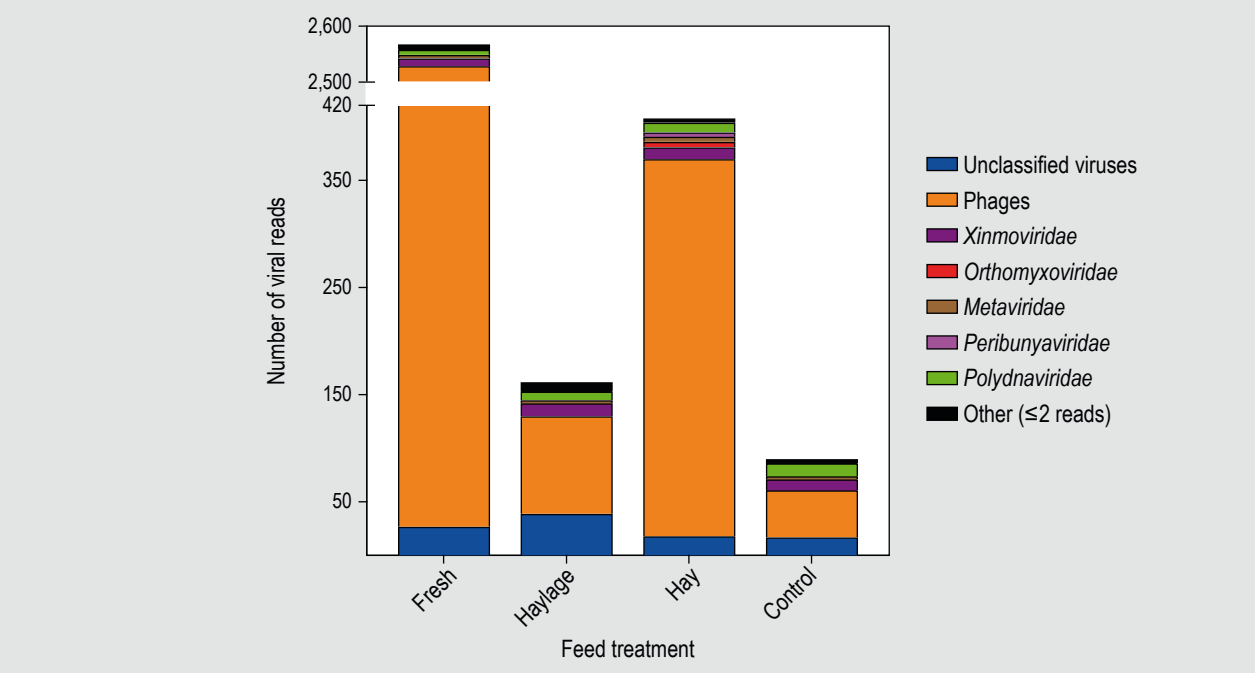

Figure 1. Taxonomic composition and number of viral reads in each feed treatment. The viral families that had two or fewer reads were grouped into 'Other'.

viral reads belonging to *Xinmoviridae* (in 3 replicates) and unclassified viruses (in all replicates) were found. These reads were, for the fresh treatment, all related to insect viruses with similar identities. The reads (detected in 3 replicates) belonging to the *Polydnaviridae* family showed 57% protein identity to CsMBV (Table S4). In the hay-treated crickets, the viral composition was rather different between the different replicates. Three replicates had a high proportion of phages (up to 177 reads), while the three other replicates had few phage reads. In the replicates with low amounts of phage reads, reads belonging to *Xinmoviridae* (Hubei chuvirus-like virus 3), *Polydnaviridae* (CsMBV) and unclassified viruses were also identified. In the unclassified virus group, there were mainly insect viral sequences matching Hubei orthoptera virus 5. In the control treatment group, the reads mainly consisted of phages, with 10-16 reads in three replicates. The majority of the viral reads in crickets fed with the control belonged to the same families (*Xinmoviridae, Polydnaviridae, Metaviridae* and unclassified viruses) identified for crickets fed with the other three treatments. Viral reads related to *Xinmoviridae* and *Polydnaviridae* were identified in three replicates, and unclassified viruses were also identified in 5 replicates (Table S4). The reads assigned to other viral families were identified in low abundance, with up to 5 reads in each feed treatment. These viral families belonged to vertebrates (*Orthomyxoviridae* and *Caliciviridae*), invertebrates (*Chuviridae*, *Rhabdoviridae and Iridoviridae*), protozoa (*Pithoviridae*), and fungi (*Partitiviridae*), some of which share both vertebrates and nonvertebrates (*Parvoviridae*, *Peribunyaviridae* and *Nairoviridae*) as their natural hosts.

The viral metagenomic workflow, a target-free approach, facilitates the characterisation of all the viruses present in a sample and allows the detection of unknown viruses, as well as the identification of possible future disease threats (Mokili *et al.*, 2012). However, potential biases associated with this workflow, in regard to sample processing, sequencing and bioinformatic errors, need to be considered (Rose *et al.*, 2016), and the identified viruses require follow-up investigations to determine their infectivity and pathogenicity. The present study shows the viral composition in crickets reared in-house and fed by different feeds and demonstrates that the majority of the viral sequences were assigned to phages and insect viruses. The large number of phages found in the crickets treated with fresh feed may be relevant to the number of crickets processed for this treatment (2 to 6) compared to other treatments (2); this may have originated from the crickets' natural gut bacteria (Cazemier *et al.*, 1997), or the drinking system available for the crickets may influence microbial contamination. Moreover, the moisture content in fresh feed may be a good environment for microbes. A previous study found higher bacteria and mould counts in late-cut fresh clover feed than in control feed, suggesting that a high water content in fresh feed can influence microbial growth (Fernandez-Cassi *et al.*, 2020). Phages have previously been identified in different invertebrates, such as mosquitoes (Ng *et al.*, 2011), crickets (De Miranda *et al.*, 2021) and wasps (Leigh *et al.*, 2018), through different viral metagenomic studies.

Viruses in crickets have previously been reported, such as cricket paralysis virus (Reinganum *et al.*, 1970) and *A. domesticus* densovirus (Maciel-Vergara and Ros, 2017). These viruses both have the ability to affect host and commercial cricket production. In a recent study, a novel iflavirus in wild and cultivated *A. domesticus* crickets from Sweden was identified (De Miranda *et al.*, 2021). Although the crickets in this study shared a similar viral flora, the number of sequences was different in each treatment. The viral sequences, specifically related to insect viruses, showed low identity (50-65%) to known viruses, indicating that they are divergent from known viruses. None of the previously reported cricket viruses were detected in this study, and no viral reads showed identity towards any known vertebrate or invertebrate pathogenic virus. However, a few reads related to viral families that contain cricket viral pathogens were identified, such as *Iridoviridae* and *Parvoviridae* (Eilenberg *et al.*, 2015).

Viruses in general are a natural part of life, and some benefit from others by a symbiotic relationship; the majority are asymptomatic to the host. It should be determined whether the detected viral reads in this study belonged to active virions that developed into a disease and could influence cricket health, the rearing facilities, and in turn, food safety aspects. This study found a limited number of viral reads, which may be attributed to rearing crickets in environmentally controlled conditions to be suitable for human consumption or technical aspects such as lower sequencing depth or no significant matches for the sequences in the BLAST database. No conclusions can be drawn regarding the effect of different feed treatments on the viral communities, as similar viral flora were shared by all the treatments. Overall, this study provides a comprehensive analysis of viruses in crickets treated with different feeds in small-scale laboratory settings, and further investigations are necessary to understand the origin of identified viruses and their impact on cricket health. Additional analyses regarding the viral flora during a larger industrial-scale rearing remain to be performed to further assess the quality and safety of edible crickets.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2021.0129

Table S1. Number of crickets used in each replicate and for each feed treatment.

Table S2. Taxonomic classification of reads at the protein level.

Table S3. Number of reads belonging to each viral family from each feed treatment.

Table S4. Number of viral reads from each feed treatment and replicate.

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Conflict of interest

The authors declare no conflict of interest.

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