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Applications of molecular diagnostics for quality control in rearing of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae for experimental use

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Spodoptera frugiperda (Smith) (Lepidoptera: Noctuidae), also known as the fall armyworm, is an economically important, widespread polyphagous pest (Montezano et al. 2018). Its resistance to various chemical insecticides and biological agents are studied frequently; therefore, often it is worthwhile to establish colonies using artificial diets (Pinto et al. 2019). Usually, insect rearing is initiated from freshly collected larvae from cultivated plants to obtain adults for subsequent cycles of reproduction and development. There is the distinct possibility that the insect population could be exposed to pathogenic bacteria or viruses at some point during the rearing process and viability could be affected. This possibility makes it essential to implement quality control procedures to ensure that larval populations, for example, can be certified as pathogen-free. We report here the initial implementation of a molecular diagnostic approach to such quality control using Illumina MiSeq sequencing of 16S rRNA genes and a metagenomic approach to detect bacteria and viruses in *S. frugiperda* larvae that ultimately died at generation F_3.

In this study during 2021, larvae of *S. frugiperda* (instars L4–L5) were collected fresh from maize field crops, transported in sterile containers, and reared using artificial diet under laboratory conditions reported previously (Zavala-Sanchéz et al. 2020). Twenty F_3 larvae were selected and reared as above. During the rearing experiment, some of the third-instar larvae died and there was concern that the cause of death was associated with bacteria or viruses present in the field-collected larvae. For the analysis, DNA was isolated individually from 2 healthy larvae and 2 dead larvae exhibiting a necrotic phenotype. The DNA was isolated using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, California, USA). The DNA samples were processed and analyzed with the ZymoBIOMICS® Targeted Sequencing Service using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, California, USA) and taxonomy assignment was performed using Uclust from Qiime v.1.9.1 (Caporaso et al. 2010) with the Zymo Research Database. For metagenomics analysis, the DNA obtained from the same 2 dead and healthy larvae were mixed and Illumina NGS workflow was prepared at Macrogen Sequencing Service (Beotkkot-ro, Geumcheon-gu, Seou, Korea). Bioinformatics analysis for metagenomics was performed in the Bacterial and Viral Bioinformatics Resource Center, Chicago, Illinois, USA (https://www.bv-brc.org/) using its pipelines accessed on 11 Jun 2022 (Wood et al. 2019). DNA sequences were deposited in National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) as BioSample accession PRJNA899064.

The results in Figure 1, showing the relative abundance of various bacterial species with 2 preparations of healthy larvae (LH1 and LH2) represented by *Enterococcus casseliflavus/flavescens/gallinarum* (Lactobacillales: Enterococcaceae) in high relative abundance (85.5% and 72.4% respectively), while *E.* mundtii (Lactobacillales: Enterococcaceae) show low relative abundance in LH2 (6%). DNA preparations from LH2 additionally showed a 21.6% relative abundance of the genus *Pseudomonas putida* (Pseudomonadales: Pseudomonadaceae). Two preparations of DNA from dead larvae (LD1 and LD2) also showed a high relative abundance of *Enterococcus casseliflavus/flavescens/gallinarum*, at 20.4% and 100% respectively, while *E. mundtii* show high relative abundance in LD1 (69.1%). Also, *Acetobacter persici* (Rhodospirillales: Acetobacteraceae) and *Pseudomonas putida* were present in the dead larvae LD1 sample with relative abundances of 9.3% and 1%, respectively. In summary, the results showed that the *Enterococcus* genus is represented in high relative abundance among larvae groups and dead larvae did not show a significant difference compared with healthy larvae. We then turned our attention to the question of viral presence in healthy and dead larvae using a metagenomic approach.

Results from metagenomic analysis showed that healthy and dead larvae harbored viruses from diverse groups (Fig. 2). Approximately 1.48% of fragments covered consisted with sequences of viruses identified in dead larvae, whereas that in healthy larvae was 0.004%. In

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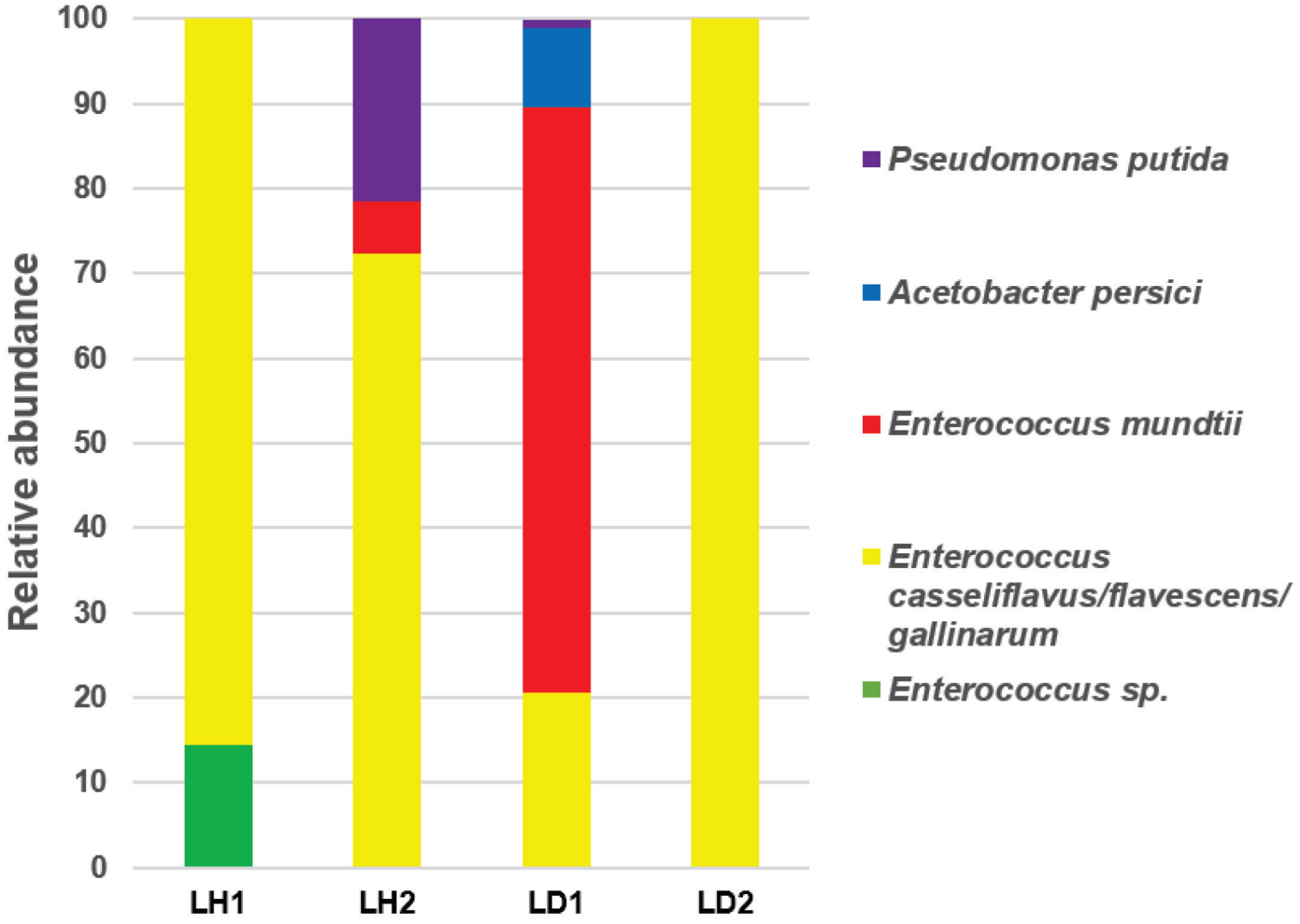

Fig. 1. Relative abundance of bacterial diversity found in samples of healthy (LH1 and LH2) and dead larvae (LD1 and LD2) of *Spodoptera frugiperda.*

dead larvae, the sequences most abundant were related to *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) nucleopolyhedrovirus with relative abundance of 77%, as well as low relative abundance of viral sequences related to *Catopsilia pomona* (Fabricius) (Lepidoptera: Pieridae) nucleopolyhedrovirus and *Hemileuca* Walker (Lepidoptera: Saturniidae) nucleopolyhedrovirus with 0.7%, respectively. In addition, the analysis detected sequences related to tomato leaf curl Laos virus and *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) nudivirus with 0.5%, respectively. On the other hand, in healthy larvae were detected lower relative abundance of viral sequences related to *Adoxophyes orana* (Fischer von Röslerstamm (Lepidoptera: Tortricidae) nucleopolyhedrovirus, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) nucleopolyhedrovirus, *Clostera anachoreta* (Denis & Schiffermüller) (Lepidoptera: Notodontidae) granulovirus and *Tipula oleracea* L. (Diptera: Tipulidae) nudivirus. The remainder of the viral sequences obtained were related to mycoviruses and phages with 20.6% in dead larvae, compared to 85% in healthy larvae (Fig. 2). Although, *S. litura* nucleopolyhedrovirus was detected in dead larvae in high relative abundance, it is necessary to conduct additional experiments to determine the cause of mortality related with this nucleopolyhedrovirus.

Our results of bacterial diversity in dead and healthy larvae are consistent with other reports, which showed that *Enterococcus* is the most common gut bacteria in Lepidoptera (Gao et al. 2019), whereas *Pseudomonas* and *Acetobacter* may fluctuate during various stages of larval development according to previous reports (Gao et al. 2019). On the other hand, metagenomics results from dead larvae show high relative abundance of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) nucleopolyhedrovirus, an *Alphabaculovirus* genus (Wang et al. 2018; Li et al. 2019), which contains insect-specific viruses with double-stranded large circular DNA genomes of 80 to 180 kb (Blissard & Theilmann 2018). The *H. zea* nudivirus is a non-occluded baculovirus, a group more recently classified as nudiviruses and include a diverse group of arthropod-specific large DNA viruses, which replicate in the nucleus in various host species (Bojko et al. 2022). All these results, and the finding of sequences related to tomato leaf curl Laos virus, a begomovirus with a circular single-stranded DNA genome and also transmitted by the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Pan et al. 2017), suggests DNA viral acquisition or persistency during insect rearing, however, more research is necessary to supports this conclusion (Pan et al. 2017). The presence of the identified viral sequences in the DNA preparations from dead larvae suggests the intriguing possibility that those viruses related to *Alphabaculovirus* genus contributed to their deaths, but additional study is required to support this hypothesis.

In conclusion, our results show that a molecular diagnostic procedure for the detection of pathogenic agents associated with *S. frugiperda* can serve as a quality control method applicable during insect rearing to prevent colony infections and avoid the spread of potential pathogens to surrounding environments.

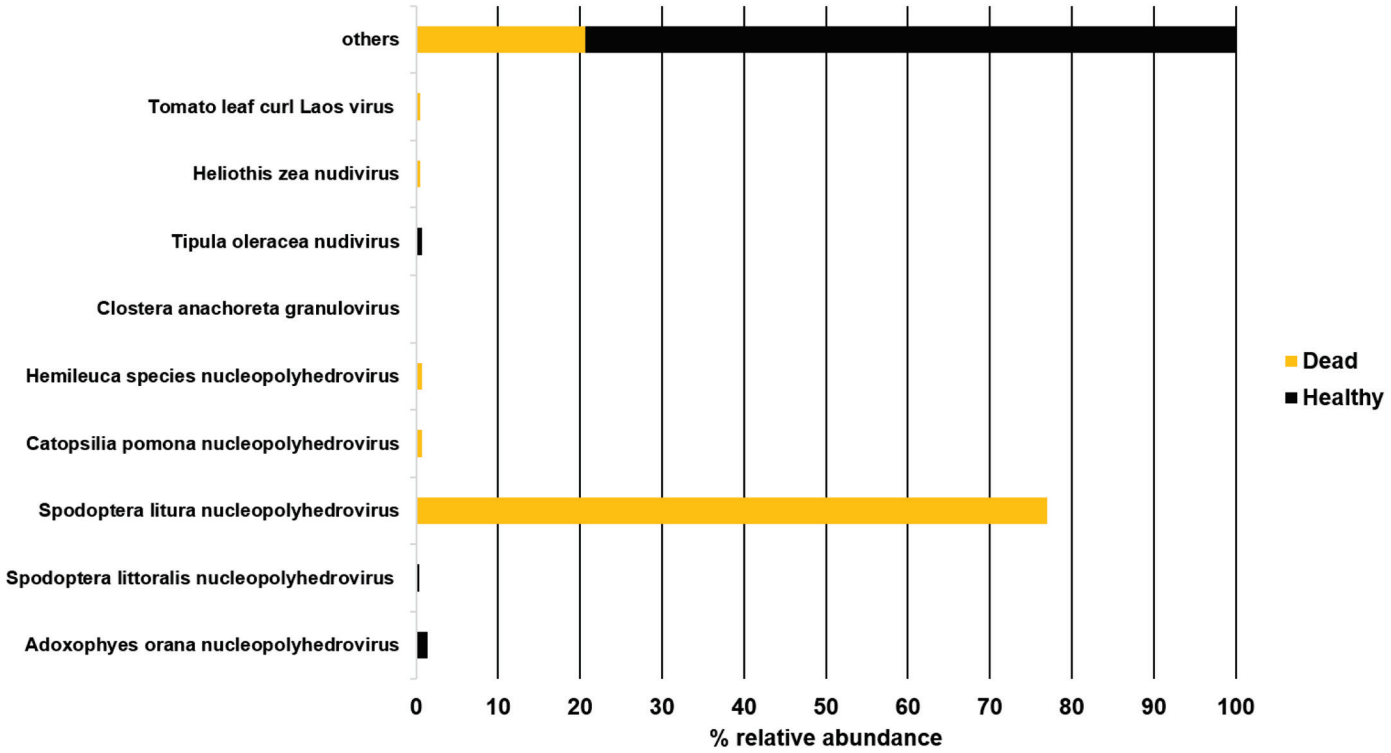

Fig. 2. Relative abundance of diversity of viruses found in the metagenomic analysis of dead and healthy larvae of *Spodoptera frugiperda*.

Summary

Illumina MiSeq sequencing of 16S rRNA genes was applied in a molecular diagnostic analysis of healthy and dead larvae obtained from a *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) colony. The results indicated the presence of *Enterococcus* (Lactobacillales: Enterococcaceae) with an average abundance of 89.2% and 94.8% in healthy and dead larvae, respectively. *Pseudomonas* (Pseudomonadales: Pseudomonadaceae) and *Acetobacter* (Rhodospirillales: Acetobacteraceae) were present in lower abundance. Additional metagenomic results revealed the presence in dead larvae of viral sequences related to *Alphabaculovirus* in high relative abundance compared with healthy larvae. Additionally, viral sequences of tomato leaf curl Laos virus, *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) nudivirus, *Clostera anachoreta* (Denis & Schiffermüller) (Lepidoptera: Notodontidae) granulovirus, and *Tipula oleracea* L. (Diptera: Tipulidae) nudivirus were detected. Our results show that molecular detection of pathogenic agents is a useful tool during insect rearing as a quality control to avoid the spread or acquisition of potential pathogens.

Key Words: *Spodoptera frugiperda*; baculovirus; molecular diagnosis; quality control

Sumario

Nuestros resultados demuestran que a través de un diagnóstico molecular en *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), usando secuenciación del 16S rDNA por Illumina MiSeq, en larvas saludables y muertas mostraron una abundancia relativa alta de *Enterococcus* (Lactobacillales: Enterococcaceae) con un promedio de 89.2% and 94.8% respectivamente. Mientras que *Pseudomonas* (Pseudomonadales: Pseudomonadaceae) y *Acetobacter* (Rhodospirillales: Acetobacteraceae) presentaron menor abundancia relativa. Adicionalmente, resultados de metagenomica mostraron en larvas muertas secuencias de virus relacionados con *Alphabaculovirus* con una abundancia relativa muy alta comparada con las larvas saludables. Se detectaron secuencias virales relacionadas al Encrespamiento amarillo de la hoja del tomate, *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) nudivirus, *Clostera anachoreta* (Denis & Schiffermüller) (Lepidoptera: Notodontidae) granulovirus and *Tipula oleracea* L. (Diptera: Tipulidae) nudivirus. Nuestros resultados demuestran que una detección molecular de agentes patogénicos es primordial durante la crianza de insectos como un control de calidad para evitar su dispersión o adquisición.

Palabras Clave: *Spodoptera frugiperda*; baculovirus; diagnóstico molecular; control de calidad

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