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Use of biocides to minimize microbial contamination in Spodoptera exigua multiple nucleopolyhedrovirus preparations

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A B S T R A C T
The presence of contaminant microbes in baculovirus-based insecticides is regulated by phytosanitary product registration authorities. We aimed to determine whether the abundance of microbes in suspensions of Spodoptera exigua multiple nucleopolyhedrovirus occlusion bodies (OBs) could be reduced by treatment with a range of biocidal compounds. The diversity of contaminant bacteria was determined by next-generation sequencing of the 16S rRNA gene. Overall, 97.9% of sequences detected were Gammaproteobacteria (mostly *Pseudomonas* spp. and Enterobacteriaceae) and 2.1% were Firmicutes (mostly *Enterococcus* spp.). Colloidal silver, benzalkonium chloride and chlorhexidine digluconate were identified as highly effective biocides. Incubation of OB suspensions with high concentrations of colloidal silver (450 mg/l) or benzalkonium chloride (6000 mg/l) resulted in marked reductions in colony forming unit counts over a 180 day period at 4 ° or 25 °C. Benzalkonium chloride and colloidal silver treatments, at either 4 or 25 °C, did not affect the insecticidal activity of OBs over an 80 day period. However, OB activity decreased following 180 days of treatment by benzalkonium chloride at either 4 or 25 °C, or by colloidal silver at 25 °C, but not at 4 °C. Counts of OBs revealed a significant decrease in OB numbers in benzalkonium chloride-treated suspensions after 180 days at both temperatures, whereas colloidal silver-treated OBs were not affected. Benzalkonium chloride also caused aggregation of OBs at the concentration tested. We conclude that biocidal compounds can markedly reduce the abundance of contaminant microorganisms in OB suspensions, and can be accompanied by reductions in OB infectivity and OB numbers in some circumstances. Future studies should focus on lower concentrations of biocides that do not affect OBs in long-term storage.

Keywords: Alphabaculovirus; SeMNPV; benzalkonium chloride; colloidal silver; chlorhexidine digluconate; occlusion body pathogenicity; stability; bacterial diversity

1. Introduction

Lepidopteran nucleopolyhedroviruses (*Alphabaculovirus*; Baculoviridae) form the active ingredient in highly effective biological insecticides for the control of field crop and forest pests (Lacey et al., 2015). The production of these viruses involves the inoculation and rearing of massive numbers of larvae, followed by harvesting and processing of virus-killed insects to extract virus occlusion bodies (OBs). Considerable efforts have been made to develop efficient systems for the *in vitro* production of these virus with limited success due to the rapid adaptation of the virus to cell culture conditions and the resulting loss of key insecticidal traits (Reid et al., 2016). As such, all virus insecticides are currently produced *in vivo* using insects reared on semi-synthetic diet (Grzywacz et al., 2014).

Following death, each virus-killed insect is rapidly colonized by the microbiota of the gut (Grzywacz et al., 1997). These microbes usually comprise species from the

Proteobacteria and Firmicutes bacterial phyla, with lower numbers of fungi (mainly Ascomycota and Basidiomycota) that are members of a highly variable microbial community that inhabits the alkaline gut of phytophagous lepidopteran larvae (Hammer et al., 2017; Chen et al., 2018). However, in the case of laboratory-reared larvae, the use of semi-synthetic diets that often contain antibiotics and preservatives can strongly influence the composition of the gut microbiota (van der Hoeven et al., 2008).

The abundance and type of microbes in OB preparations are subject to regulation by phytosanitary product registration authorities, who define the upper limits for these microorganisms (usually specified at 10^8 colony forming units (CFU)/mL of formulated product) and restrict the presence of vertebrate and plant pathogens in baculovirus-based insecticides (OECD, 2011). Microbial contamination of OB preparations can also reduce the stability of virus insecticides in long-term storage (Burges and Jones, 1998). As many bacterial contaminants have a similar size and density as OBs, efforts to remove these by filtration and differential centrifugation, including zonal centrifugation, can be inefficient, time-consuming, costly in terms of labor and lead to significant loss of OBs (Ignoffo and Couch, 1981; Shapiro, 1986; Grzywacz et al., 1997; Hunter-Fujita et al., 1998). An alternative approach, involving the use of ultra-high pressure treatments, was effective but also costly and time-consuming (Butz et al., 1995).

Baculovirus formulations often include bacteriostatic and fungistatic agents such as benzoates, sorbates or parabens as preservatives (Jones and Burges, 1998), but the use of biocidal compounds to kill contaminant microbes has received little attention from researchers in baculovirus production. To our knowledge, a single exception to this is a study in which the abundance of bacteria in preparations of purified Cydia pomonella granulovirus OBs was markedly reduced by treatment with 0.4% sodium pyrithione and 0.1% dodecylamine (triamine), without significant loss of insecticidal activity, whereas occlusion derived virions released from granulovirus OBs were completely deactivated (Krieg et al., 1979).

Biocides are compounds that kill a broad spectrum of bacterial and fungal microbes, and are widely used in cosmetics, pharmaceuticals and paints, and have numerous clinical, culinary and industrial applications as disinfectants (Rossmoore, 1995). The mode of action of these substances differs among the different compounds, but often involves disruption of microbial cell integrity or cell metabolic processes.

In order to identify biocidal compounds that were highly effective at reducing microbial contaminants, without adverse effects on the insecticidal activity or stability of OBs, we performed a detailed characterization of the bacterial community present in OB preparations. We then screened a range of different types of biocides for their efficacy and examined the effects of high concentrations of the selected compounds on the insecticidal

activity and stability of OBs stored under refrigeration or at room temperature. For this we used preparations of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV), a virus that has an established history of use as the basis for biological insecticides in many countries (Smits and Vlak, 1988; Hunter-Fujita et al., 1998; Bianchi et al., 2000; Lasa et al., 2007).

2. Materials and methods

2.1. Insect and virus production

Larvae of *S. exigua* were obtained from a laboratory colony maintained in the Instituto de Ecología AC, Xalapa, Mexico. The colony was reared on a semi-synthetic diet (Supplementary material, Table S1), under controlled conditions of 25 ± 2 °C temperature, 70 ± 5% relative humidity (RH) and 12:12 light-dark photoperiod. Each liter of larval diet contained 1g potassium sorbate, 1.6 g methyl paraben and 4.4 ml of 4% formaldehyde solution to reduce microbial proliferation. Adults were fed *ad libitum* on a 10% (wt/vol) sucrose solution. A sample of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) occlusion bodies (OBs) was kindly provided by P. Támez-Guerra (Universidad Autónoma de Nuevo León, Mexico) and was used to inoculate *S. exigua* fourth instars by the droplet feeding method (Hughes et al., 1986). OBs were extracted from virus killed larvae by trituration in sterile distilled water, filtered through a fine metal mesh (80 µm pore size) and stored at -20 °C until use.

2.2. Production of experimental batches of OBs

The production of experimental batches of OBs involved the inoculation of three independent batches of 320-400 larvae. Each batch comprised four groups of 80-100 *S. exigua* larvae, at 24-30 hours after molting to the fourth instar. Each group of larvae was inoculated by placing insects in a ventilated plastic box (340 × 190 × 60 mm) with a strip of diet (230 ×10 ×2 mm) previously treated with 1 x 10^9 OBs, estimated to result in a 95% prevalence of lethal polyhedrosis. Following a 24 h period of feeding, when the treated diet had been consumed completely, a new piece of OB-free diet (230 ×10 ×10 mm) was placed in the box and rearing continued at 25 ± 1 °C and 60 ± 10% RH in continuous darkness. At six days after inoculation, dead larvae were collected using entomological tweezers and stored in 50 ml plastic tubes at -20 °C until processed.

To extract OBs, each group of infected larvae was thawed, homogenized in sterile distilled water using a ceramic mortar and the resulting slurry was filtered twice through steel mesh (80 µm pore size) to remove debris. The OBs from the four groups of larvae were pooled to form a single batch, counted in triplicate using a Neubauer Improved chamber (Hawksley, Lancing, UK) and the OB concentration was adjusted to 1.0×10^9 - 1.1×10^9 OBs/ml, in a total volume of 600 ml, using sterile distilled water. Each final OB suspension was placed in a l000 ml sterile glass jar and stored in darkness at 4 °C for 48 h until used in experiments.

2.3. Metagenomics analysis of microbial community

Total DNA was extracted from the OB suspension of each of the three batches. For this, 15 ml of each OB suspension was filtered under vacuum through sterile filter paper (Whatman No. 3) under sterile conditions. Each filter paper was cut into small pieces, placed in a 1.5 microcentrifuge tube and DNA extraction was performed using the DNeasy Blood & Tissue Kit® (Qiagen, USA) and following the manufacturer's instructions. The quantity and quality of the extracted DNA were verified by electrophoresis in 1% agarose and measured in a spectrophotometer (Biospec-nano, Shimadzu Scientific Instruments, Japan).

Amplicon library preparation and genomic sequencing were performed by Macrogen Inc. (Seoul, South Korea). Amplicons of the V3 region of the 16S rDNA gene were amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG) and 518R (5'-GTATTACCGCGGCTGCTGG). Sequencing was performed on a GS-FLX Titanium platform, generating over 163,000 paired-end reads with an average length of 422 bp. Sequencing datasets were deposited in the Sequence Read Archive Database of the NCBI with the accession number PRJNA603778.

Quality analysis of raw paired-end reads was performed with PRINSEQ v.0.20.4 (Schmieder and Edwards 2011). Reads shorter than 50 bp or with a quality score lower than 20 were discarded from the analysis. High quality paired-end reads were joined using QIIME v.1.8.0 (Caporaso et al., 2010). Detection and removal of chimeras were conducted with MOTHUR v.1.25.0 (Schloss et al., 2009). Sequences were then clustered in Operational Taxonomic Units (OTUs) at 97% similarity, using QIIME under the default settings (Caporaso et al., 2010). A representative sequence was randomly selected within each OTU and a taxonomic category was assigned to each OTU using the Greengenes 13_8 reference database (DeSantis et al., 2006). The OTUs corresponding to chloroplast, mitochondrial and archaeal DNA were removed from the OTU table. A rarefaction curve based on Chao1 index was drawn from the filtered OTUs in QIIME v.1.8.0.

2.4. Biocide effects on microbial contamination: initial screening

Ten different commercial biocidal products were assessed for their activity against aerobic microbes present in OBs suspensions: i) benzalkonium chloride (alkylbenzyldimethylammonium chloride, 80%, Golden Bell, Mexico), ii) iodopovidone (10%, Laboratorios Jaloma SA de CV, Mexico), iii) colloidal silver (0.35%, Mercancías Salubles SA de CV, Mexico), iv) copper oxide nanopowder, <50 nm particle size (Sigma-Aldrich, USA), v) chlorhexidine digluconate (≥99.5%, 1,6-Bis(N5-[p-chlorophenyl]-N1-biguanido)hexane, Sigma-Aldrich, Switzerland), vi) methylisothiazolinone (2-methyl-4-isothiazolin-3-one, ≥95%, Sigma-Aldrich, Germany), vii) Triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol, >99%, Sigma-Aldrich, USA), viii) azadirachtin (3%, Biokrone

SA de CV, Mexico), ix) phthaldialdehyde solution (benzene-1,2-dicarbaldehyde, ≥99.9%, Sigma-Aldrich, USA), x) peracetic acid (39% in acetic acid, Sigma-Aldrich, USA).

The range of concentrations of each biocide tested was defined by reference to published literature that quantified their efficacy against Gram positive and Gram negative bacteria. Once a reference concentration had been identified, we performed preliminary tests involving 24 h treatment of newly-prepared OB suspensions with a ten-fold lower concentration and ten-fold to one hundred-fold higher concentrations of each biocide. If a biocide was found to be ineffective in preliminary tests, it was still included in the detailed tests in order to demonstrate its reduced efficacy compared to that of the other compounds tested.

Detailed screening involved the following procedure. A 500 µl volume of OB suspension was placed in a 1.5 ml microtube and mixed with the predetermined concentration of each biocidal compound. In all cases an identical sample of OB suspension without biocide was included as control. Microcentrifuge tubes were placed in an orbital shaker at 150 rpm and a temperature of 30 ± 1 °C for 24 h, after which each sample was diluted (1/10 and 1/100 in sterile water) and a 100 µl volume of each dilution was inoculated on LB agar (Broth Lennox, BD Bioxon Agar, Sigma Aldrich) in Petri plates. Sterile glass beads (5 mm diameter) were used to spread bacteria. Plates were incubated at 30 ± 1 °C for 24 h and the numbers of colony forming units were then counted. CFU counts that exceeded 100 colonies per plate were classified as >100 CFU. Three plates (replicates) were inoculated simultaneously for each sample.

2.5. Biocide effects on microbial contaminants and insecticidal activity of OBs

Three biocides that significantly reduced the microbial load in the initial screening assay were tested to evaluate their influence on the insecticidal activity of OBs. For this, the three biocides were each mixed with triplicate samples of 500 µl of OB suspension (1×10^9 OBs) at the concentrations that proved most effective for suppression of microbial contaminants in the previous screening assay: 6000 mg/l benzalkonium chloride, 450 mg/l colloidal silver and 2000 mg/l chlorhexidine digluconate. Following treatment, OB + biocide suspensions were incubated at 30 ± 1 °C for 24 h in continuous agitation at 150 rpm and were then used for CFU counts and insect bioassays. The addition of 1% Tween 80 was used to overcome the aggregation of OBs in the benzalkonium chloride treatment.

Aerobic CFU counts on biocide-treated OB suspensions were evaluated following the methodology described in the initial screening process, using LB agar plates inoculated with 100 µl of OB suspension that had been diluted 10-fold and 100-fold. All dilutions were prepared and plated using triplicate samples. Plates were incubated at 30 ± 1 °C for 24 h and CFU counts were performed. Control OB suspensions without biocides were treated identically.

Biocide-treated OB suspensions were assayed in second instar *S. exigua* larvae inoculated by the droplet feeding technique (Hughes et al., 1986). Groups of 30 newly molted larvae were starved overnight and then allowed to feed on droplets of 10% (wt/vol) sucrose, 0.01% (vol/vol) fluorella blue food dye and a concentration of 6.2×10^3 OBs/ml. This OB concentration was previously determined to result in 50% mortality for this virus strain. A similar group of control larvae were treated identically but fed on a solution of sucrose and food dye alone. Groups of 24 larvae that ingested the suspension within 15 min were transferred individually to a 24 well tissue culture plate containing pieces of diet and reared at 24 ± 1 °C. Virus induced mortality was observed at seven days post-inoculation and confirmed by microscopic observation of OBs. The bioassay was performed on three occasions for each batch of OB suspension.

2.6. Effects of biocide treatment on OB suspensions stored at two temperatures

Two effective biocides, benzalkonium chloride and colloidal silver, were selected to evaluate their effects on microbial contaminants and insecticidal activity of OBs suspension stored for six months at refrigerated or ambient temperatures. Chlorhexidine digluconate was excluded from this test due the high cost of this substance. For benzalkonium chloride and colloidal silver treatments, a new batch of OBs was produced with a concentration of 1.05×10^9 OBs/ml, as described in the section on production of experimental batches of OBs. Before biocide treatment, the microbial load (CFU/ml) and insecticidal activity in second instars were evaluated in triplicate samples following the methodology described in section 2.5. Two samples of 10 ml were treated with biocide, one with 6000 mg/l benzalkonium chloride and the other with 450 mg/l colloidal silver. A third sample of 10 ml was used as an untreated control. The microbial load and insecticidal activity of these three 10 ml samples were again incubated at 30 ± 1 °C for 24 h after treatment. Each OB suspension was then divided into two 5 ml samples that were stored at 4 ± 1.3 °C in a laboratory refrigerator or 25 ± 1 °C in a laboratory incubator. Microbial contaminants and insecticidal activity were evaluated in each 5 ml sample after 80 and 180 days. The number of OBs in each sample was counted in triplicate using a Neubauer Improved chamber using a phase contrast microscope at the beginning and end of the experiment.

2.7. Statistical analysis

Larval mortality in controls and biocide treatments was compared by t-test for benzalkonium chloride and chlorhexidine digluconate and by Wilcoxon rank sum test with continuity correction for colloidal silver. CFU counts were also subjected to a Wilcoxon test for colloidal silver, but were zero in the other treatments that were not subjected to statistical analysis. The effects of biocide treatment, temperature and sample time on log CFU counts, log OB counts and percentage of larval mortality in bioassays were examined by three-way analysis of variance in an R-based package (Jamovi, 2019). The normality of

log-transformed and percentage mortality values was assessed by Shapiro–Wilk test. Treatment means were compared by Tukey's test ($P<0.05$).

3. Results

3.1. Metagenomics analysis of the bacterial community

After quality checks and OTU filtering, we obtained a total of 51,266 reads for batch 1, 48,321 reads for batch 2, and 46,514 reads for batch 3. The rarefaction curves based on Chao1 index confirmed that sequencing depth was sufficient (Supplementary Figure S1).

The richness of operational taxonomic units (OTUs), akin to bacterial molecular species, was 163, 156 and 183 for batches 1, 2 and 3, respectively, with a total of 214 OTUs across all batches. Bacteria present in the OBs suspensions mainly belonged to the phyla Proteobacteria (97.9% of sequences), specifically to the Gammaproteobacteria class, and in lesser proportion to Firmicutes (2.1%). The Firmicutes phylum was dominated by the Enterococcaceae family (1.6% of sequences) whilst the Proteobacteria phylum was dominated by the Pseudomonadaceae (78.4%) and Enterobacteriaceae (19.1%) families. The most prevalent organism by far was *Pseudomonas viridiflava* that represented 59.7 - 71.0% of all 16S rRNA gene sequences detected (Fig. 1). Other relatively abundant organisms were *Morganella morganii*, *Pseudomonas* spp. and *Providencia* spp. that each represented approximately 10% of the bacterial community. The other organisms were present at low levels (mostly <2% of sequences detected) (Fig. 1).

In terms of richness, 95 of the 214 identified OTUs were assigned to Pseudomonads, in particular to unidentified *Pseudomonas* spp. (43 OTUs identified in total), *P. viridiflava* (25 OTUs) and *P. veronii* (11 OTUs) (Fig. 2). In contrast, the highest richness of Enterobacteriales, a typically dominant bacterial order in the digestive tract of insects, was observed for *M. morganii* (32-34 OTUs identified in each batch of OBs), followed by unidentified Enterobacteriaceae, *Providencia* spp. and *Enterococcus haemoperoxidus* and *E. casseliflavus*.

3.2. Biocide effects on microbial contamination: initial screening

Of the ten biocides assessed, only 6000 mg/l benzalkonium chloride, 450 mg/l colloidal silver and 2000 mg/l chlorhexidine digluconate resulted in a marked reduction or complete elimination of the aerobic microbial load (Table 1). All three compounds showed similar efficacy at the dilutions of 1/10 and 1/100 with CFU counts of less than 1000 CFU/ml in all cases, i.e., <10 CFU in a 100 µl volume of the 1/100 dilution. In contrast, OB preparations treated with any of the seven remaining biocides retained counts of over 100 CFU per plate, so were not considered for additional experiments.

3.3. Biocide effects on microbial contaminants and insecticidal activity of OBs

The aerobic microbial load in OB preparations was quantified and observed to decrease from approximately 5×10^8 CFU/ml in control preparations to between zero and 10 CFU/ml in preparations treated with benzalkonium chloride, colloidal silver, or chlorhexidine digluconate, at the concentrations identified as effective in the screening assay (Table 2).

None of the biocides had a significant effect on the insecticidal activity of OBs after 24 h of incubation. In all cases, the prevalence of lethal polyhedrosis of second instars varied between 40.3 and 60.6% in control groups (Table 2), and was similar to the mortality (41.2 - 52.3%) of larvae that consumed OBs that had been treated with benzalkonium chloride ($t = 1.412$; df = 16; $P = 0.177$), colloidal silver (Wilcoxon $W = 53.5$; $P = 0.251$; N = 18), or chlorhexidine digluconate ($t = 0.913$; df = 16; $P = 0.375$).

3.4. Storage stability of OB preparations with selected biocides

3.4.1. Microbial contaminants

The mean (± SE) aerobic CFU count of OB preparations prior to treatment was 7.4 $(\pm 1.3) \times 10^8$ CFU/ml. Microbial load was significantly affected by biocidal treatment ($F = 3446.5$; df = 1, 36; $P < 0.001$), sample time ($F = 101.5$; df = 1, 36; $P < 0.001$), and incubation temperature ($F = 31.2$; df = 1, 36; $P < 0.001$). The microbial load of control OB preparations increased slightly after 24 h incubation at 30 °C (1.6×10^9 CFU/ml, shown as log $10^{9.193 \pm 0.059}$ in Fig. 3AB), and did not change significantly during 180 days of incubation at 4 °C, whereas at 25 °C microbial load decreased by approximately two logarithms during the same period (Fig. 3B). The benzalkonium chloride treatment reduced CFU counts to zero at all time points and was not included in the statistical analysis. The microbial load of colloidal silver-treated OB preparations was reduced to 2.5×10^3 CFU/ml (log $10^{3.399 \pm 0.059}$) at 24 h post-treatment and decreased by approximately one logarithm in the 4 °C treatment, compared to a complete elimination of microbial contaminants in the 25 °C treatment at the end of the 180 day incubation period (Fig. 3AB).

3.4.2. Insecticidal activity

Bioassays performed using *S. exigua* second instars that consumed an LC_{50} concentration of inoculum (1.05×10^9 OBs/ml), prior to biocide treatment of OB preparations, resulted in a mean (±SE) mortality of 47.2 ± 3.0%. Virus-induced mortality was significantly affected by biocidal treatment ($F = 13.473$; df = 2, 36; $P < 0.001$), and sample time ($F = 41.5$; df = 2, 36; $P < 0.001$), but not by incubation temperature ($F = 2.771$; df = 1, 36; $P = 0.105$). At 24 h post-treatment, the prevalence of mortality did not differ significantly in any of the biocide treatments or the control (Fig. 4AB). The control OB preparations retained insecticidal activity without significant decreases at 4 °C and 25 °C during the 180 day period. Benzalkonium-treated OBs were significantly less pathogenic

(18.9 ± 2.8% mortality) at 180 days post-treatment compared with the control treatment. Colloidal silver-treated OBs at 4 °C retained insecticidal activity similar to that of the control, but not at 25 °C at which larval mortality at 180 days was reduced to 26.4 ± 4.2% (Fig. 4AB).

3.4.3. Occlusion body counts

OB counts were performed at the start (24 h post-treatment) and following 180 days incubation (Fig. 5AB). OB counts were significantly affected by biocidal treatment (F = 88.0; df = 2, 24; P <0.001), sample time (F = 168.5; df = 1, 24; P <0.001), and incubation temperature (F = 8.136; df = 1, 24; P <0.009). OB counts in the control were unchanged following incubation at 4 °C, but were slightly reduced in the control at 25 °C (Fig. 5AB). OB counts were reduced by approximately half a logarithm (~65% reduction) in the benzalkonium chloride treatment at both temperatures after 180 days. This reduction was apparently due to the disappearance of OBs during the experiment. In contrast, OB counts remained unchanged in the colloidal silver treated preparations for the duration of the study.

4. Discussion

The bacterial composition of SeMNPV OB suspensions was characterized by analysis of 16S rRNA gene sequences. Three highly effective biocidal compounds were identified that controlled bacterial contaminants for the duration of the study. The insecticidal activity of biocide-treated OBs declined after 180 d incubation except in one treatment combination (colloidal silver, 4 °C).

Previous estimates of the abundance of aerobic microbial contaminants in OB suspensions have been in the region of 10^7 - 10^8 CFU/ml based on standard culturing methods (Podgwaite et al., 1983; Grzywacz et al., 1997; Lasa et al., 2008; Ruiz et al., 2015). Numbers of bacteria may increase rapidly after death as the larval cadaver becomes colonized (Grzywacz et al., 1997; Ramirez-Arias et al., 2019), so that the abundance of aerobic microbes measured in control OB suspensions in the present study (~5×10^8 CFU/ml) likely reflects the fact that virus-killed cadavers were collected after all insects had died from virus disease. All previous studies, without exception, have reported yeasts and molds as minor components in the microbiota of OB preparations, typically in the range 10^3 - 10^5 CFU/ml (Grzywacz et al., 1997; Lasa et al., 2008; Ruiz et al., 2015). For this reason we focused on the dominant bacterial components of OB suspensions for the 16S rRNA-based analyses, rather than fungal components that would have required analysis of 18S rRNA gene sequences.

Contaminants are considered a quality control issue as phytosanitary product registration authorities set limits on the quantity and type of microbial contaminants that are permitted in virus-based insecticides (Shapiro 1986; Jenkins and Grzywacz 2000;

Grzywacz and Moore 2017). Most of the microbial community present in OBs suspensions is likely to be derived from the gut microbiome that colonizes the larval cadaver as the tissues break down following death. The midgut of phytophagous lepidopteran larvae is highly alkaline (Berenbaum 1980) and represents a specific habitat for a community of microbes that are adapted to such conditions (Broderick et al., 2004). The composition of the lepidopteran gut community depends on multiple dietary, physiological and environmental factors, many of which interact. These include insect species (Phalnikar et al., 2018; Jones et al., 2019), development stage and niche along the digestive tract (Tang et al., 2012; Jones et al., 2019), the food plant species and associated microbial community (Broderick et al., 2004; Mason and Raffa 2014; Jones et al., 2019), plant defensive compounds (Mason et al., 2019), the soil microbiome in which the food plant grows (Hannula et al., 2019), the insect's immune response (Crava et al. 2015; Caccia et al., 2016), and the presence of parasites and pathogens (Caccia et al., 2016; Polenogova et al., 2019), including nucleopolyhedroviruses (Jakubowska et al., 2013).

Analysis of 16S rRNA gene sequences directly from OB suspensions provided a culture-independent method for characterizing the bacterial community. Overall, 97.9% of sequences were Gammaproteobacteria dominated by Pseudomonadaceae and Enterobacteriaceae families. The most prevalent organism, *Pseudomonas viridiflava*, represented two-thirds of all 16S rRNA gene sequences detected. Other relatively abundant organisms were *Morganella morganii*, *Pseudomonas* spp. and *Providencia* spp. (Fig. 2). Interestingly, *P. viridiflava* is a well-recognized pathogen of multiple plant species (Sarris et al., 2012) that has been isolated from lepidopteran larvae (Khoja et al., 2006) and the oral secretions of a beetle (Cardoza et al., 2006). Given its phytopathogenic status, it would be interesting to determine whether *P. viridiflava* is a common component of OB suspensions used to produce biological insecticides. Other pseudomonads have been reported from the larval cuticle (Tang et al., 2012) and gut (Shao et al., 2014) of *Spodoptera littoralis*, whereas *Morganella morganii* is a normally harmless commensal species present in the gut of humans and other vertebrates, although it may cause opportunistic clinical infections on occasions (Liu et al., 2016). This organism has been isolated from the dipteran larval gut (Jeon et al., 2011) and as a lethal pathogen of mass-reared fruit flies (Salas et al., 2017). The Gram-positive Firmicutes phylum represented just 2.1% of 16S rRNA gene sequences and was dominated by the Enterococcaceae family. Indeed, *Enterococcus* spp. have been reported to be abundant and constant members of the gut community in *Lymantria dispar*, *Manduca sexta* and *S. littoralis* larvae (Broderick et al., 2004; Brinkmann et al., 2008; Teh et al., 2016), and from other insect orders (Cox and Gilmore 2007; Lehman et al., 2009). We detected no major human pathogens, such as species of *Salmonella*, *Shigella*, *Vibrio*, or occasional pathogens such as *Bacillus cereus* or *Staphylococcus aureus*, a common inhabitant of human skin. In contrast, previous studies have emphasized the abundance of *Enterococcus* spp., Enterobacteriaceae and *Bacillus* spp. (Grzywacz et al., 1997), but are in

agreement with our findings in reporting very low levels of *S. aureus*, and an absence of *Salmonella*, *Shigella*, and *Vibrio* species (Grzywacz et al., 1997; Lasa et al., 2008). We presume that the principal differences among these studies are derived from the different origins of the insect colonies, experimental virus inocula used and the marked differences in bacterial identification techniques, based on conventional culture methods and biochemical reactions (Grzywacz et al., 1997; Lasa et al., 2008), compared to our direct molecular approach that would not necessarily be expected to reflect closely the community composition determined from the culture-based methods reported previously.

The bacterial community composition is also likely to reflect the addition of antibiotics and bacteriostatic substances to reduce the prevalence of opportunistic bacterial pathogens and spoilage of the semi-synthetic diets used to rear larvae. The diet used in the present study contained sorbate, methyl paraben and formaldehyde to control microbial contaminants (Supplemental material, Table S1), whereas that of Lasa et al. (2008) contained sorbate, methyl paraben, and two antibiotics (streptomycin, tetracycline), and that of Grzywacz et al. (1997) contained only methyl paraben (McKinley et al., 1984).

Of the ten biocidal products screened, three stood out in terms of their antimicrobial activity across a range of concentrations, namely colloidal silver, benzalkonium chloride and chlorhexidine diglutonate. These compounds differ in their mode of action. Colloidal silver binds to key functional groups of enzymes, particularly thiol groups, resulting in the disruption of the cell membrane, the release of potassium ions and cell death (Jung et al., 2008), whereas benzalkonium chloride is a cationic surfactant that compromises cellular permeability by dissociation of cell membrane lipid bilayers, resulting in leakage of cellular contents followed by the disruption of proteins and nucleic acids and cell death (Tezel and Pavlostathis 2015). Chlorhexidine digluconate appears to collapse the bacterial cell membrane causing leakage of low molecular weight cell components and rapid death (Cheung et al., 2012). However, we did not evaluate this compound in detail due to its elevated cost. To treat one liter of OB suspension with chlorhexidine digluconate would cost approximately US$14.20 compared to US$2.28 for treatment with colloidal silver and US$0.24 for treatment with benzalkonium chloride (Mexican peso - US dollar exchange rate was 22.5:1 at time of publication). The concentrations of benzalkonium chloride (6000 mg/l) and colloidal silver (450 mg/l) that we used were over ten-fold higher than the concentrations at which these compounds are typically used (Tien et al., 2008; Tezel and Pavlostathis 2015). The present study therefore represents a stringent, worst-case test of biocide compound effects on OB activity and stability over an extended period of incubation. Future studies should focus on their efficacy at lower concentrations.

Both benzalkonium chloride and colloidal silver had immediate antimicrobial activity that was sustained over the 180 day incubation period, with zero CFU counts in the benzalkonium chloride treatment and decreasing counts in the colloidal silver treatment, irrespective of temperature (Fig. 3). Control OB suspensions did not show a significant

reduction in insecticidal activity during the 180 day period at either temperature, whereas the biocide-treated OBs were as active as the control suspensions up to 80 days, but resulted in significantly reduced mortality in the 180 day samples, except for the colloidal silver treatment at 4 °C (Fig. 4).

The clumping of OBs in the benzalkonium chloride treatment was an issue that may have significantly influenced the infectivity of OBs. We overcame this issue by adding Tween 80 to the OB suspension prior to bioassays and for accurate OB counting. OB counts after 180 days of incubation were reduced by approximately 65% (half a logarithm) in benzalkonium chloride treated suspensions at both 4 °C and 25 °C, compared to a slight reduction in the control at 25 °C and no significant changes in the colloidal silver treatment (Fig. 5). The reduction in insecticidal activity of benzalkonium-treated OBs appears to be a direct result of the presence of fewer OBs in treated OB suspensions. Benzalkonium chloride may have caused OB clumping through its interaction with the polyhedron envelope or OB-associated proteins. In a previous study we observed a loss of insecticidal activity, a ~35% reduction in OB counts and a break down of viral genomes in SeMNPV OB preparations stored at 25 °C for 18 months, whereas insecticidal activity was fully retained under refrigerated and frozen storage conditions (Lasa et al., 2008).

The reasons why OBs were lost in the benzalkonium chloride treatment are unclear, although we postulate that the stability of the polyhedrin matrix may have been reduced by exposure to this compound, which was mildly alkaline (pH 8.8) at the concentration tested, and the presence of chloride anions that facilitate the dissolution of polyhedrin in alkaline solution (Whitt & Manning, 1987). In contrast, in the control, the loss of OBs at 25 °C may have been due to degradation by the alkaline protease enzyme present in OBs produced in insects (Wood, 1980; Boogaard et al., 2017), an enzymatic process that is likely to have been slowed or halted at 4°C. In the colloidal silver treatment the OB-associated alkaline protease may have been inactivated as this biocide attacks key functional groups of enzymes (Jung et al., 2008), so that silver-treated OBs remained intact for the duration of the study. Clearly these hypotheses can only be validated through further study.

Previous attempts to control microbial contaminants have focused on clean practices during rearing of infected larvae, harvesting infected insects prior to or immediately following death and the use of bacteriostatic agents such as sorbate and benzoate salts or glycerol in OB formulations (Behle and Birthisel 2014; Grzywacz and Moore 2017). An additional, but largely overlooked factor, is the presence of contaminants in the OB inoculum used to infect larvae, which can markedly affect the abundance and community composition of contaminant microorganisms that subsequently colonize virus-killed insects (G. Bouwer, pers. comm.).

In conclusion, it was clear that including high concentrations of benzalkonium chloride or colloidal silver in OB suspensions dramatically reduced the abundance of contaminant microorganisms, but did not improve the retention of insecticidal activity of

OBs in storage compared to that of the control suspensions, which had not lost activity after 180 days. Indeed, the results of the present study are consistent with prior assertions that microbial contaminants are not detrimental to the stability of mature OBs for periods of months (Grzywacz et al., 1997; Lasa et al., 2008), although immature OBs lacking a polyhedron envelope may be more susceptible to microbial degradation (Ramirez-Arias et al., 2019). However, both biocidal compounds reduced microbial contaminants for at least 180 days and may find applications as adjuvants in OB formulations, or biocide coatings inside bioinsecticide product containers. The use of lower concentrations of the biocides should reduce costs and might reduce the prevalence of OB aggregation observed in benzalkonium chloride-treated suspensions.

Acknowledgements

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Appendix A. Supplemental data

Supplementary data to this article can be found in: Table S1, Fig. S1

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Table 1. Colony forming units (CFU) counts in plates inoculated with OB suspensions alone or previously treated with different biocide and concentrations. OB suspensions were diluted 1/10 or 1/100 prior to plating.

Biocide	**Concentration (mg/l)**	**CFU/plate**	
		1/10	1/100
Benzalkonium chloride	Control	>100	>100
	6	>100	>100
	60	>100	>100
	600	>100	>100
	6000	0	0*
Iodopovidone	Control	>100	>100
	6.2	>100	>100
	62	>100	>100
	620	>100	>100
	6200	>100	>100
Colloidal silver	Control	>100	>100
	0.45	>100	>100
	4.5	>100	>100
	45	>100	>100
	450	6	1*
Copper oxide	Control	>100	>100
	1.6	>100	>100
	16	>100	>100
	160	>100	>100
	1600	>100	>100
Chlorhexidine digluconate	Control	>100	>100
	2	>100	>100
	20	>100	>100
	200	>100	>100
	2000	0	0*
Methylisothiazolinone	Control	>100	>100
	5	>100	>100
	50	>100	>100
	500	>100	>100
	5000	>100	>100
Triclosan	Control	>100	>100
	3	>100	>100
	30	>100	>100
	300	>100	>100
	3000	>100	>100
Azadirachtin	Control	>100	>100
	15	>100	>100

	150	>100	>100
	1500	>100	>100
	15000	>100	>100
Phthaldialdehyde	Control	>100	>100
	5	>100	>100
	50	>100	>100
	500	>100	>100
	5000	>100	>100
Peracetic acid	Control	>100	>100
	6	>100	>100
	60	>100	>100
	600	>100	>100
	6000	>100	>100

*Reduction of aerobic microorganisms to less than 1 x 10^3 CFU/ml based on a sampled volume of 100 µl diluted 1/100 prior to plating onto agar.

Table 2. Microbial load in colony forming units (CFU)/ml and percentage of larval mortality for control (OBs alone) and OB preparations treated with biocides for 24 h.

Biocide	Concentration (mg/l)	Microbial load ± SE (CFU/ml)		Mortality ± SE (%)	
		OBs alone	OBs + biocide	OBs alone	OBs + biocide
Benzalkonium chloride	6000	5.2 (±1.4)×10^8	0 ± 0	60.6 ± 3.1a	52.3 ± 5.0a
Colloidal silver	450	4.8 (±2.2)×10^8a	10.0 ± 3.3b	44.0 ± 2.1a	41.2 ± 3.1a
Chlorhexidine	2000	5.1 (±3.7)×10^8	0 ± 0	40.3 ± 2.7a	43.5 ± 2.3a

Values followed by different letters differ significantly for pairwise comparisons of treatment and controls, Wilcoxon test on CFU values (*P* <0.05, colloidal silver) or t-test on mortality values (P<0.05). Statistical analyses were not performed for microbial loads with zero values, i.e., benzalkonium chloride and chlorhexidine digluconate.

Figure legends

Fig. 1. Relative abundance of operational taxonomic units (OTUs) of bacteria detected by 16S rRNA gene sequence analysis on three batches of SeMNPV OB suspensions. Values next to bars indicate percentage values.

Fig. 2. Numbers of operational taxonomic units (OTUs) of bacteria detected by 16S rRNA gene sequence analysis on three batches of SeMNPV OB suspensions.

Fig. 3. Logarithm of counts of colony forming units (mean CFU ±SE) in control and colloidal silver-treated OB suspensions following 24 h, 80 d, or 180 d incubation at (A) 4 °C or (B) 25 °C. Columns headed by identical letters did not differ significantly for comparisons of biocide treatment (upper case) and incubation period (lower case) (ANOVA, Tukey $P > 0.05$). CFU counts were zero in all benzalkonium chloride-treated samples (values not shown)

Fig. 4. Percentage of mortality (mean±SE) in larvae that consumed control and biocide-treated OB suspensions that had been subjected to 24 h, 80 d, or 180 d incubation at (A) 4 °C or (B) 25 °C. Columns headed by identical letters did not differ significantly for comparisons of biocide treatments (upper case) and incubation period (lower case) (ANOVA, Tukey $P > 0.05$).

Fig. 5. Logarithm of virus occlusion body counts (mean±SE) in control and biocide-treated OB suspensions following 24 h or 180 d incubation at (A) 4 °C or (B) 25 °C. Columns headed by identical letters did not differ significantly for comparisons of biocide treatments (upper case) and incubation period (lower case) (ANOVA, Tukey $P > 0.05$).

Fig 1.

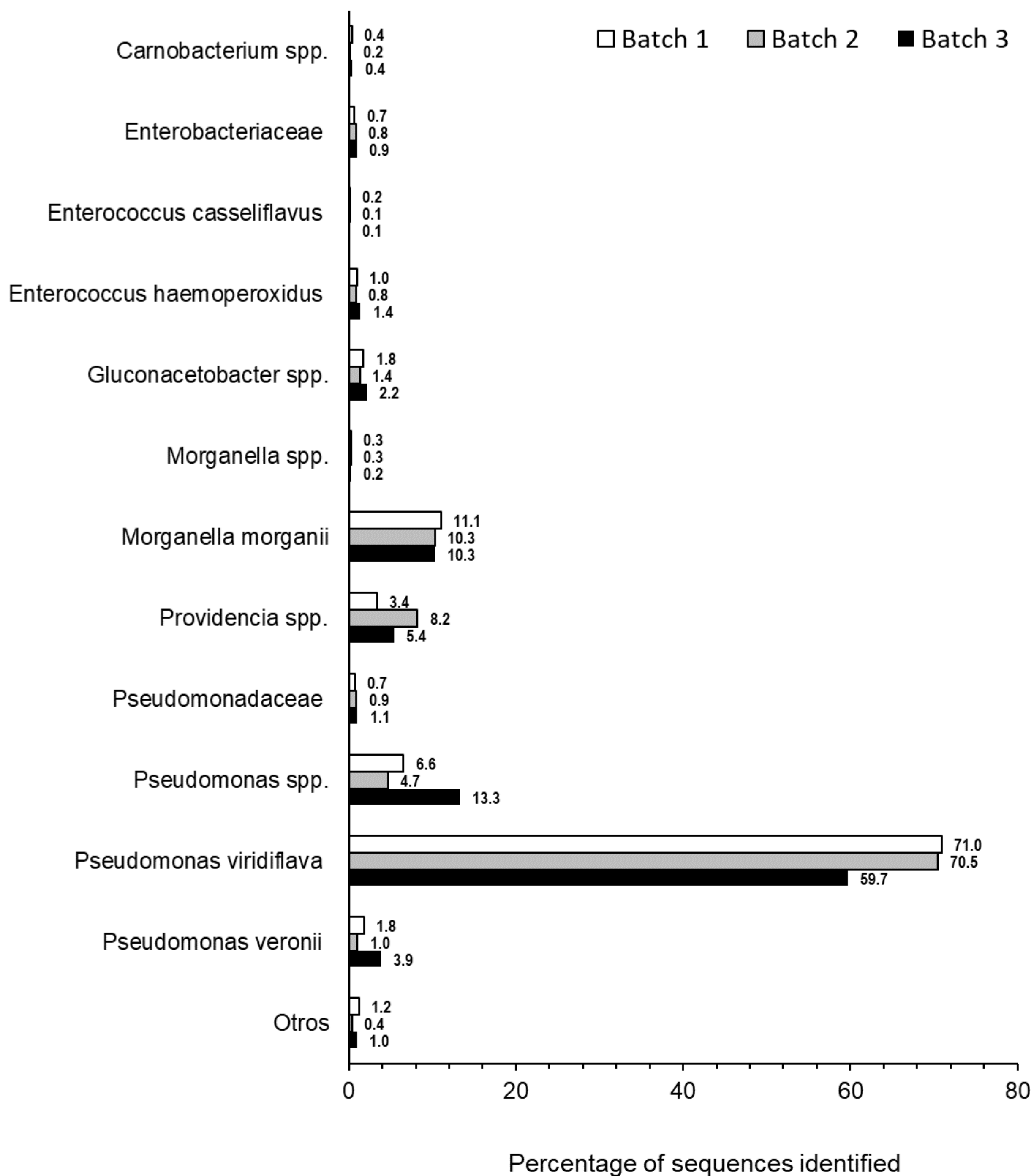


Fig. 2

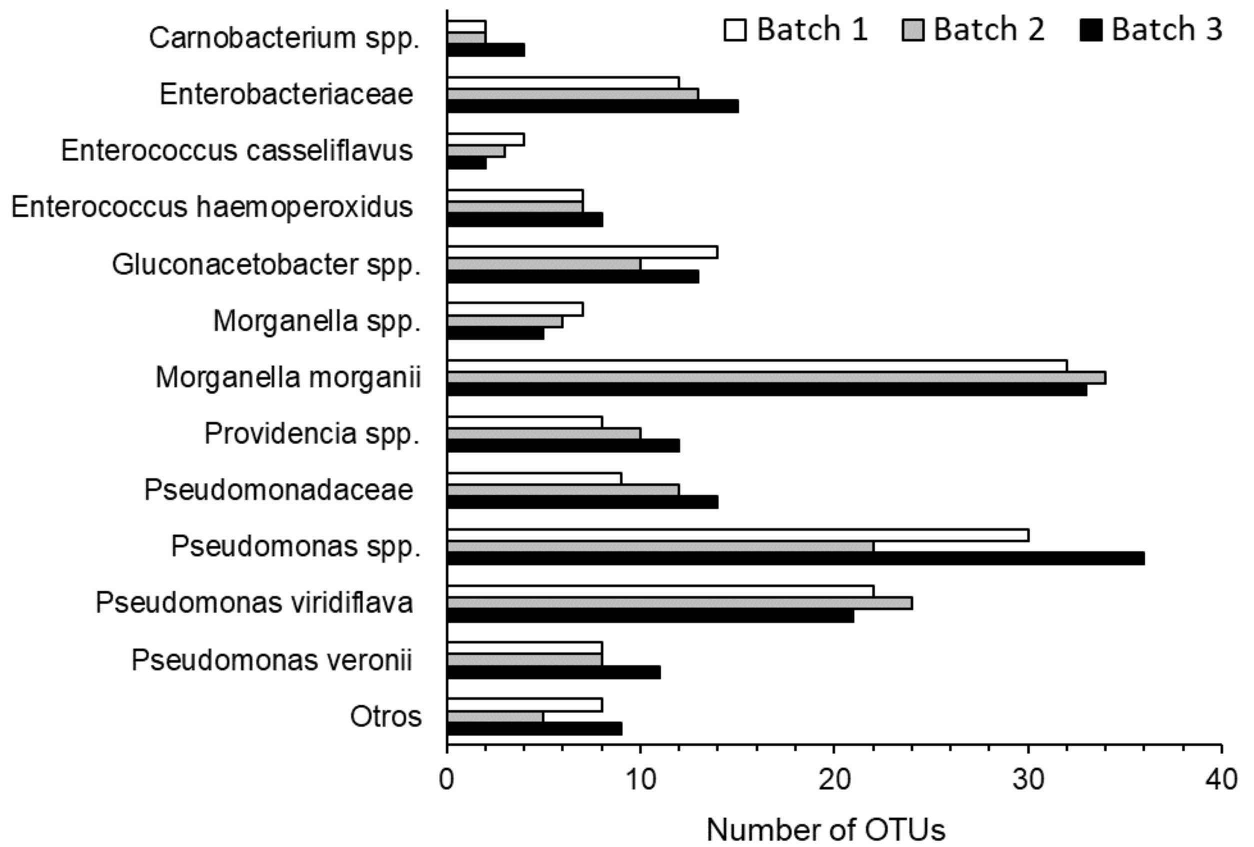
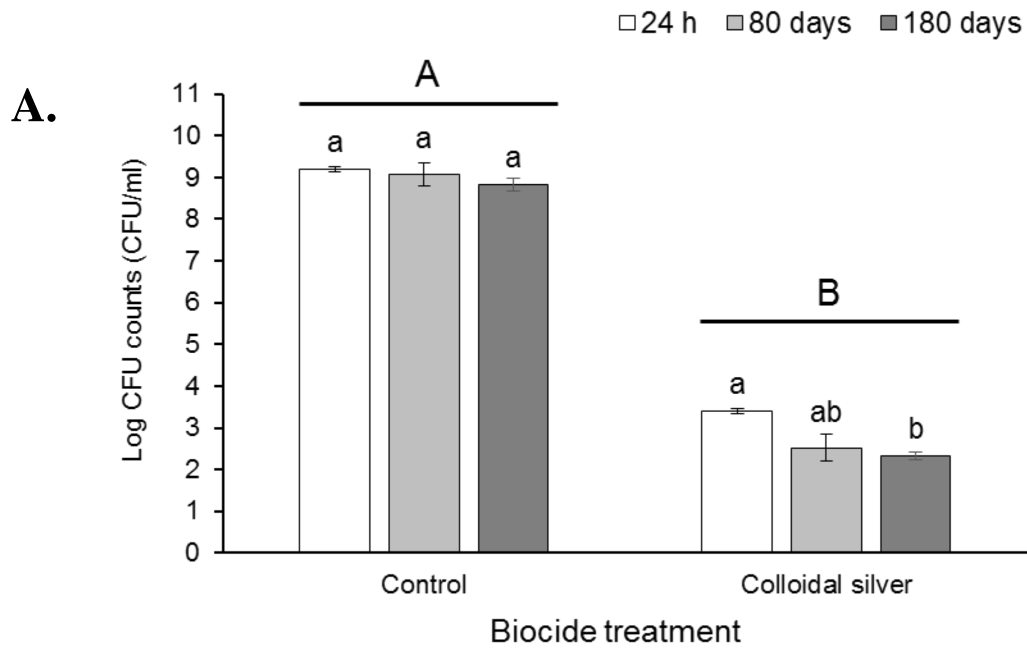


Fig. 3

A.



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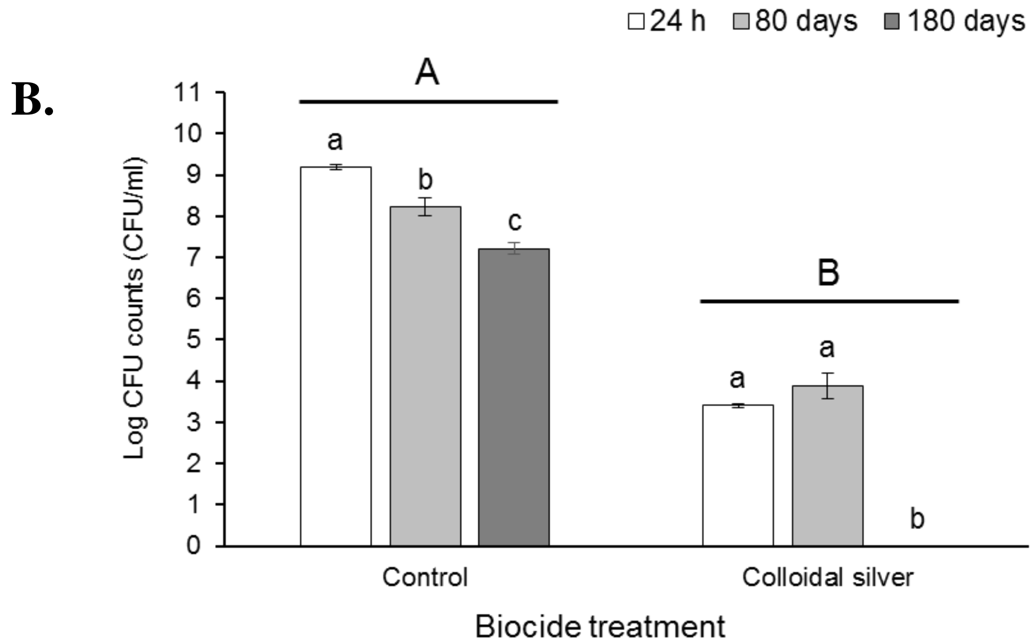
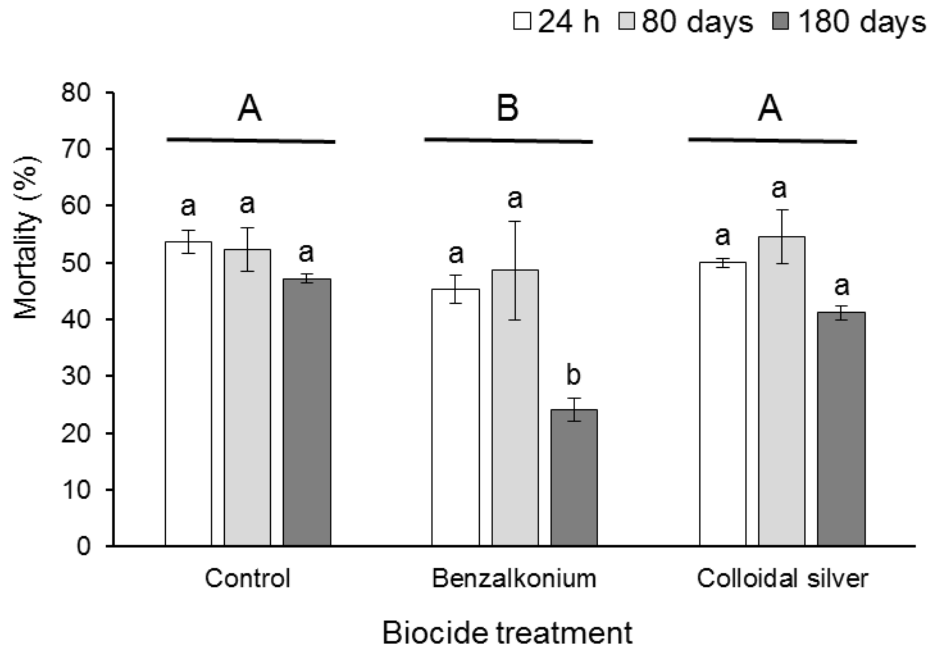


Fig. 4

A.



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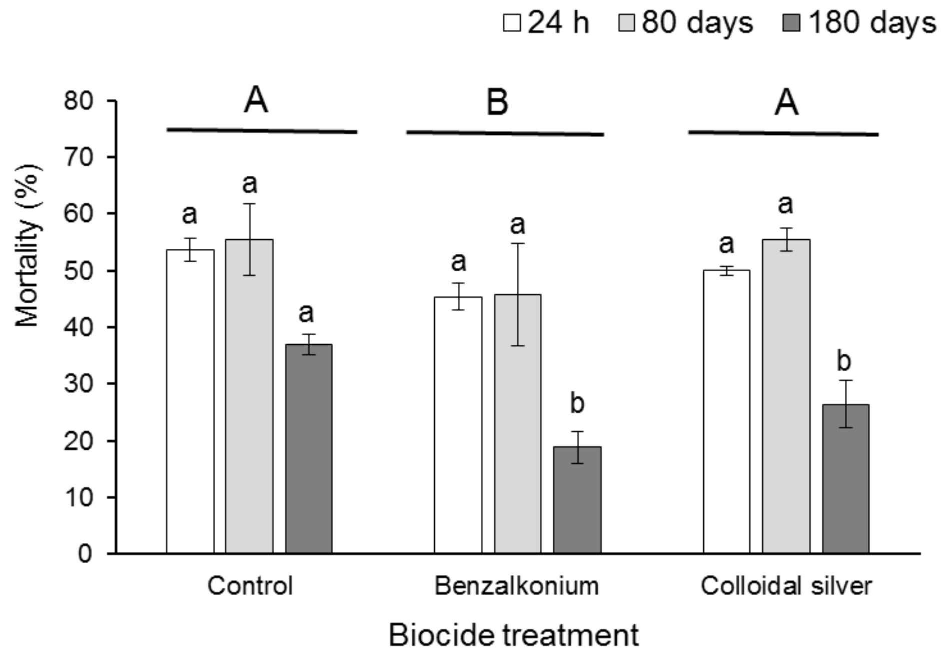
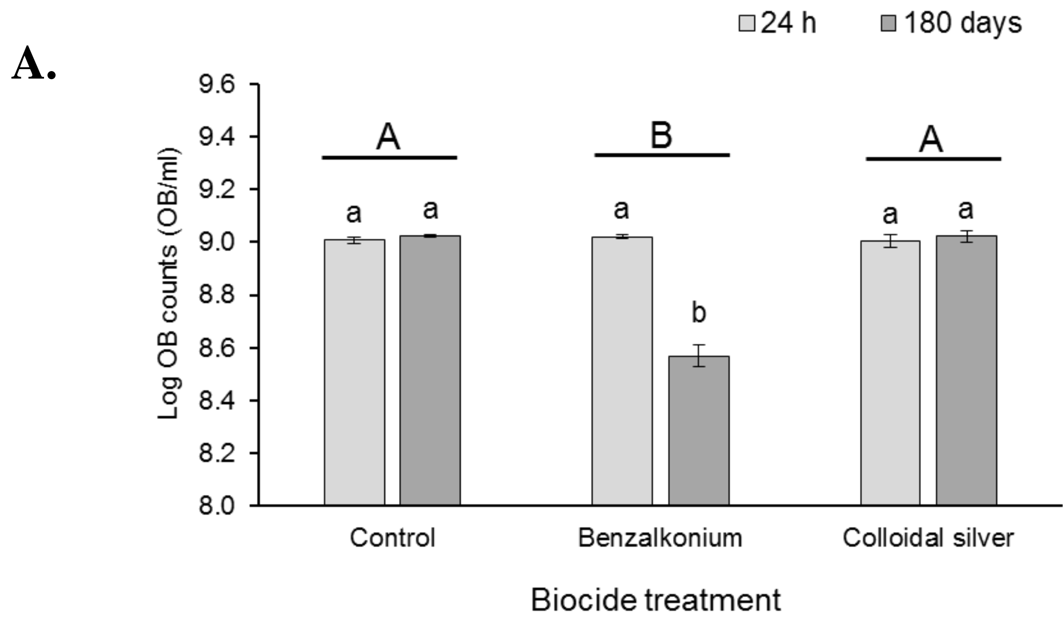


Fig. 5

A.



B.