sustainability

MDPI

Article

Assessment of Antifungal/Anti-Oomycete Activity of Frass Derived from Black Soldier Fly Larvae to Control Plant Pathogens in Horticulture: Involvement of *Bacillus velezensis*

Ghazaleh Arabzadeh [1], Maxime Delisle-Houde [2], Grant W. Vandenberg [1], Nicolas Derome [3], Marie-Hélène Deschamps [1], Martine Dorais [2], Antony T. Vincent [1] and Russell J. Tweddell [2,*]

[1] Département des Sciences Animales, Université Laval, Quebec, QC G1V 0A6, Canada; ghazaleh.arabzadeh.1@ulaval.ca (G.A.); grant.vandenberg@fsaa.ulaval.ca (G.W.V.); marie-helene.deschamps@fsaa.ulaval.ca (M.-H.D.); antony.vincent@fsaa.ulaval.ca (A.T.V.)
[2] Département de Phytologie, Université Laval, Quebec, QC G1V 0A6, Canada; maxime.delisle-houde.1@ulaval.ca (M.D.-H.); martine.dorais@fsaa.ulaval.ca (M.D.)
[3] Département de Biologie, Université Laval, Quebec, QC G1V 0A6, Canada; nicolas.derome@bio.ulaval.ca
* Correspondence: russell.tweddell@fsaa.ulaval.ca

Abstract: Frass, the residual material resulting from the bioconversion of organic matter by black soldier fly larvae (BSFL), has gained attention as a sustainable alternative to conventional fertilizers due to its high nutrient content. Additionally, frass has been found to possess antifungal properties, which can help control plant pathogens affecting horticultural crops. In this study, frass from BSFL reared on the Gainesville diet, a universally employed reference/control diet, was investigated in vitro for its effect on the growth of seven important fungal/oomycete pathogens. Dual culture overlay assays clearly showed that fresh Gainesville diet extract, as well as BSFL frass extract derived from this diet, contained microorganisms producing compound(s) that strongly inhibit(s) the mycelial growth of fungal/oomycete plant pathogens. Fungi and bacteria were then isolated from the fresh Gainesville diet and BSFL frass using the serial dilution technique. Among the different fungi/bacteria isolated, the isolate GV1-11 in Gainesville diet and FGV15-6 in frass demonstrated strong antifungal/anti-oomycete activity. Both isolates were genetically identified by whole-genome sequencing as *Bacillus velezensis*, a bacterium used as a biocontrol agent, strongly suggesting that *B. velezensis*, which is present in the Gainesville diet, can survive the process of BSFL rearing and is one of the key factors contributing to the observed antifungal and anti-oomycete activity in the resulting frass. This work underlines the importance of the inherent microbial characteristics of feedstocks on the antifungal/anti-oomycete activity of frass and points out the possibility of exploiting frass to control plant pathogens affecting horticultural crops.

Keywords: frass; black soldier fly; *Hermetia illucens*; Gainesville diet; *Bacillus velezensis*; antifungal activity

check for updates

Citation: Arabzadeh, G.; Delisle-Houde, M.; Vandenberg, G.W.; Derome, N.; Deschamps, M.-H.; Dorais, M.; Vincent, A.T.; Tweddell, R.J. Assessment of Antifungal/Anti-Oomycete Activity of Frass Derived from Black Soldier Fly Larvae to Control Plant Pathogens in Horticulture: Involvement of *Bacillus velezensis*. *Sustainability* **2023**, *15*, 10957. https://doi.org/10.3390/su151410957

Academic Editor: Sean Clark

Received: 23 May 2023
Revised: 29 June 2023
Accepted: 7 July 2023
Published: 13 July 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Hermetia illucens L. (Diptera:Stratiomyidae), commonly known as black soldier fly, has recently attracted attention in the agrifood industry. The black soldier fly larvae (BSFL) can efficiently transform waste organic residues into valuable larval biomass, which could be used as a feed in poultry, aquaculture, and livestock diets [1,2]. The conversion of waste streams into high-quality feed ingredients by BSFL is an ideal approach for establishing a circular economy by upcycling low value organic residues [3,4]. It has been estimated that BSFL can transform 1000 kg of fruit and vegetable waste into about 125 kg of fresh larval biomass and about 250 kg of frass, the main secondary product resulting from this bioprocess [5]. Frass is composed of larval excrement, larval exuviae from molting events, and undigested food waste [6]. Considering its high nutrient content [7–10], its significant microbial diversity [11,12], and the large quantities generated [5], frass is currently poorly valorized, and its potential applications need to be further investigated.

Several studies have investigated the potential use of frass as biofertilizers and have reported that frass has a similar mineral composition (N-P-K) to other biofertilizers used for growing plants [7–10,13,14]. In a field-scale experiment conducted with cowpea plant (*Vigna unguiculata* (L.) Walp.), the use of BSFL frass as a biofertilizer was shown to significantly reduce the incidence of Fusarium wilt disease caused by *Fusarium oxysporum* [15]. The reduction of Fusarium wilt incidence was hypothetically associated with the presence of fragments of chitin remaining in frass biofertilizers, with chitin and chitosan (a linear polysaccharide resulting from the deacetylation of chitin) being shown to be potent elicitors of plant defense mechanisms [15]. A recent publication reported the antifungal activity of frass, more specifically, frass obtained from BSFL reared on the universally employed reference/control Gainesville diet, against *F. oxysporum* [7], suggesting that frass could suppress plant disease by exerting a direct effect on pathogenic fungi.

The objective of the work reported herein was to further study (1) the antifungal/anti-oomycete activity (antagonistic activity) of frass produced by BSFL reared on the Gainesville diet using plant pathogenic fungi and oomycetes, and (2) the mechanism behind the antifungal/anti-oomycete activity of BSFL frass.

2. Materials and Methods

2.1. Plant Pathogenic Fungi and Oomycetes

Fungi (*Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *F. oxysporum*, *Botrytis cinerea*, *Alternaria solani*) and oomycetes (*Pythium ultimum*, *Phytophthora capsici*) were supplied by the Laboratoire d'expertise et de diagnostic en phytoprotection (Ministère de l'Agriculture, des Pêcheries et de l'Alimentation (MAPAQ), Québec, QC, Canada). Fungi and oomycetes were grown on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) for 2 weeks at room temperature (22.5 °C). They were subcultured every two weeks.

2.2. Gainesville Diet

BSFL were reared on the reference laboratory Gainesville house fly diet, which consists of 50% wheat bran (Rudolph, Québec, QC, Canada), 30% alfalfa meal (La Coop, Lévis, QC, Canada), and 20% cornmeal (Shah Trading Company, Montréal, QC, Canada) [16], formulated with water added to 70% moisture level.

2.3. Frass

BSFL frass was produced in the Laboratoire de recherche en sciences aquatiques (LARSA) of Université Laval (Québec, QC, Canada). Cardboard containing eggs clutches was suspended above 160 g of Gainesville in containers (17.5 cm × 19 cm) covered with a double net for 24 h. The containers were incubated (27 °C, 70% relative humidity) at a photoperiod of 12:12 (L:D) for 4 days. Four-day old larvae were sieved, and 800 larvae were manually counted and transferred to each of three containers with 800 g of Gainesville diet. Containers were incubated at 27 °C, 70% relative humidity, and in complete darkness for one week.

2.4. Extracts of Frass/Gainesville Diet

In order to prepare the frass extract, frass (10 g) was added to physiological saline solution (100 mL; 0.5% NaCl) and placed under agitation (150 rev/min) at 27 °C for 60 min before being passed through 8 layers of sterile cheesecloth. Gainesville diet extract was prepared according to the same procedure.

2.5. Effect of Extracts on Plant Pathogen Mycelial Growth (Dual Culture Overlay Assay)

A first layer (20 mL) of PDA 10% supplemented with 13.5 g/L of CRITERION™ Agar (Hardy Diagnostics, Santa Maria, CA, USA) containing different concentrations of frass (0% (control), 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, and 1% filtered (0.22 µm)) or Gainesville diet (0% (control), 0.001%, and 1%) extracts was poured into petri dishes. Extracts were added once the PDA had been cooled to 48 °C. Petri dishes were then

incubated at room temperature in darkness for 48 h and then covered with 10 mL of PDA 10% supplemented with 13.5 g/L of CRITERION™ Agar (second layer) and incubated at 4 °C for 24 h in darkness. A PDA plug (10 mm diameter) of actively growing mycelium of each plant pathogen was deposited on the second layer (in the center). After 7 days of incubation (in darkness) at room temperature, the mycelium radial growth of each phytopathogen was measured with a ruler and expressed as the percent inhibition of radial growth according to the following equation: {[mycelium radial growth (control) − mycelium radial growth (extract treatment)]/mycelium radial growth (control)} × 100. Each treatment was carried out in triplicate petri dishes.

2.6. Isolation of Bacteria and Fungi from Gainesville Diet and Frass Extracts

Gainesville diet and frass extracts were separately serial diluted and 100 µL of each dilution (1×10^{-1} to 1×10^{-7}) was spread on tryptic soy agar (TSA; Becton, Dickinson and Company) for bacteria and on PDA for fungi. The petri plates were incubated at 27 °C for 24 h (TSA) or at room temperature for 72 h (PDA). Bacterial and fungal colonies with distinct morphological characteristics were subcultured two times on TSA (bacteria) or PDA (fungi) using the streaked technique to obtain pure cultures of each isolate. Bacterial isolates were cultivated overnight in tryptic soy broth (TSB; Becton, Dickinson and Company) under agitation (150 rev/min) at 27 °C and preserved at −85 °C in 15% *w*/*v* glycerol (VWR International, West Chester, PA, USA). Fungal isolates were preserved on PDA at 4 °C.

2.7. Screening Isolates for Antagonistic Activity against Plant Pathogens (Dual Culture on Agar)

PDA plugs (10 mm diameter) of actively growing mycelium of each fungal isolate and each plant pathogen were placed face to face (separated by a space of 30 mm) on PDA in triplicate and incubated for 7 days at room temperature. The presence or absence of an inhibition zone between the fungal isolate and each plant pathogen was then evaluated visually.

Bacterial isolates were individually cultivated overnight in TSB as previously described. For each isolate, 100 µL of the bacterial culture was spread on one side of PDA and a PDA plug (10 mm diameter) of actively growing mycelium of each plant pathogen was placed on the other side. After 7 days of incubation (at room temperature), the presence or absence of an inhibition zone between the bacterial isolate and each plant pathogen was evaluated visually. Each isolate was tested in triplicate for each plant pathogen.

2.8. Identification of Isolates GV1-11 and FGV15-6

2.8.1. DNA Extraction and Whole Genome Sequencing

Genomic DNA was extracted from a TSB overnight culture of isolates using the QIAGEN DNeasy Blood and Tissues kit (Qiagen Inc., Montréal, QC, Canada) as indicated in the manufacturer's protocol for Gram-positive bacteria. DNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation and sequencing were performed at the plateforme d'analyses génomiques of the Institut de biologie intégrative et des systèmes (IBIS; Université Laval). Briefly, Genomic DNA (500 ng in 55 µL) was mechanically fragmented for 40 sec using a Covaris M220 ultrasonicator (Covaris, Woburn, MA, USA) using default settings. Fragmented DNA was transferred to a PCR tube and library synthesis was performed with the NEB Next Ultra II (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions. A TruSeq HT adapter (Illumina, San Diego, CA, USA) was used to barcode the library, which was then sequenced in 4% of an Illumina MiSeq 300 base pair paired-end run (600 cycle, v3 kit, Illumina, San Diego, CA, USA).

2.8.2. Genomic Sequence Analysis

The de novo assembly of the paired-end reads was conducted using the Shovill tool in the online Galaxy platform [17], which is a pipeline for Illumina paired end reads based around SPAdes [18]. Species identification was performed using the type strain genome server (TYGS) (https://tygs.dsmz.de/; accessed on 19 April 2023) for a whole genome-

based taxonomic analysis [19]. To determine the closest strain genomes to the GV1-11 strain, two complementary approaches were employed. Firstly, the MASH algorithm, which is a fast approximation of intergenomic relatedness was used for the comparison of the GV1-11 genome with all of the strain genomes that are available in the TYGS database [20], to select the ten strains with the smallest MASH distances. A second approach was based on the 16S rDNA gene sequences determined using an additional set of ten closely related type strains. These were extracted from the GV1-11 genome using RNAmmer [21], and each sequence was subsequently compared using BLAST [22] against the 16S rDNA gene sequences available in the TYGS database, which currently consist of 18,893 type strains. This was used as a proxy to find the best 50 matching type strains (according to the bitscore) to the GV1-11 genome. The Genome BLAST Distance Phylogeny approach (GBDP), with the 'coverage' algorithm and distance formula d5, was subsequently used to calculate precise distances [23], which were finally employed to determine the 10 closest type strain genomes to the GV1-11 genome. The determination of closely related type strains to FGV15-6 was done based on the provided genome data and the manually selected type strains. For both isolates, GBDP and accurate intergenomic distances, as inferred by the 'trimming' algorithm and distance formula d5, were used for all pairwise comparisons among the set of genomes, on the basis of which the phylogenomic inferences were derived [23]. One hundred distance replicates were calculated each. Using the recommended settings, GGDC 3.0 was used to calculate digital DNA–DNA hybridization (DDH) values and confidence intervals [23,24]. The subsequent intergenomic distances were employed to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1, including SPR postprocessing [25]. One hundred pseudo-bootstrap replicates each inferred the branch support. The trees were rooted at the midpoint [26] and visualized with PhyD3 [27]. A 70% dDDH radius around each of the 13 type strains for GV1-11 and 10 type strains for FGV15-6 was used as previously described to perform the type-based species clustering [19], and a 79% dDDH threshold was used as previously described to perform the subspecies clustering [28].

2.9. Effect of Bacillus Velezensis on Plant Pathogen Mycelial Growth

The effect of *B. velezensis* on the mycelial growth of each plant pathogen was determined using dual culture overlay assay. The assay was conducted as described previously except that the first layer contained the bacterial isolate (1×10^7 CFU mL^{-1}) cultivated overnight at 27 °C in TSB under agitation (150 rev min^{-1}). The experiment was conducted with four replicates.

3. Results

3.1. Effect of Extracts on Plant Pathogen Mycelial Growth

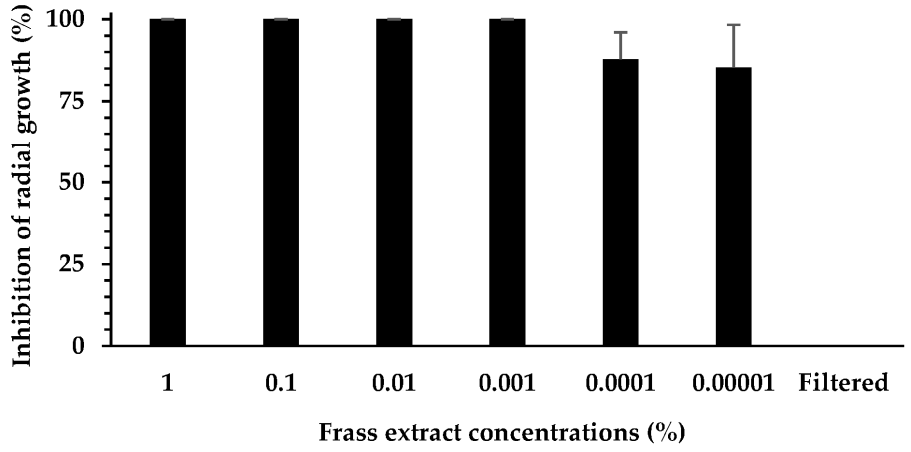
Frass extract caused a strong inhibition of mycelial growth for all plant pathogens at each concentration tested (Figure 1). A complete inhibition of mycelial growth was observed for *A. solani* and *P. ultimum* at a concentration of 1% (Figure 1c,g), for *P. capsici* and *F. oxysporum* at a concentration of 0.001% (Figure 1a,d), and for *S. sclerotiorum* at a concentration of 0.00001% (Figure 1e). Filtered frass extract caused a very weak mycelial growth inhibition (0.63% to 4.87%) or no mycelial growth inhibition (Figure 1).

Fresh Gainesville diet and frass extracts at both concentrations tested (0.001% and 1%) caused similar inhibition of mycelial growth for each plant pathogen (Figure 2a,b). Fresh Gainesville diet and frass extracts caused complete inhibition of mycelial growth for *S. sclerotiorum*, *P. capsici*, and *F. oxysporum* at both concentrations tested (Figure 2a,b) and for *P. ultimum* at a concentration of 1% (Figure 2a).

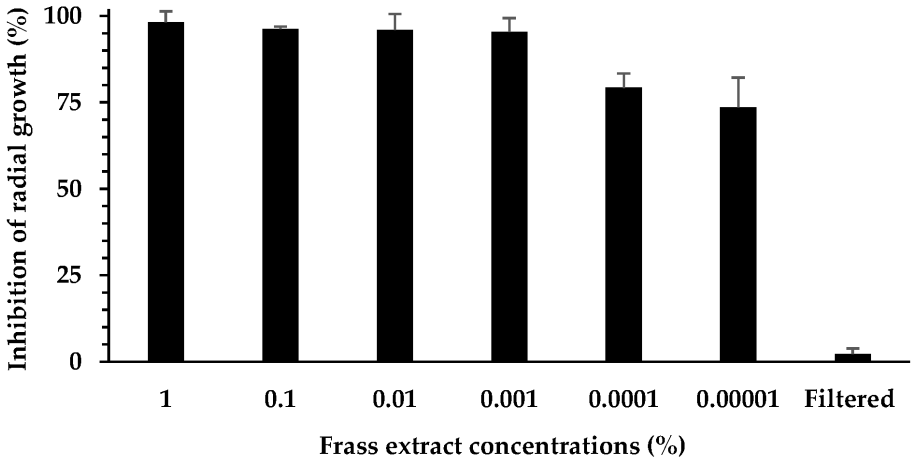
3.2. Screening Isolates for Antagonistic Activity against Plant Pathogens (Dual Culture on Agar)

Among the bacterial and fungal isolates (showing distinct morphological characteristics on agar) obtained from fresh Gainesville diet and frass, only isolate GV1-11 in Gainesville diet and isolate FGV15-6 in frass caused an inhibition zone against all the plant pathogens in dual culture on agar.

(**a**)



(**b**)



(**c**)

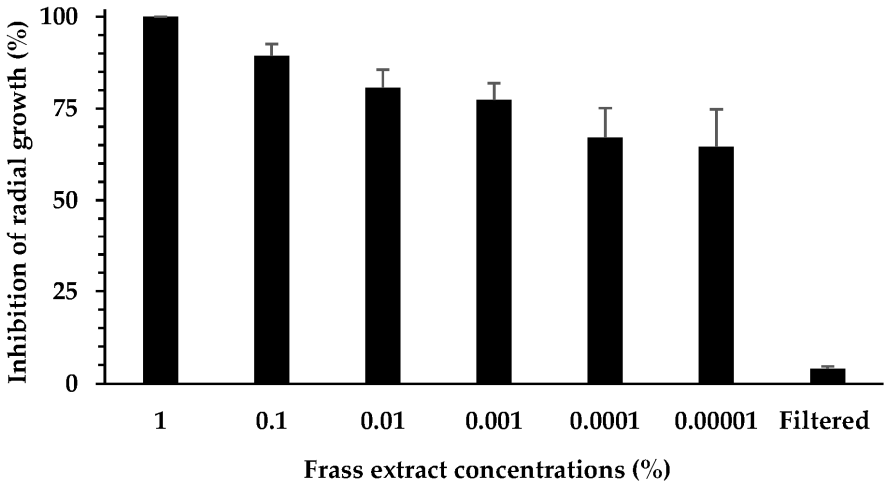
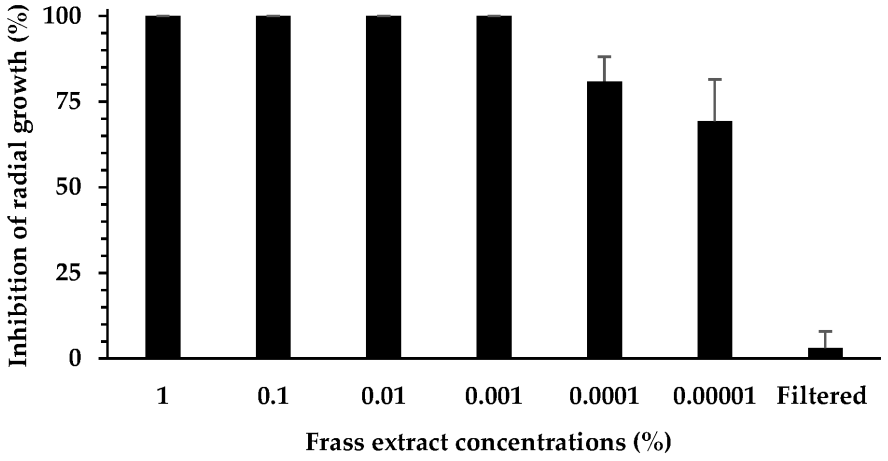
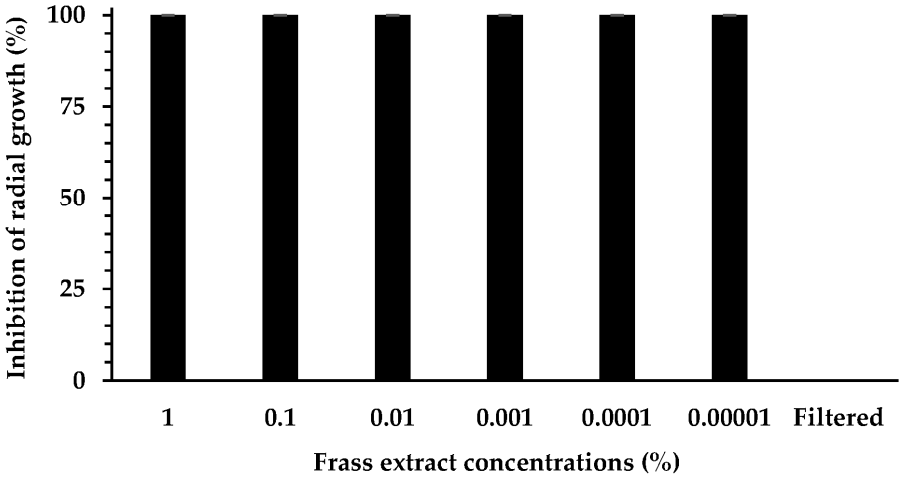


Figure 1. *Cont.*

(**d**)



(**e**)



(**f**)

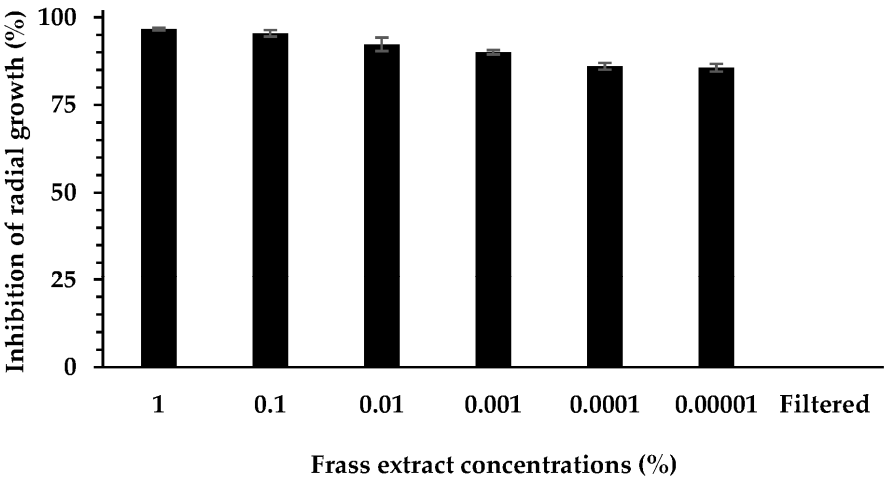


Figure 1. *Cont.*

(**g**)

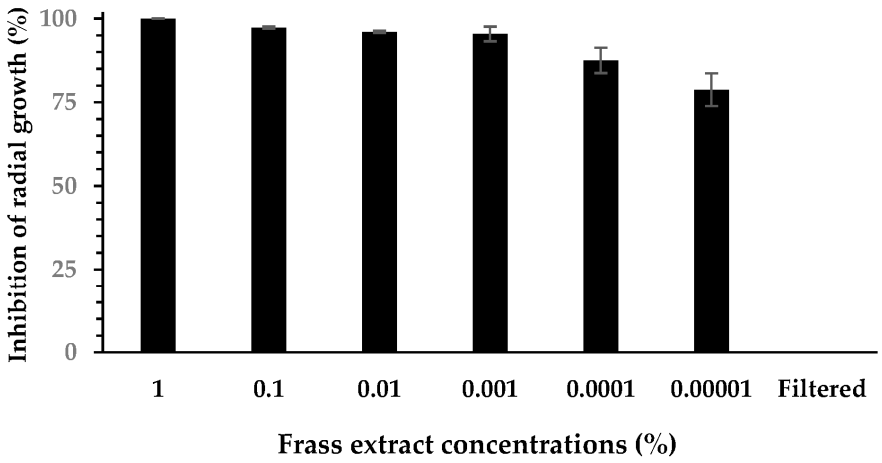


Figure 1. Effect of filtered (0.22 µm) frass extract (1%) and non-filtered frass extract at different concentrations on the mycelial growth of *Phytophthora capsici* (**a**), *Botrytis cinerea* (**b**), *Alternaria solani* (**c**), *Fusarium oxysporum* (**d**), *Sclerotinia sclerotiorum* (**e**), *Rhizoctonia solani* (**f**), and *Pythium ultimum* (**g**) using dual culture overlay assay. Each value represents the mean of three replicates ± standard deviation.

3.3. Identification of Isolates

Isolates GV1-11 and FGV15-6 showed some of the characteristics of bacteria from the *Bacillus* genus, i.e., beige and white-cream colonies when cultivated on TSA and rod shapes when observed under light microscopy. Based on the results of digital DNA–DNA hybridization analysis, the clustering yielded 10 (GV1-11) and 7 species clusters (FGV15-6), and the novel organism revealed a maximum identity similarity of 88.2% (GV1-11) and 87.8% (FGV15-6) with *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 (Figure 3 and Table 1), which was recently reclassified as *B. velezensis* [29]. Moreover, isolates GV1-11 and FGV15-6 were located in 1 of 10 and 1 of 7 subspecies clusters, respectively. The type strain genome server (TYGS) predicted that both strains belong to known species *B. velezensis*.

(**a**)

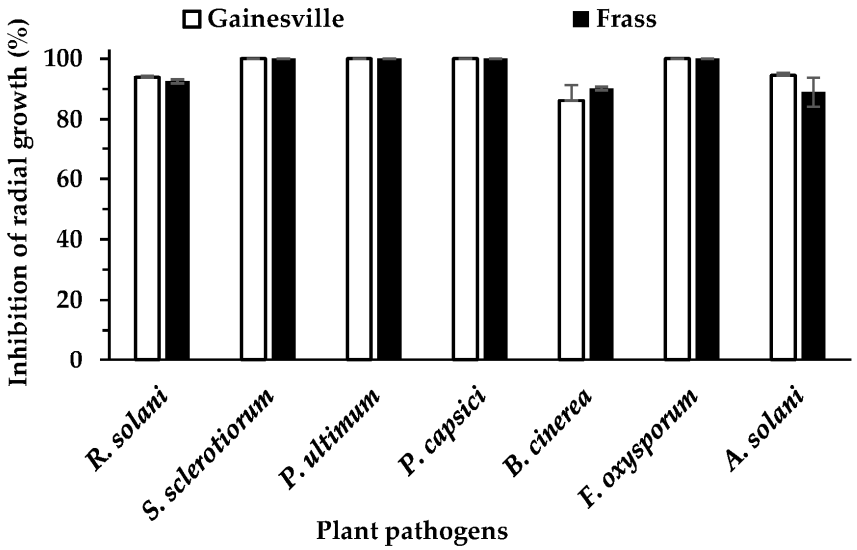


Figure 2. *Cont.*

(**b**)

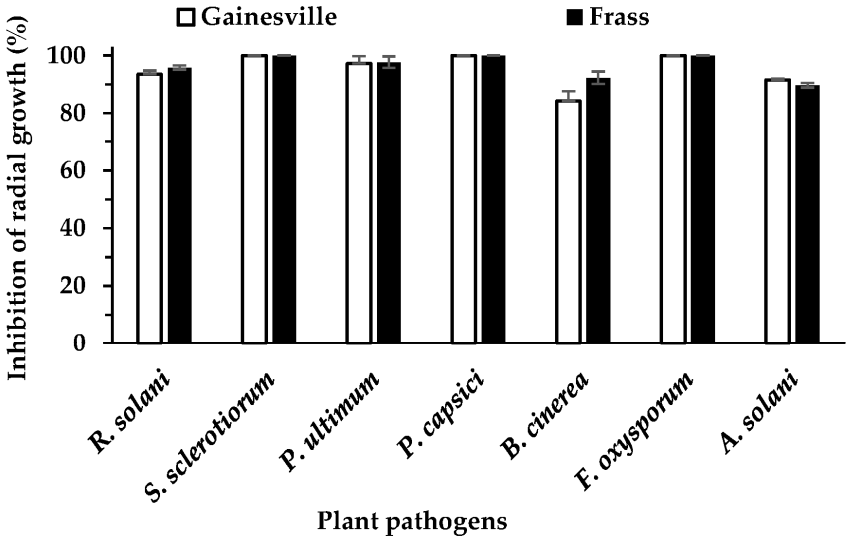


Figure 2. Effect of frass extract and Gainesville diet extract at two concentrations (1% (**a**) and 0.001% (**b**)) on the mycelial growth of *Phytophthora capsici*, *Botrytis cinerea*, *Alternaria solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Pythium ultimum* using dual culture overlay assay. Each value represents the mean of three replicates ± standard deviation.

Table 1. Pairwise comparisons of GV1-11 and FGV15-6 genomes vs. type-strain genomes using the TYGS comparison server with the second formula.

	GV1-11			FGV15-6		
	dDDH	**C.I.**	**G+C Content Difference**	**dDDH**	**C.I.**	**G+C Content Difference**
***Bacillus* Strain**	**(d4, in %)**	**(d4, in %)**	**(in %)**	**(d4, in %)**	**(d4, in %)**	**(in %)**
B. amyloliquefaciens subsp. *plantarum* FZB42	88.2	[85.7–90.3]	0.63	87.8	[85.3–89.9]	0.59
B. velezensis NRRL B-41580	84.4	[81.7–86.8]	0.47	84.1	[81.4–86.6]	0.43
B. methylotrophicus KACC 13105	83.3	[80.5–85.8]	0.59	83.0	[80.2–85.5]	0.55
B. vanillea XY18	56.5	[53.8–59.2]	0.48	57.2	[54.5–60.0]	0.44
B. siamensis KCTC 13613	56.2	[53.4–58.9]	0.49	56.9	[54.1–59.6]	0.45
B. amyloliquefaciens DSM 7	55.5	[52.7–58.2]	0.24	56.1	[53.4–58.8]	0.20
B. nakamurai NRRL B-41091	30.7	[28.3–33.2]	0.58	31.7	[29.3–34.2]	0.62
B. spizizenii TU-B-10	20.7	[18.5–23.1]	2.02	22.3	[20.0–24.7]	2.06
B. axarquiensis NRRL B-41617	20.6	[18.4–23.0]	2.05	22.1	[19.9–24.6]	2.09
B. subtilis NCIB 3610	20.6	[18.4–23.1]	2.45			
B. rugosus SPB7	20.5	[18.3–22.9]	2.72	22.0	[19.7–24.4]	2.76
B. vallismortis DV1-F-3	20.4	[18.1–22.8]	2.08			
B. mojavensis KCTC 3706	20.3	[18.0–22.7]	2.18			

3.4. Effect of B. velezensis on Plant Pathogen Mycelial Growth (Dual Culture Overlay Assay)

Dual culture overlay assays were conducted with *B. velezensis*. The bacterium was shown to cause a very strong inhibition of mycelial growth for *S. sclerotiorum* (100%), *A. solani* (96%), and *B. cinerea* (93.1%), a strong inhibition for *P. ultimum* (78.6%) and *R. solani* (71.3%), and a moderate inhibition for *F. oxysporum* (36.2%) and *P. capsici* (21.6%) (Figure 4).

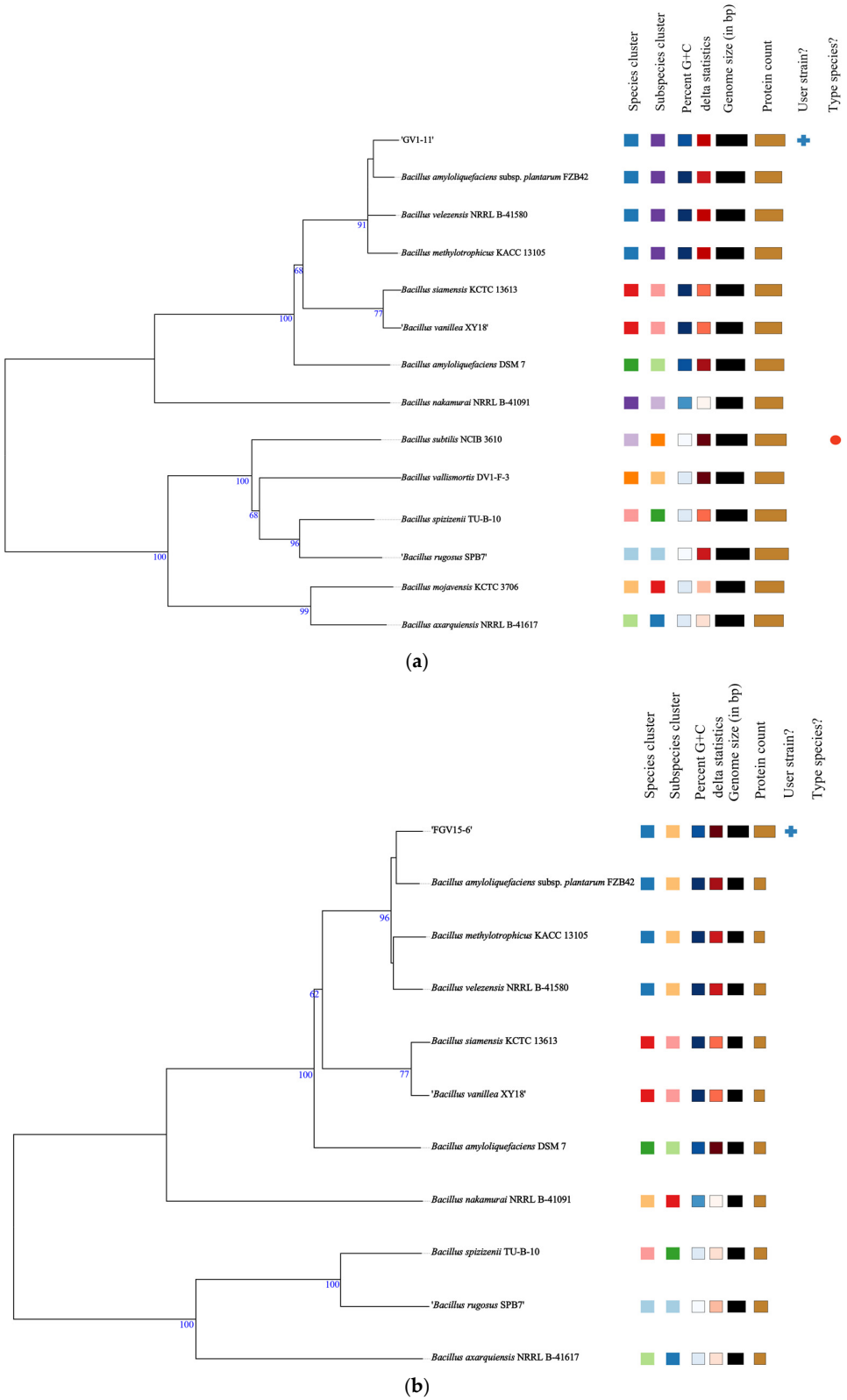


Figure 3. Whole-genome-based phylogenetic tree highlighting the position of the GV1-11 (**a**) and FGV15-6 (**b**) strains relative to other closely related bacterial taxa. Tree developed with FastME 2.1.6.1 [25] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above the branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 79.1% (**a**) and 78.5% (**b**). The tree was rooted at the midpoint [26].

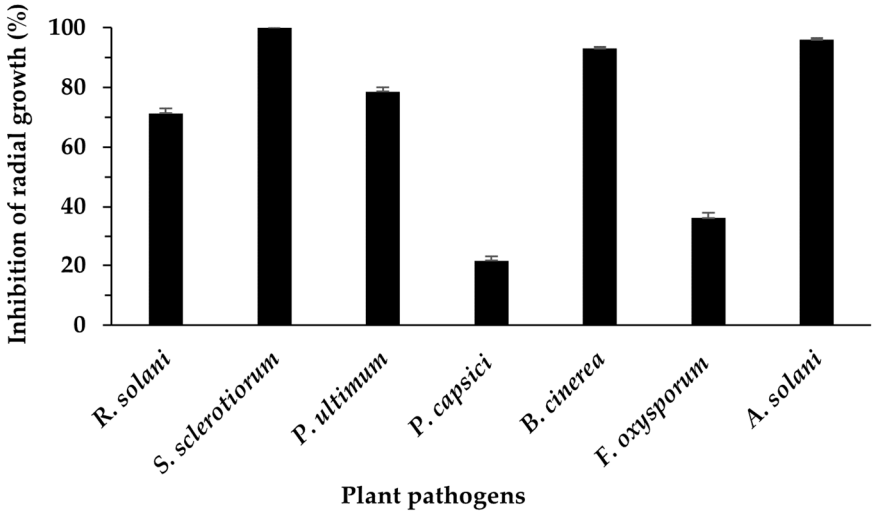


Figure 4. Effect of *Bacillus velezensis* on mycelial growth of *Phytophthora capsici*, *Botrytis cinerea*, *Alternaria solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Pythium ultimum*, based on dual culture overlay assay. Each value represents the mean of four replicates ± standard deviation.

4. Discussion

Frass, an important byproduct of the BSFL bioconversion process, is regarded as a highly valuable source of nutrients for horticultural crops; it provides slow release of macro- and micronutrients, minimizing nutrient runoff to the environment [30]. In this respect, several studies have investigated the possibility of upcycling frass for use as a biofertilizer and have reported its utility as an organic fertilizer [8,14,31–33]. A recent study showed that frass biofertilizer amendment reduces the death of cowpea plants due to *F. oxysporum* [15] and that frass, more specifically, frass obtained from BSFL reared on the Gainesville diet, exerts antifungal activity against *F. oxysporum* [7], suggesting that the beneficial effects of frass amendment for horticultural crops go beyond a simple valuable source of nutrients.

In this study, the effect of frass extract obtained from BSFL reared on the Gainesville diet on the mycelial growth of seven important plant pathogens was evaluated in vitro using the dual culture overlay assay, a well-established technique to test microorganisms for their ability to produce antimicrobial compounds/antibiotics [34]. Dual culture overlay assays clearly showed that the frass extract contained microorganism-producing compound(s) that strongly inhibit(s) the mycelial growth of fungal plant pathogens of the phyla Ascomycota (*A. solani*, *B. cinerea*, *F. oxysporum*, *S. sclerotiorum*) and Basidiomycota (*R. solani*), as well as plant pathogens of the class oomycetes (*P. capsici*, *P. ultimum*) in the Chromista kingdom. It seems that regardless of the extract concentrations (0.00001–1%) applied to the first layer of agar, the concentrations of antifungal/anti-oomycete compound(s) produced by the microorganisms and their diffusion in the second layer resulted in a strong inhibitory effect on the mycelial growth of the tested plant pathogens. Filtering the extract through a 0.22 µm filter allowed us to remove bacteria/fungi and other microorganisms in a procedure called cold sterilization. The extract free from microorganisms was thereafter shown to have no antagonistic activity against the tested plant pathogens. The lack of antagonistic activity of the 0.22-µm-filtered extract showed that (1) antifungal/anti-oomycete compound(s), if present in the extract, is (are) not in concentration(s) high enough to affect the growth of the tested plant pathogens, and (2) microorganisms present in the extract are responsible for its antagonistic activity. Dual culture overlay assays conducted with fresh Gainesville diet revealed that the Gainesville diet itself contains microorganism(s) producing compound(s) that strongly inhibit(s) the mycelial growth of the tested plant pathogens. It strongly suggests that microorganism(s) present in the Gainesville diet is (are) the main factor responsible for the observed antagonistic activity of the frass.

In order to identify the microorganism(s) involved in the antagonistic activity against the tested plant pathogens, fungi and bacteria were isolated from fresh Gainesville diet and

frass using the serial dilution technique. Among the different fungi/bacteria isolated, only the isolates GV1-11 in Gainesville diet and FGV15-6 in frass were shown to exert strong antifungal/anti-oomycete activity against all the pathogens tested; as such, these isolates were then sequenced. The results of digital DNA–DNA hybridization analysis revealed that GV1-11 and FGV15-6 have 88.2% and 87.8% identity, respectively, with *B. amyloliquefaciens* subsp. *plantarum* FZB42 (Figure 3 and Table 1), which is high enough to support the claim that these are the same species. This strongly suggests, on the one hand, that the bacterium present in the Gainesville diet survives the BSFL rearing process and, on the other hand, that the bacterium is one of the primary factors contributing to the observed antifungal/oomycete activity in the resulting frass.

According to phylogenomic analysis, Dunlap et al. [29] reclassified *B. amyloliquefaciens* subsp. *plantarum* FZB42 as a later heterotypic synonym of *B. velezensis* NRRL B-41580 [29]. The results of dDDH (digital DNA–DNA hybridization), ANI (average nucleotide identity), TETRA (tetranucleotide), and AAI (average amino acid identity) comparisons of *B. amyloliquefaciens* with the *B. amyloliquefaciens plantarum* FZB42, *Bacillus methylotrophicus*, and *B. velezensis* also revealed that FZB42 was not *B. amyloliquefaciens*, but rather, *B. velezensis* [35]. Hence, isolates GV1-11 and FGV15-6 were predicted by the type strain genome server (TYGS) to be *B. velezensis* species, as shown in the first two records in Table 1.

B. velezensis FZB42, a bacterium belonging to the *Bacillus subtilis* species complex [35], is known for its beneficial effects on plant growth and disease suppression in several crops [36–41]. Strain FZB42, which has been gradually accepted as the model Gram-positive plant-growth-promoting and biocontrol rhizobacterium, is used commercially as biofertilizer and as a biocontrol agent in agriculture [42,43].

According to the results of whole genome sequencing, the *B. velezensis* FZB42 genome has nine giant gene clusters, representing about 10% of the whole genome, which play a role in the synthesis of antimicrobial molecules [44]. Three of these nine gene clusters are involved in synthesizing cyclic lipopeptides surfactins, bacillomycin-D (a member of the iturin family), and fengycins [45]. Among the plethora of antimicrobial compounds produced by the bacteria of the *B. subtilis* group [46,47], lipopeptides have been reported to be key compounds for biocontrol activity [48], while fengycins and iturins are known for their strong antifungal properties [49–52]. In the present study, it is very likely that *B. velezensis* exerted its antagonistic activity against the tested pathogens through the production of fengycins and iturins. It is interesting to note that while the bacterium showed moderate antagonistic activity against *P. capsici* and *F. oxysporum*, extracts obtained from frass and fresh Gainesville diet showed strong antagonistic activity against the latter. This suggests that other factors of chemical or microbial nature are involved in the antifungal/anti-oomycete activity of frass. It cannot be excluded that larval gut microorganisms [53] present in larval excreta contributed to the antagonistic activity of frass against the tested plant pathogens.

This study reveals the strong antagonistic activity of BSFL frass against several plant pathogenic fungi and oomycetes and strongly suggests that the presence of *B. velezensis* in the Gainesville diet used to rear BSFL is the main contributing factor to this activity. This work underlines the importance of the inherent microbial characteristics of feedstock for rearing BSFL on the antifungal/anti-oomycete activity of frass and points out the possibility of exploiting BSFL frass to control plant pathogens affecting horticultural crops.

Over the past decade, the use of BSFL as a food and feed in agricultural systems has been generating increasing interest within the scientific community. This interest will continue, considering that rearing BSFL provides a sustainable solution for organic waste management (efficiently upcycling waste materials to a nutrient-rich larval biomass, which could then be used as animal feed ingredients), offering sustainable alternatives for traditional animal husbandry for feeding a growing world population. In this context, the quantity of frass produced will increase in the future. The present study opens new avenues of research on the valorization of BSFL frass for the control of plant pathogens that may eventually lead to a decrease in the use of conventional pesticides. Embracing frass valorization for the control of plant pathogens aligns with the principles of sustainable

development, circular economy concepts, and reducing dependency on non-renewable resources and conventional pesticides.

Author Contributions: Conceptualization, G.A., M.D.-H., G.W.V., A.T.V. and R.J.T.; methodology, G.A., M.D.-H. and M.-H.D.; software, G.A. and A.T.V.; validation, N.D., M.-H.D., M.D. and A.T.V.; formal analysis, G.A.; data curation, G.A.; writing—original draft preparation, G.A., M.D.-H. and R.J.T.; writing—review and editing, G.W.V., N.D., M.-H.D., M.D., A.T.V. and R.J.T.; supervision, G.W.V. and R.J.T.; project administration, G.W.V.; funding acquisition, G.W.V. All authors have read and agreed to the published version of the manuscript.

Funding: Premier Tech Technologies Ltd. and Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG) [grant number: STPGP 521402].

Data Availability Statement: Available on request.

Acknowledgments: The authors thank Jérôme St-Cyr and Brian Boyle (Plateforme d'analyses **génomiques**, IBIS, Université Laval) for their collaborations and the staff at the LARSA. The authors also thank Julien Vivancos from the MAPAQ for providing the fungi and oomycetes.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cullere, M.; Tasoniero, G.; Giaccone, V.; Miotti-Scapin, R.; Claeys, E.; De Smet, S.; Dalle Zotte, A. Black soldier fly as dietary protein source for broiler quails: Apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. *Animal* **2016**, *10*, 1923–1930. [CrossRef] [PubMed]
2. Veldkamp, T.; Bosch, G. Insects: A protein-rich feed ingredient in pig and poultry diets. *Anim. Front.* **2015**, *5*, 45–50.
3. Diener, S.; Zurbrügg, C.; Tockner, K. Conversion of organic material by black soldier fly larvae: Establishing optimal feeding rates. *Waste Manag. Res.* **2009**, *27*, 603–610. [CrossRef] [PubMed]
4. Nguyen, T.T.X.; Tomberlin, J.K.; Vanlaerhoven, S. Ability of black soldier fly (Diptera:Stratiomyidae) larvae to recycle food waste. *Environ. Entomol.* **2015**, *44*, 406–410. [CrossRef]
5. Ravi, H.K.; Degrou, A.; Costil, J.; Trespeuch, C.; Chemat, F.; Vian, M.A. Larvae mediated valorization of industrial, agriculture and food wastes: Biorefinery concept through bioconversion, processes, procedures, and products. *Processes* **2020**, *8*, 857. [CrossRef]
6. Schmitt, E.; de Vries, W. Potential benefits of using *Hermetia illucens* frass as a soil amendment on food production and for environmental impact reduction. *Curr. Opin. Green Sustain. Chem.* **2020**, *25*, 100335. [CrossRef]
7. Arabzadeh, G.; Delisle-Houde, M.; Tweddell, R.J.; Deschamps, M.-H.; Dorais, M.; Lebeuf, Y.; Derome, N.; Vandenberg, G. Diet composition influences growth performance, bioconversion of black soldier fly larvae: Agronomic value and in vitro biofungicidal activity of derived frass. *Agronomy* **2022**, *12*, 1765. [CrossRef]
8. Beesigamukama, D.; Mochoge, B.; Korir, N.K.; Fiaboe, K.K.M.; Nakimbugwe, D.; Khamis, F.M.; Subramanian, S.; Dubois, T.; Musyoka, M.W.; Ekesi, S.; et al. Exploring black soldier fly frass as novel fertilizer for improved growth, yield, and nitrogen use efficiency of maize under field conditions. *Front. Plant Sci.* **2020**, *11*, 574592. [CrossRef]
9. Beesigamukama, D.; Subramanian, S.; Tanga, C.M. Nutrient quality and maturity status of frass fertilizer from nine edible insects. *Sci. Rep.* **2022**, *12*, 7182. [CrossRef]
10. Ding, M.-Q.; Yang, S.-S.; Ding, J.; Zhang, Z.-R.; Zhao, Y.-L.; Dai, W.; Sun, H.-J.; Zhao, L.; Xing, D.; Ren, N.; et al. Gut microbiome associating with carbon and nitrogen metabolism during biodegradation of polyethene in *Tenebrio* larvae with crop residues as co-diets. *Environ. Sci. Technol.* **2023**, *57*, 3031–3041. [CrossRef]
11. Bruno, D.; Bonelli, M.; De Filippis, F.; Di Lelio, I.; Tettamanti, G.; Casartelli, M.; Ercolini, D.; Caccia, S. The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. *Appl. Environ. Microbiol.* **2019**, *85*, e01864-18. [CrossRef]
12. Jiang, C.-L.; Jin, W.-Z.; Tao, X.-H.; Zhang, Q.; Zhu, J.; Feng, S.-Y.; Xu, X.-H.; Li, H.-Y.; Wang, Z.-H.; Zhang, Z.-J. Black soldier fly larvae (*Hermetia illucens*) strengthen the metabolic function of food waste biodegradation by gut microbiome. *Microb. Biotechnol.* **2019**, *12*, 528–543. [CrossRef]
13. Penhallegon, R. *Nitrogen-Phosphorus-Potassium Values of Organic Fertilizers*; Oregon State University, Extension Service: Corvallis, OR, USA, 2003.
14. Temple, W.D.; Radley, R.; Baker-French, J.; Richardson, F. *Use of Enterra Natural Fertilizer (Black Soldier Fly Larvae Digestate) as a Soil Amendment*; Enterra Feed Corporation: Vancouver, BC, Canada, 2013.
15. Quilliam, R.S.; Nuku-Adeku, C.; Maquart, P.; Little, D.; Newton, R.; Murray, F. Integrating insect frass biofertilisers into sustainable peri-urban agro-food systems. *J. Insects Food Feed* **2020**, *6*, 315–322. [CrossRef]
16. Hogsette, J.A. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. *J. Econ. Entomol.* **1992**, *85*, 2291–2294. [CrossRef] [PubMed]

17. Afgan, E.; Baker, D.; van den Beek, M.; Blankenberg, D.; Bouvier, D.; Cech, M.; Chilton, J.; Clements, D.; Coraor, N.; Eberhard, C.; et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res.* **2016**, *44*, W3–W10. [CrossRef] [PubMed]
18. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [CrossRef]
19. Meier-Kolthoff, J.P.; Göker, M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat. Commun.* **2019**, *10*, 2182. [CrossRef]
20. Ondov, B.D.; Treangen, T.J.; Melsted, P.; Mallonee, A.B.; Bergman, N.H.; Koren, S.; Phillippy, A.M. Mash: Fast genome and metagenome distance estimation using MinHash. *Genome Biol.* **2016**, *17*, 132. [CrossRef] [PubMed]
21. Lagesen, K.; Hallin, P.; Rødland, E.A.; Staerfeldt, H.-H.; Rognes, T.; Ussery, D.W. RNAmmer: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* **2007**, *35*, 3100–3108. [CrossRef]
22. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinform.* **2009**, *10*, 421. [CrossRef]
23. Meier-Kolthoff, J.P.; Auch, A.F.; Klenk, H.-P.; Göker, M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinform.* **2013**, *14*, 60. [CrossRef]
24. Meier-Kolthoff, J.P.; Carbasse, J.S.; Peinado-Olarte, R.L.; Göker, M. TYGS and LPSN: A database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Res.* **2022**, *50*, D801–D807. [CrossRef] [PubMed]
25. Lefort, V.; Desper, R.; Gascuel, O. FastME 2.0: A comprehensive, accurate, and fast distance-based phylogeny inference program. *Mol. Biol. Evol.* **2015**, *32*, 2798–2800. [CrossRef] [PubMed]
26. Farris, J.S. Estimating phylogenetic trees from distance matrices. *Am. Nat.* **1972**, *106*, 645–668. [CrossRef]
27. Kreft, Ł.; Botzki, A.; Coppens, F.; Vandepoele, K.; Van Bel, M. PhyD3: A phylogenetic tree viewer with extended phyloXML support for functional genomics data visualization. *Bioinformatics* **2017**, *33*, 2946–2947. [CrossRef] [PubMed]
28. Meier-Kolthoff, J.P.; Hahnke, R.L.; Petersen, J.; Scheuner, C.; Michael, V.; Fiebig, A.; Rohde, C.; Rohde, M.; Fartmann, B.; Goodwin, L.A.; et al. Complete genome sequence of DSM 30083(T), the type strain (U5/41(T)) of *Escherichia coli*, and a proposal for delineating subspecies in microbial taxonomy. *Stand. Genom. Sci.* **2014**, *9*, 2. [CrossRef] [PubMed]
29. Dunlap, C.A.; Kim, S.-J.; Kwon, S.-W.; Rooney, A.P. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp. *plantarum* and "*Bacillus oryzicola*" are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics. *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 1212–1217. [PubMed]
30. Shaviv, A. Advances in controlled-release fertilizers. *Adv. Agron.* **2001**, *71*, 1–49.
31. Beesigamukama, D.; Mochoge, B.; Korir, N.K.; Fiaboe, K.K.M.; Nakimbugwe, D.; Khamis, F.M.; Subramanian, S.; Wangu, M.M.; Dubois, T.; Ekesi, S.; et al. Low-cost technology for recycling agro-industrial waste into nutrient-rich organic fertilizer using black soldier fly. *Waste Manag.* **2021**, *119*, 183–194. [CrossRef] [PubMed]
32. Klammsteiner, T.; Turan, V.; Fernández-Delgado Juárez, M.; Oberegger, S.; Insam, H. Suitability of black soldier fly frass as soil amendment and implication for organic waste hygienization. *Agronomy* **2020**, *10*, 1578. [CrossRef]
33. Setti, L.; Francia, E.; Pulvirenti, A.; Gigliano, S.; Zaccardelli, M.; Pane, C.; Caradonia, F.; Bortolini, S.; Maistrello, L.; Ronga, D. Use of black soldier fly (*Hermetia illucens* (L.), Diptera: Stratiomyidae) larvae processing residue in peat-based growing media. *Waste Manag.* **2019**, *95*, 278–288. [CrossRef] [PubMed]
34. Sánchez-Hidalgo, M.; Pascual, J.; de la Cruz, M.; Martín, J.; Kath, G.S.; Sigmund, J.M.; Masurekar, P.; Vicente, F.; Genilloud, O.; Bills, G.F. Prescreening bacterial colonies for bioactive molecules with Janus plates, a SBS standard double-faced microbial culturing system. *Antonie Van Leeuwenhoek* **2012**, *102*, 361–374. [CrossRef] [PubMed]
35. Fan, B.; Blom, J.; Klenk, H.-P.; Borriss, R. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* Form an "Operational Group *B. amyloliquefaciens*" within the *B. subtilis* Species Complex. *Front. Microbiol.* **2017**, *8*, 22.
36. Chowdhury, S.P.; Dietel, K.; Rändler, M.; Schmid, M.; Junge, H.; Borriss, R.; Hartmann, A.; Grosch, R. Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. *PLoS ONE* **2013**, *8*, e68818. [CrossRef]
37. Elanchezhiyan, K.; Keerthana, U.; Nagendran, K.; Prabhukarthikeyan, S.R.; Prabakar, K.; Raguchander, T.; Karthikeyan, G. Multifaceted benefits of *Bacillus amyloliquefaciens* strain FBZ24 in the management of wilt disease in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. *Physiol. Mol. Plant Pathol.* **2018**, *103*, 92–101. [CrossRef]
38. Schmiedeknecht, G.; Bochow, H.; Junge, H. Use of *Bacillus subtilis* as biocontrol agent. II. Biological control of potato diseases. *J. Plant Dis. Prot.* **1998**, *105*, 376–386.
39. Sylla, J.; Alsanius, B.W.; Krüger, E.; Reineke, A.; Strohmeier, S.; Wohanka, W. Leaf microbiota of strawberries as affected by biological control agents. *Phytopathology* **2013**, *103*, 1001–1011. [CrossRef]
40. Talboys, P.J.; Owen, D.W.; Healey, J.R.; Withers, P.J.; Jones, D.L. Auxin secretion by *Bacillus amyloliquefaciens* FZB42 both stimulates root exudation and limits phosphorus uptake in *Triticum aestivum*. *BMC Plant Biol.* **2014**, *14*, 51. [CrossRef]
41. Yao, A.V.; Bochow, H.; Karimov, S.; Boturov, U.; Sanginboy, S.; Sharipov, A.K. Effect of FZB 24® *Bacillus subtilis* as a biofertilizer on cotton yields in field tests. *Arch. Phytopathol. Plant Prot.* **2006**, *39*, 323–328. [CrossRef]
42. Chowdhury, S.P.; Hartmann, A.; Gao, X.; Borriss, R. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42—A review. *Front. Microbiol.* **2015**, *6*, 780. [CrossRef]

43. Fan, B.; Wang, C.; Song, X.; Ding, X.; Wu, L.; Wu, H.; Gao, X.; Borriss, R. *Bacillus velezensis* FZB42 in 2018: The gram-positive model strain for plant growth promotion and biocontrol. *Front. Microbiol.* **2018**, *9*, 2491. [CrossRef] [PubMed]
44. Chen, X.H.; Koumoutsi, A.; Scholz, R.; Eisenreich, A.; Schneider, K.; Heinemeyer, I.; Morgenstern, B.; Voss, B.; Hess, W.R.; Reva, O.; et al. Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* **2007**, *25*, 1007–1014. [CrossRef] [PubMed]
45. Chen, X.H.; Koumoutsi, A.; Scholz, R.; Schneider, K.; Vater, J.; Süssmuth, R.; Piel, J.; Borriss, R. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. *J. Biotechnol.* **2009**, *140*, 27–37. [CrossRef]
46. Caulier, S.; Nannan, C.; Gillis, A.; Licciardi, F.; Bragard, C.; Mahillon, J. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. *Front. Microbiol.* **2019**, *10*, 302. [CrossRef]
47. Stein, T. *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Mol. Microbiol.* **2005**, *56*, 845–857. [CrossRef] [PubMed]
48. Bouchard-Rochette, M.; Machrafi, Y.; Cossus, L.; Nguyen, T.T.A.; Antoun, H.; Droit, A.; Tweddell, R.J. *Bacillus pumilus* PTB180 and *Bacillus subtilis* PTB185: Production of lipopeptides, antifungal activity, and biocontrol ability against *Botrytis cinerea*. *Biol. Control* **2022**, *170*, 104925. [CrossRef]
49. Arrebola, E.; Jacobs, R.; Korsten, L. Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. *J. Appl. Microbiol.* **2010**, *108*, 386–395. [CrossRef]
50. DeFilippi, S.; Groulx, E.; Megalla, M.; Mohamed, R.; Avis, T.J. Fungal competitors affect production of antimicrobial lipopeptides in *Bacillus subtilis* strain B9-5. *J. Chem. Ecol.* **2018**, *44*, 374–383. [CrossRef]
51. Gao, L.; Han, J.; Liu, H.; Qu, X.; Lu, Z.; Bie, X. Plipastatin and surfactin coproduction by *Bacillus subtilis* pB2-L and their effects on microorganisms. *Antonie Van Leeuwenhoek* **2017**, *110*, 1007–1018. [CrossRef]
52. Gong, Q.; Zhang, C.; Lu, F.; Zhao, H.; Bie, X.; Lu, Z. Identification of bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against *Aspergillus flavus*. *Food Control* **2014**, *36*, 8–14. [CrossRef]
53. Klammsteiner, T.; Walter, A.; Bogataj, T.; Heussler, C.D.; Stres, B.; Steiner, F.M.; Schlick-Steiner, B.C.; Arthofer, W.; Insam, H. The core gut microbiome of black soldier fly (*Hermetia illucens*) larvae raised on low-bioburden diets. *Front. Microbiol.* **2020**, *11*, 993. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.