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Synergistic Insecticidal Effect of *Photorhabdus luminescens* and *Bacillus thuringiensis* against Fall Armyworm (*Spodoptera frugiperda*)

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Abstract: Combining microbial products with different mechanisms of action can produce synergistic insecticidal effects and slow down the development of resistance. This study evaluated the insecticidal activity of mixtures containing the commercial *Photorhabdus luminescens* (Pl) strain ATCC 29,999, the local isolate Pl 2103-UV, and the commercial *Bacillus thuringiensis* subsp. *kurstaki* (Bt) ABTS-351 against *Spodoptera frugiperda*. As the proportion of Bt increased in the mixtures, insecticidal activity increased, with the synergistic ratios reaching 1.98 for ATCC 29,999 and 5.29 for 2103-UV at a1:5 Pl:Bt ratio, representing approximately twofold and fivefold reductions, respectively, in the Bt dosage required for mortality. Hemolymph analysis revealed the highest Pl bacterial loads in the 1:5 treatments within the *S. frugiperda* hemocoel. Histopathology also showed exacerbated midgut vacuolation in the 1:5 ATCC 29,999:Bt treatment. Whole-genome analysis showed that 2103-UV produced more porins, potentially contributing to its higher insecticidal activity. This study demonstrated that Bt assists the invasion of Pl into the hemocoel and enhances synergistic insecticidal efficiency. The findings provide a reference for integrating Pl with other microbial products to sustainably manage significant and severe lepidopteran pests.

Keywords: entomopathogenic nematode symbiotic bacteria; combination formulations; histopathology section; pesticide reduction

Citation: Chang, T.-Y.; Hsieh, C.; Wu, L.-H. Synergistic Insecticidal Effect of *Photorhabdus luminescens* and *Bacillus thuringiensis* against Fall Armyworm (*Spodoptera frugiperda*). *Agriculture* **2024**, *14*, 864. https://doi.org/10.3390/agriculture14060864

Academic Editors: Danai Gkizi and Eirini Karanastasi

Received: 26 April 2024
Revised: 23 May 2024
Accepted: 29 May 2024
Published: 30 May 2024

1. Introduction

Taiwan is situated at the intersection of the tropics and subtropics, and is characterized by a hot and humid climate. Due to its geographical location and climatic characteristics, a broad variety of crops can be cultivated in Taiwan [1–3]. Crops are often grown in polycultures and intensive cultivation systems. These factors have led to numerous types of pests and diseases, and crops are frequently affected by harmful organisms, which can cause large-scale pest and disease outbreaks [4,5]. In order to control pests, diseases, and weeds with the aim of reducing produce losses and maintaining yield, farmers usually rely on chemical pesticides, because they are easy to use, act quickly, and have a wide range of applications in the field. However, the irrational use of chemical pesticides or a lack of knowledge about their application can lead to several issues. These include acceleration of the development of pest resistance to pesticides, environmental pollution, and increases in pesticide residues in agricultural products. To address these issues, biopesticides made from microorganisms can be used. These are specific to certain pests, environmentally friendly, and effective in solving pest problems, thus providing a solution that balances environmental protection and control efficacy [6–10].

As widely recommended microbial pesticides for controlling lepidopteran pests, *Bacillus thuringiensis* (Bt, *B. thuringiensis* subsp. *kurstaki*) products are also available in Taiwan.

These biopesticides have been widely used in crop management with good control efficacy, making them some of the most successful microbial plant protection products [11–14]. However, with the broad use of Bt, many target pests have developed resistance, such as the fall armyworm (*Spodoptera frugiperda*) and the diamondback moth (*Plutella xylostella*). Additionally, due to the extensive use of Bt in transgenic crops in countries such as Brazil, Argentina, and the United States, the allele frequency of resistance to the Cry1F protein in some *S. frugiperda* populations has become as high as 0.29, confirming the development of resistance. The high migratory ability of *S. frugiperda* also poses a significant challenge for resistance management [15–17].

Researchers have demonstrated that *Photorhabdus luminescens* (Pl), an intestinal symbiotic bacterial *Heterorhabditis* species of entomopathogenic nematodes, can secrete various metabolites, such as proteases, lipases, chitinases, protease inhibitors, antibiotics, hemolysins, and lipopolysaccharides. Pl possesses insecticidal, acaricidal, and antimicrobial properties, showing great potential for research and development in plant pest and disease management [18–21].

Combining microbial pesticides is a strategy for mitigating and delaying the development of pesticide resistance and enhancing pest control efficacy. Combining two or more microbial products with different modes of action can prevent the occurrence of resistance while also enhancing control effects [22]. A previous study incorporated a culture of *Photorhabdus temperata* subsp. *Temperate* into an artificial diet, which was fed to third-instar *Spodoptera exigua* larvae. While treatment with Bt alone resulted in 5% mortality, the combined Bt and *P. temperata* treatment resulted in a significantly higher mortality rate of 65%, demonstrating a synergistic enhancement in insecticidal activity [23]. However, different combinations of microbes, ratios, or mixing methods may also result in antagonistic interactions. For instance, Wu et al. (2022) [24] reported that co-culturing Bt and Pl reduced insecticidal activity compared with Bt alone. This reduction was attributed to Pl inhibiting Bt growth, indicating resource or growth competition during co-culture. When Bt and Pl were cultured separately before mixing, the insecticidal activity surpassed that of Bt alone. This enhancement may be attributed to Pl metabolites interfering with the insect humoral immune response by inhibiting phospholipase A2 (PLA2) activity, which is necessary for producing the eicosanoid immune precursor arachidonic acid (AA) [25]. Consequently, this inhibition leads to a reduction in phenoloxidase (PO) activity. Pl can also suppress gene expression of the dual oxidases Duox and Nox in the synthesis pathway of reactive oxygen species (ROSs), reducing ROS generation and counteracting the killing of Bt. Additionally, Bt-induced midgut perforation facilitates the spread of Pl in the gut and hemocoel, leading to septicemia [26–29].

This study evaluated the synergistic control efficacy and potential mechanisms of commercial Bt ABTS-351 and the commercial US Pl strain ATCC 29,999, as well as the locally isolated Pl strain 2103-UV, against *S. frugiperda*. We tested different Pl:Bt ratios (1:1, 1:3, and 1:5) through combined application trials to comprehensively investigate the synergistic effects of Bt and Pl against *Spodoptera frugiperda* and to elucidate the underlying mechanisms. This study had the following objectives: 1. to investigate how increasing the proportion of Bt affects the insecticidal efficiency of the mixtures; 2. to utilize hemolymph assays to confirm whether Pl infection levels in *S. frugiperda* hemocoel increase with higher Bt ratios; 3. to compare histopathology to examine differences in pathological effects and insecticidal efficiency against *S. frugiperda* between ATCC 29,999, 2103-UV, and their Bt mixtures; and 4. to perform next-generation sequencing to analyze the phylogenetic relationship between ATCC 29,999 and 2103-UV, assess the diversity of insecticidal protein genes, and identify potential factors contributing to the synergy with Bt.

This study serves as the first case of using both *Bacillus thuringiensis* and *Photorhabdus luminescens* for the combined control of *Spodoptera frugiperda*. Hemolymph assays were applied to understand the possible mechanisms of synergistic effects between Bt and Pl, and histopathological techniques were used to investigate potential pathways for synergistic pathological effects between Pl and Bt. This aligns with the goal of sustainable pest management by consistently reducing the use of chemical pesticides.

2. Materials and Methods

2.1. Insect Rearing

Cultivation of the *S. frugiperda* colony was performed in incubators at 25 °C ± 2 °C and a relative humidity of 65% ± 5% under a 12 h light–12 h dark photoperiod [30]. The colony was obtained from the population established at the Beneficial Insect Ecological Adaptation Research Laboratory, Department of Plant Medicine, National Pingtung University of Science and Technology, collected from the corn field at the Smart Farm of National Pingtung University of Science and Technology (N22° 38′ 32.3″, E120° 36′ 16.4″). Third-instar *S. frugiperda* larvae were reared individually in 3 × 3 cm^2 rearing containers.

2.2. Strains and Medium Culture Conditions

Photorhabdus luminescens strain Pl ATCC 29,999 and *P. luminescens* strain Pl 2103-UV were both acquired from the Department of Biological Science and Technology at National Kaohsiung Normal University. The bacterial strains Pl ATCC 29,999 and Pl 2103-UV were cultured on a Luria–Bertani agar (LA) plate and incubated at 30 °C for two days. This was used as the source of inoculum. To prepare the cultures of Pl ATCC 29,999 and Pl 2103-UV, a colony of Pl from the LA plate was inoculated into 100 mL of Luria–Bertani broth (LB) and incubated at 30 °C at 200 rpm for 120 h. The bacterial culture concentrations were diluted with sterile water (SW) until the culture's optical density (OD) at 600 nm reached 0.2, equivalent to 1 × 10^7 colony-forming units (CFU)/mL. Three media were used in this study: nutrient bromothymol blue agar (NBTA) [2.3% nutrient agar (Difco, Detroit, MI, USA), 2.5 × 10^{-3}% bromothymol blue (Merck, Rahway, NJ, USA), and 4 × 10^{-3}% 2,3,5-triphenyl-tetrazolium (Sigma-Aldrich, St. Louis, MO, USA)], Luria–Bertani agar (Difco, Detroit, MI, USA), and Luria–Bertani broth (LB), which contained peptone (10 g/L), yeast extract (5 g/L), and NaCl (5 g/L) [24].

The commercial *Bacillus thuringiensis* subsp. *kurstaki* (Bt) ABTS-351, with an active ingredient content of 23.7%, containing a total amount of 16,000 IU/MG of microbial fermentation solids, spores, and insecticidal protein toxins, was a wettable powder (WP) formulation (Sumitomo Chemical Taiwan Co., Ltd., Taipei, Taiwan).

2.3. Oral Toxicity

Individual third-instar *S. frugiperda* larvae were reared in 3 × 3 cm^2 rearing containers, each containing 1 × 1 cm^2 of corn leaf. To test the oral toxicity of different mixing ratios of Pl ATCC 29,999, Pl 2103-UV, and Bt against the third-instar *S. frugiperda* larvae, based on the experiment described in [23], we hypothesized that as the concentration of Bt in the mixture increased, it would enhance the infection of Pl in the hemocoel, as well as the insecticidal activity. Therefore, we set the Pl:Bt mixing ratios to 1:1, 1:3, and 1:5. The mixed solutions were sequentially diluted four times to obtain 2×, 4×, 8×, and 16× dilutions for each mixing ratio. For the 1:1 ratio, the 2× dilution contained 296.25 ppm of Bt and 5 × 10^6 CFU/mL of Pl; the 4× dilution contained 148.13 ppm of Bt and 2.5 × 10^6 CFU/mL of Pl; the 8× dilution contained 74.06 ppm of Bt and 1.25 × 10^6 CFU/mL of Pl; and the 16× dilution contained 37.03 ppm of Bt and 6.25 × 10^6 CFU/mL of Pl. For the 1:3 ratio, the 2× dilution contained 444.38 ppm of Bt and 2.5 × 10^6 CFU/mL of Pl; the 4× dilution contained 222.19 ppm of Bt and 1.25 × 10^6 CFU/mL of Pl; the 8× dilution contained 111.09 ppm of Bt and 6.25 × 10^5 CFU/mL of Pl; and the 16× dilution contained 55.55 ppm of Bt and 3.13 × 10^5 CFU/mL of Pl. For the 1:5 ratio, the 2× dilution contained 493.75 ppm of Bt and 1.7 × 10^6 CFU/mL of Pl; the 4× dilution contained 246.88 ppm of Bt and 8.5 × 10^5 CFU/mL of Pl; the 8× dilution contained 123.44 ppm of Bt and 4.25 × 10^5 CFU/mL of Pl; and the 16× dilution contained 61.72 ppm of Bt and 2.13 × 10^5 CFU/mL of Pl. For the Bt treatment alone, Bt was sequentially diluted five times to obtain 1×, 2×, 4×, 8×, and 16× dilutions. The 1× dilution contained 2370 ppm Bt, the 2× dilution contained 1185 ppm Bt, the 4× dilution contained 592.5 ppm Bt, the 8× dilution contained 296.25 ppm Bt, and the 16× dilution contained 148.13 ppm Bt (Table S1). Leaves soaked in LB medium were used as a control. Corn leaves were immersed in these mixed solutions to provide food for

S. frugiperda. Freshly soaked corn leaves were replenished daily for three days. Every 24 h, the number of dead insects was recorded, and the cumulative mortality rate over 72 h was documented. The median lethal time (LT_{50}) and the relationship between the concentration, logarithm, and mortality rate were calculated. Subsequently, probit analysis was used to calculate the median lethal concentration (LC_{50}) of each mixing ratio treatment. LC_{50} values were determined based on the dosage of Bt (in ppm). Single treatments with Pl ATCC 29,999, Pl 2103-UV, and Bt were used as control groups. Each treatment consisted of three replicates, with 10 larvae per replicate.

2.4. Leaf Consumption

Thirty larvae were singly placed in a 6 × 6 cm^2 rearing container containing 4 × 5 cm^2 of corn leaf. To investigate the sublethal effects of Pl on *S. frugiperda*, cultures of Pl ATCC 29,999 and Pl 2103-UV were incubated for 24, 72, and 120 h. Corn leaves were then dipped into the bacterial cultures and provided to the *S. frugiperda* larvae for feeding. Leaves soaked in LB medium were used as control. After one day of feeding, the corn leaves were removed and weighed to determine the amount consumed. This process was repeated by providing new corn leaf discs dipped in the bacterial cultures and continued for three days. Untreated leaves were used as blanks to evaluate weight loss due to natural water evaporation. Each treatment consisted of three replicates, with 10 larvae per replicate. The leaf consumption was calculated using the following mathematical equation.

$$\text{Leaf consumption (g)} = \text{Leaf weight before feeding} - \text{Leaf weight after feeding}$$

2.5. Hemolymph Assay

To further investigate the synergistic effects of Pl and Bt, we observed the infection status of Pl ATCC 29,999 in the bodies of *S. frugiperda* after treatment with mixed solutions of Pl ATCC 29,999 and Bt ABTS-35 (at ratios of 1:1, 1:3, and 1:5). According to a method modified from [23], the mixed solutions of Pl ATCC 29,999 and Bt ABTS-351 were prepared as follows: the 1:1 mixture contained 148.13 ppm of Bt and 5 × 10^6 CFU/mL of Pl; the 1:3 mixture contained 222.19 ppm of Bt and 2.5 × 10^6 CFU/mL of Pl; and the 1:5 mixture contained 245.89 ppm of Bt and 1.7 × 10^6 CFU/mL of Pl. Pl ATCC 29,999 treatment alone was used as the control group, representing the infection status of Pl ATCC 29,999 in the hemocoel under Pl ATCC 29,999 treatment alone. Fifth-instar *S. frugiperda* larvae were fed with corn leaves soaked in mixed solutions to facilitate operations and reduce post-treatment mortality rates. These solutions were provided for feeding. After 24, 48, 72, and 96 h of feeding, 10 µL of blood lymph was extracted from the forelegs of *S. frugiperda* using a 10 µL microsyringe (HAMILTON® 80330, Dogger, Taipei, Taiwan) with a 0.3 mm needle. The extracted lymph was transferred to a 0.2 mL PCR tube and serially diluted 10,000 times using sterile, autoclaved water. Subsequently, 100 µL of the hemolymph was spread onto nutrient broth bromothymol blue agar (NBTA) as a solid culture medium using a spiral plater (DWS Whitley WASP Touch, Genmall Biotechnology Co., Ltd., Taipei, Taiwan) and incubated for 48 h. The number of blue-green colonies of Pl ATCC 29,999 was counted to assess the infection status of Pl ATCC 29,999 in the hemocoel of *S. frugiperda* at different mixing ratios (1:1, 1:3, and 1:5). Three plates of NBTA culture medium were prepared for each mixing ratio treatment, with a total of five larvae per treatment.

2.6. Preparation and Sectioning of Midgut Tissues of Spodoptera frugiperda

Sample preparation and staining were performed using a method modified from [24]. The samples and control groups (untreated samples) that received Pl treatment, Bt treatment, and Pl + Bt combined treatment at a ratio of 1:5 were first immersed in 10% formalin for 24 h. Subsequently, they were dehydrated with different concentrations of alcohol (70%, 85%, 95%, and 100%) for about 20–25 h each. Thereafter, they were subjected to a two-hour clearing process using a xylene substitute (Sub-X, Leica, Tech Quality Instrument Co., Ltd., Taipei, Taiwan), which was repeated three times. Subsequently, the samples underwent a

two-hour paraffinization process using wax tissue infiltration pellets (Paraplast, Leica, Tech Quality Instrument Co., Ltd., Taipei, Taiwan), and this was repeated three times. Finally, the paraffinized insect samples were embedded into cassette tapes (Cassettes, Leica, Tech Quality Instrument Co., Ltd., Taipei, Taiwan) using fresh Paraplast and allowed to solidify. Once completed, tissue pathology sections (5 μm) were obtained using a manual rotary microtome (Histocore biocut, Leica, Tech Quality Instrument Co., Ltd., Taipei, Taiwan). Then, the sections were washed and stained with hematoxylin and eosin for histopathological analysis to localize the effects of the toxin.

A light microscope (SAGE VISION, Taipei, Taiwan) was used to examine the midgut symptoms on the completed glass slide samples. The image sensor (SONY, Exmor RS CMOS sensor with a USB 3.0 interface, SAGE VISION, Taipei, Taiwan) of the SAGE VISION C20 camera was used to photograph all slide samples. Five symptom photos for each treatment in which the midgut cavity occupied 50% of the total image area were chosen. The average number of vesicles in each treatment was quantified using ImageJ (version 1.53a) (https://imagej.nih.gov/ij/index.html, accessed on 7 March 2024) to observe the synergistic effects of Pl ATCC 29,999, Pl 2103-UV, and Bt. The number of vesicles in the untreated *S. frugiperda* gut was used as a control, representing the number of vesicles in healthy *S. frugiperda* bodies.

2.7. The Phylogenetic Analysis and Comparison of Pore-Forming Proteins between the Commercialized Strain Pl ATCC 29,999 from the United States and the Indigenous Strain Pl 2103-UV

The complete genome sequencing results of Pl 2103-UV were cross-referenced with those of other photobacteria available in the NCBI database, including ATCC 29,999 (BioProject Accession: PRJNA257857), and they were compared (Table 1) to reconstruct a phylogenetic relationship tree. Furthermore, the porins generated by Pl 2103-UV and Pl ATCC 29,999 were compared separately to discuss potential reasons for the different synergistic effects of the two strains.

Table 1. There were 14 reference genomes in this phylogenetic analysis. The NCBI website has 14 genomes, which are listed in the table.

Organism Name	Stain	Assembly
Photorhabdus luminescens subsp. *luminescens*	DSM 3368	GCA_001083805.1
Photorhabdus luminescens subsp. *mexicana*	MEX47-22	GCA_004348775.1
Photorhabdus luminescens	ATCC 29,999	GCA_900102985.1
Photorhabdus luminescens	HIM3	GCA_002204205.1
Photorhabdus luminescens	LN2	GCA_000767775.1
Photorhabdus luminescens	H4	GCA_002969005.1
Photorhabdus luminescens	H1	GCA_002968995.1
Photorhabdus luminescens subsp. *sonorensis*	Caborca	GCA_006239335.1
Photorhabdus luminescens	H3	GCA_002968975.1
Photorhabdus luminescens	H5	GCA_002969055.1
Photorhabdus luminescens	NBAII HiPL101	GCA_000798635.2
Photorhabdus luminescens	NBAII H75HRPL105	GCA_000826725.2

2.8. Statistical Analysis

All statistical data are presented as the mean ± standard deviation. Data on the mortality, leaf consumption, hemolymph assay, and number of vesicles were first subjected to a normality test (Shapiro–Wilk test) to confirm normal distribution. After confirming normality ($p > 0.05$), a variance homogeneity test (Levene test) was conducted to ensure the homogeneity of variance ($p > 0.05$), and a one-way analysis of variance (ANOVA) was used to detect differences in mean values among groups. Post hoc tests were performed using Tukey's honestly significant difference test (Tukey's HSD) to examine group differences. Probit analysis was conducted using the ecotox package in the statistical software R (version 4.2.1). The Bt concentrations were logarithmically transformed, and the survival rates were introduced into the Probit model for parameter estimation. Using the estimated parameters, the concentration logarithm value corresponding to a probit value of 5 (corresponding to a

50% survival rate) was calculated and then converted to obtain the estimated LC_{50} value. Generalized linear models (GLMs) were used to evaluate the linear regression relationship between the relative concentration of Bt in the mixed solution and mortality. The synergistic ratio (SR) between Bt and Pl, referenced from [31], was calculated by dividing the LC_{50} obtained from the single treatment with Bt by the LC_{50} obtained from mixed treatment, where SR > 1 indicated synergistic effects, and SR < 1 indicated antagonistic effects. All statistical analyses were performed using the R-4.2.1 software [32]. The SR of Pl and Bt in each mixing ratio treatment was calculated using the following mathematical equation:

$$SR = \frac{LC_{50} \text{ of Bt alone}}{LC_{50} \text{ of the mixture (Pl + Bt)}}$$

3. Results

3.1. Oral Toxicity

3.1.1. The Oral Toxicity of *Photorhabdus luminescens* ATCC 29,999 and *Photorhabdus luminescens* 2103-UV against *Spodoptera frugiperda*

After being cultured for different periods of time (24, 72, and 120 h), the bacterial strains Pl ATCC 29,999 and Pl 2103-UV were provided to third-instar *S. frugiperda* larvae using the leaf-soaking method for feeding. For the treatments with Pl ATCC 29,999 or Pl 2103-UV cultured for 24 or 72 h, the mortality rate of *S. frugiperda* after three days did not significantly differ from the value of 0% obtained in the control group. However, in the treatment with Pl ATCC 29,999 or Pl 2103-UV cultured for 120 h, better insecticidal effects were observed in comparison with the control group ($F_{6,209} = 6.15$, $p = 2.48 \times 10^{-3}$) (Figure 1).

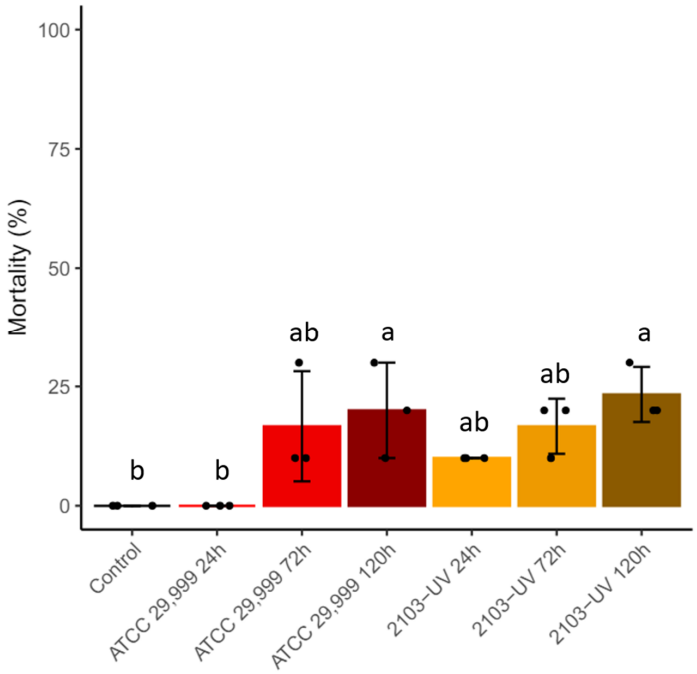

Figure 1. The mortality of third-instar *Spodoptera frugiperda* larvae caused by different culture durations (cultured for 24, 72, and 120 h, respectively) of *Photorhabdus luminescens* (Pl) ATCC 29,999 and 2103-UV. The *x*-axis represents the different treatments, and the *y*-axis represents the mortality (%). The black bar represents the control with the LB culture medium. Cultures of Pl ATCC 29,999 for 24, 72, and 120 h are represented by bars ranging from light red to dark red; cultures of Pl 2103-UV for 24, 72, and 120 h are represented by bars ranging from light yellow to brown. Cultures of Pl ATCC 29,999 and 2103-UV for 120 h increased the mortality rate of *S. frugiperda* ($F_{7,209} = 6.15$, $p = 2.48 \times 10^{-3}$). Each treatment consisted of 10 larvae per replicate, with three replicates. Different letters (a, b) indicate significant differences ($p < 0.05$) as measured with Tukey's HSD test.

3.1.2. Sublethal Effects of *Photorhabdus luminescens* ATCC 29,999 and *Photorhabdus luminescens* 2103-UV on *Spodoptera frugiperda*

Treatment with Pl ATCC 29,999 cultured for 24, 72, or 120 h progressively inhibited leaf consumption by third-instar *S. frugiperda* larvae. In the treatment with Pl ATCC 29,999 cultured for 120 h, the average leaf mass loss was 0.08 ± 0.01 g, which was significantly lower than that of the control group at 0.13 ± 0.03g. This treatment inhibited the feeding behavior of third-instar *S. frugiperda* larvae and exhibited a sublethal effect (Figure 2). On the other hand, Pl 2103-UV cultured for 24, 72, or 120 h did not significantly affect the leaf consumption of third-instar *S. frugiperda* larvae ($F_{7,209}$ = 14.4, $p = 7.97 \times 10^{-6}$), indicating that there were different pathogenic mechanisms between the two Pl strains.

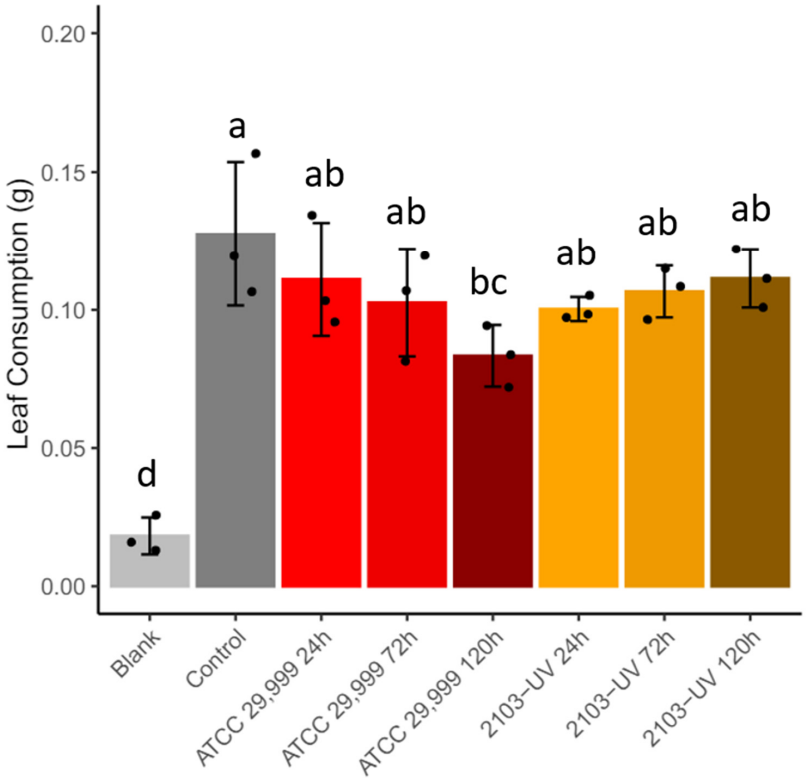

Figure 2. *Photorhabdus luminescens* (Pl) ATCC 29,999 and 2103-UV affected the leaf consumption of third-instar larvae of *Spodoptera frugiperda*. The *x*-axis represents the different treatments, and the *y*-axis represents the leaf consumption (g). The control group consisted of a control and a blank. The dark gray bars represent the control with the LB culture medium, and the light gray bars represent the blank with the weight loss due to the natural evaporation of water from the leaves under normal circumstances. Cultures of Pl ATCC 29,999 for 24, 72, and 120 h are represented by bars ranging from light red to dark red; cultures of Pl 2103-UV for 24, 72, and 120 h are represented by bars ranging from light yellow to brown. Cultures of Pl ATCC 29,999 for 120 h reduced the leaf consumption of *S. frugiperda* ($F_{7,209}$ = 14.4, $p = 7.97 \times 10^{-6}$). Each treatment consisted of 10 larvae per replicate, with three replicates. Different letters (a, b, c, d) indicate significant differences ($p < 0.05$) as measured with Tukey's HSD test.

3.1.3. The Oral Toxicity of Different Mixing Ratios (Pl:Bt) of 1:1, 1:3, and 1:5 for *Photorhabdus luminescens* ATCC 29,999, *Photorhabdus luminescens* 2103-UV, and *Bacillus thuringiensis* subsp. *kurstaki* against *Spodoptera frugiperda*

When Pl ATCC 29,999 and Pl 2103-UV were cultured for 120 h and mixed with commercial Bt at ratios of 1:1, 1:3, and 1:5, the LC_{50} values against *S. frugiperda* were lower than that of Bt alone (164 ppm), regardless of whether it was Pl ATCC 29,999 or Pl 2103-UV mixed with Bt at the above ratios. At the 1:1 mixing ratio, the 3-day LC_{50} was 154 ppm for Pl ATCC 29,999 and 132 ppm for Pl 2103-UV. At the 1:3 mixing ratio, the 3-day LC_{50} was 99 ppm for Pl ATCC 29,999 and 62 ppm for Pl 2103-UV. It can be observed that as the

proportion of Bt increased in the mixed solution, the insecticidal effect also increased. The 1:5 mixing ratio showed the most significant impact, with the 3-day LC_{50} being 83 ppm when Pl ATCC 29,999 was mixed with Bt at a 1:5 ratio, and 31 ppm when Pl 2103-UV was mixed with Bt at a 1:5 ratio. By dividing the LC_{50} of Bt alone by the LC_{50} of the mixed treatment (Pl + Bt), the synergistic ratio (SR) was obtained. The SR values were 1.98 and 5.29 when Pl ATCC 29,999 and Pl 2103-UV were mixed with Bt at a ratio of 1:5, respectively, indicating that the combined treatment of Bt and Pl was able to reduce the necessary Bt dose for achieving 50% mortality by approximately twofold and fivefold (Table 2).

Table 2. Synergistic effects of a mixed treatment of *Photorhabdus luminescens* ATCC 29,999, *Photorhabdus luminescens* 2103-UV, and *Bacillus thuringiensis* subsp. *kurstaki* (Bt) on the third-instar larvae of *Spodoptera frugiperda*, in which the median lethal concentration (LC_{50}) of Bt was estimated.

Treatment	Slope ± SE	LC_{50} (ppm) at 3 d	95% CI	χ^2	Synergistic Ratio
Pl, ATCC 29,999	NA	NA	NA	NA	—
Pl, 2103-UV	NA	NA	NA	NA	—
Bt	1.13 ± 0.28	164	49.78–277.96	2.71	—
ATCC 29,999 + Bt (1:1)	0.50 ± 0.34	154	79.50–7458.42 *	0.51	1.06
ATCC 29,999 + Bt (1:3)	1.60 ± 0.38	99	57.28–139.92	6.24	1.66
ATCC 29,999 + Bt (1:5)	0.82 ± 0.35	83	1.26–159.59	1.16	1.98
2103-UV + Bt (1:1)	0.65 ± 0.35	132	57.32–448.82	2.28	1.24
2103-UV + Bt (1:3)	0.36 ± 0.34	62	43.7–83.22	0.83	2.65
2103-UV + Bt (1:5)	0.71 ± 0.37	31	2.28–51.63 *	0.93	5.29

Pl: *Photorhabdus luminescens*, Bt: *Bacillus thuringiensis* subsp. *kurstaki*. The synergistic ratio (SR) between Bt and Pl was evaluated using the method referenced in [31], where the LC_{50} obtained from the Bt treatment was divided by the LC_{50} obtained from the mixed treatment, resulting in the calculation of the SR. SR > 1 indicates synergistic interaction; SR < 1 indicates antagonistic interaction. * The data were modified to enable the calculation of the 95% CI, and the raw data were adjusted to exclude irrational numbers.

3.1.4. The Relationship between the *Bacillus thuringiensis* subsp. *kurstaki* Concentration in the Mixed Solution and the Insecticidal Effect

Pl ATCC 29,999 and Pl 2103-UV were mixed with Bt at ratios of 1:1, 1:3, and 1:5 for insecticidal testing. As the concentration of Bt increased in the mixed solution, the synergistic insecticidal effect improved. The results of the linear regression analysis for the Bt concentration versus the mortality rate at each mixing ratio of Pl ATCC 29,999 or Pl 2103-UV with Bt are shown in Figure 3. The mortality rates of the 1:1 to 1:5 mixing ratios of Pl ATCC 29,999 with Bt were relatively low for the treatments with lower Bt concentrations (the first two concentrations counted from the left in Figure 3b–d), at around 40–56%. As the concentration of Bt increased, the mortality rates showed a positive correlation with the Bt concentration, especially with the 1:3 mixing ratio, which had the highest slope (1.6 ± 0.38). This suggests that the insecticidal efficacy improved significantly with increasing Bt concentration (Figure 3c). The mortality caused by the mixed solution of Pl ATCC 29,999 and Bt had a positive correlation with the concentration of Bt. In the mixed solutions of Pl 2103-UV and Bt with ratios of 1:1–1:5, the mortality rates ranged from 53% to 64% at lower concentrations (the first two concentrations in Figure 3e–g). However, for all mixing ratios of Pl 2103-UV with Bt, the slopes ranged from 0.36 to 0.71. This indicated that as the concentration of Bt increased, there was no significant improvement in the insecticidal efficacy. This suggested a weaker correlation between the mixed solution of Pl 2103-UV with Bt and the concentration of Bt. By analyzing the mortality data of low-concentration treatments for each mixing ratio, it was shown that the mortality rates of the mixed solutions of Pl 2103-UV and Bt at low concentrations were higher than those of Pl ATCC 29,999 with Bt ($F_{5,17} = 17.1$, $p = 4.27 \times 10^{-5}$), especially for the 1:5 ratio, where the low-concentration treatment could cause over 60% mortality (Figure 4). This indicated that the mixed solution of Pl 2103-UV and Bt exhibited higher insecticidal efficacy, even at low concentrations. Since the insecticidal efficacy did not significantly increase with an increase in the Bt concentration, the slopes were lower than those of the mixed solutions of Pl ATCC 29,999 and Bt.

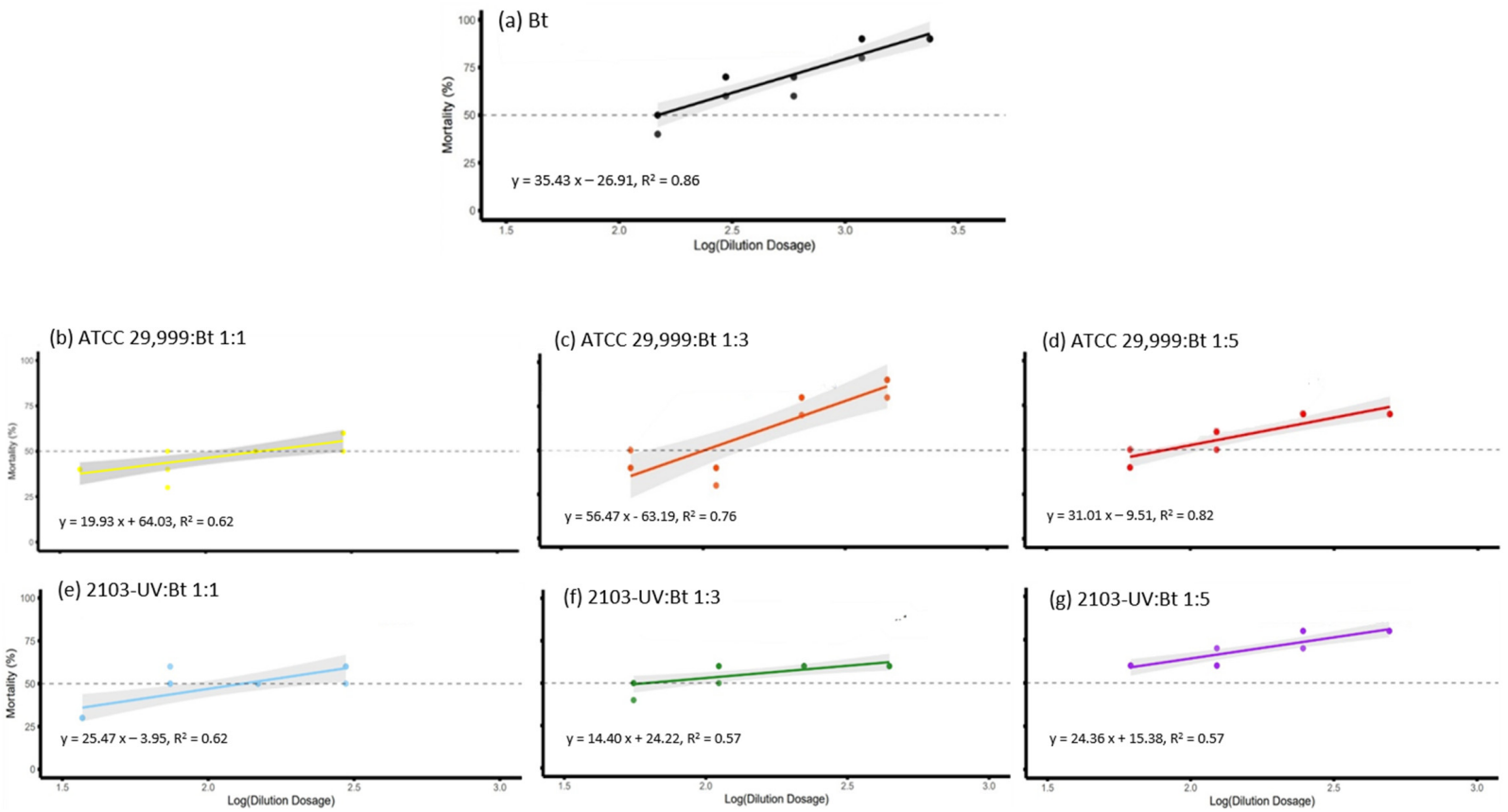

Figure 3. Correlation between the mortality rates of third-instar *Spodoptera frugiperda* larvae and the concentration of *Bacillus thuringiensis* subsp. *kurstaki* (Bt) in the mixed solution with different mixing ratios of the *Photorhabdus luminescens* (Pl) ATCC 29,999, 2103-UV, and *Bacillus thuringiensis* subsp. *kurstaki* treatments. Pl and Bt were mixed in the ratios of 1:1, 1:3, and 1:5, and the mixture was provided for consumption by third-instar *S. frugiperda* larvae. The *x*-axis represents the logarithm (base 10) of the concentration of Bt in the mixture (ppm), and the *y*-axis represents the mortality (%). The linear regression lines of different colors represent different treatments. The dashed line represents the 50% mortality threshold for the tested *S. frugiperda* individuals. The shaded area reflects the 95% confidence level, which was estimated using the ggplot2 R package. (**a**) Bt treatment, Slope = 1.13, $R^2 = 0.86$, $p = 6.82 \times 10^{-7}$; (**b**) Pl ATCC 29,999 + Bt 1:1, Slope = 0.5, $R^2 = 0.62$, $p = 2.27 \times 10^{-3}$; (**c**) Pl ATCC 29,999 + Bt 1:3, Slope = 1.6, $R^2 = 0.76$, $p = 2.12 \times 10^{-4}$; (**d**) Pl ATCC 29,999 + Bt 1:5, Slope = 0.82, $R^2 = 0.82$, $p = 5.54 \times 10^{-5}$; (**e**) Pl 2103-UV + Bt 1:1, Slope = 0.65, $R^2 = 0.62$, $p = 2.41 \times 10^{-3}$; (**f**) Pl 2103-UV + Bt 1:3, Slope = 0.36, $R^2 = 0.57$, $p = 6.81 \times 10^{-7}$; (**g**) 2103-UV + Bt 1:5, Slope = 0.71, $R^2 = 0.81$, $p = 6.81 \times 10^{-7}$.

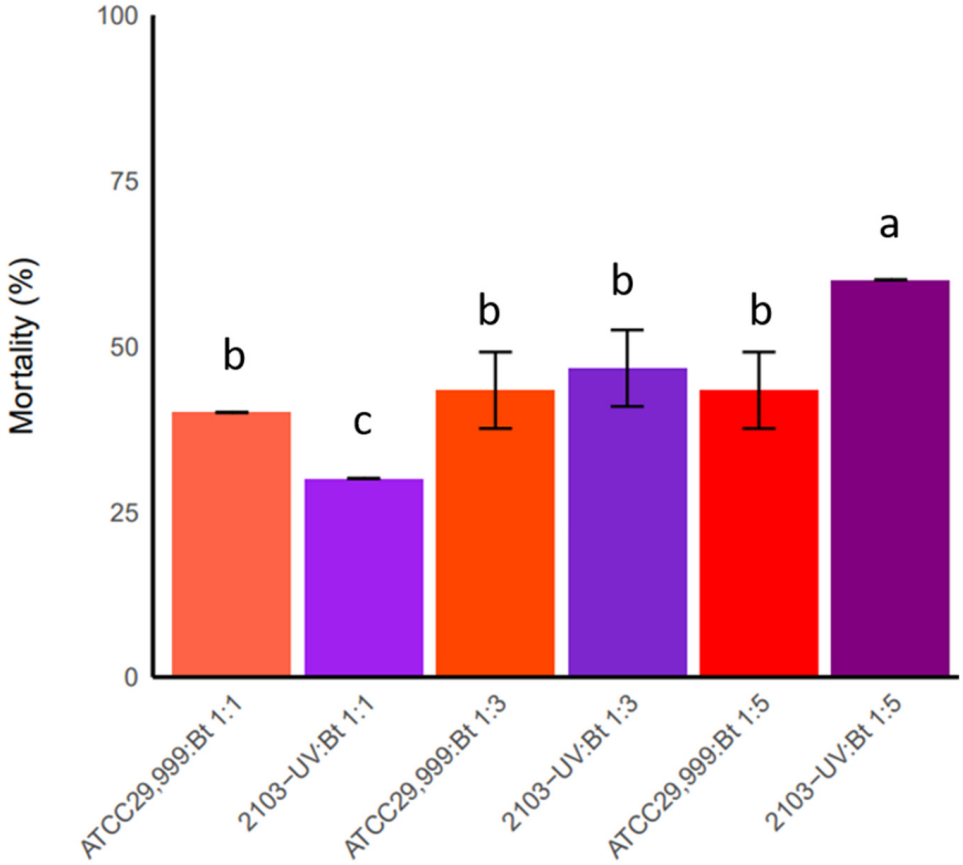

Figure 4. Mortality of third-instar *Spodoptera frugiperda* larvae treated with low doses of different mixing ratios of *Photorhabdus luminescens* (Pl) ATCC 29,999, 2103-UV, and *Bacillus thuringiensis* subsp. *kurstaki* (Bt). The *x*-axis represents the different treatments, and the *y*-axis represents the mortality (%). Pl ATCC 29,999 and Bt in ratios of Pl:Bt 1:1, 1:3, and 1:5 at low concentrations are represented by bars ranging from light red to dark red; Pl 2103-UV and Bt in ratios of Pl:Bt 1:1, 1:3, and 1:5 at low concentrations are represented by bars ranging from light purple to dark purple. The mortality rate of the Pl 2103-UV and Bt mixture was higher in the low-concentration treatment than that of Pl ATCC 29,999 and Bt ($F_{5,17} = 17.1$, $p = 4.27 \times 10^{-5}$). Each treatment consisted of 10 larvae per replicate, with three replicates. Different letters (a, b, c) indicate significant differences ($p < 0.05$) as measured with Tukey's HSD test.

3.1.5. The Effects of Different Mixing Ratios (Pl:Bt) of 1:1, 1:3, and 1:5 of *Photorhabdus luminescens* ATCC 29,999, *Photorhabdus luminescens* 2103-UV, and *Bacillus thuringiensis* subsp. *kurstaki* on the Median Lethal Time (LT_{50}) for *Spodoptera frugiperda*

Pl ATCC 29,999 and Pl 2103-UV were mixed with commercial Bt at ratios of 1:1, 1:3, and 1:5, and the median lethal time (LT_{50}) for *S. frugiperda* was observed for each treatment. Compared with the control group (Figure 5a), regardless of whether the mixed solution contained Pl ATCC 29,999 or Pl 2103-UV with Bt, as the density of Bt increased in the mixed solution, there was no significant difference in the killing speed, with the LT_{50} being achieved at around 36 h (Figure 5b–g).

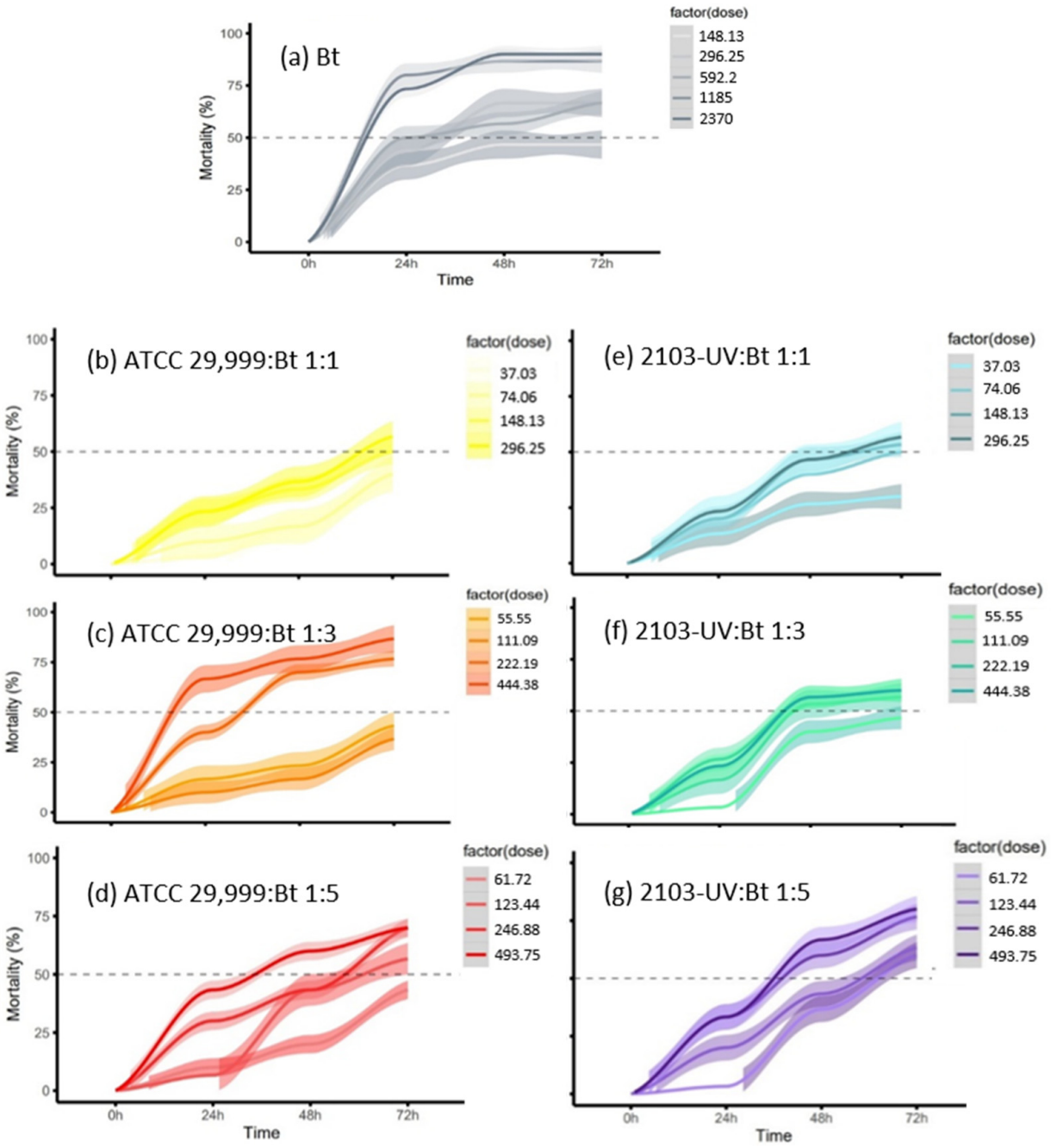

Figure 5. The LT_{50} (median lethal time) of third-instar *Spodoptera frugiperda* with treatments at different mixing ratios of *Photorhabdus luminescens* (Pl) ATCC 29,999, 2103-UV, and *Bacillus thuringiensis* subsp. *kurstaki* (Bt). Pl and Bt were mixed in ratios of 1:1, 1:3, and 1:5, and the mixture was provided for consumption by third-instar *S. frugiperda* larvae. The *x*-axis represents the time after treatment (hours), and the *y*-axis represents the mortality rate (%). Curves of different colors and shades represent the concentrations of Bt (ppm) present in the blended liquids with different mixing ratios. The dashed line represents the 50% mortality threshold for the tested *S. frugiperda* individuals. The shaded area reflects the 95% confidence level, which was estimated using the ggplot2 R package. (**a**) Bt treatment; (**b**) Pl ATCC 29,999 + Bt at 1:1; (**c**) Pl ATCC 29,999 + Bt at 1:3; (**d**) Pl ATCC 29,999 + Bt at 1:5; (**e**) Pl 2103-UV + Bt at 1:1; (**f**) Pl 2103-UV + Bt at 1:3; (**g**) Pl 2103-UV + Bt at 1:5.

3.2. Infection in the Hemolymph of Spodoptera frugiperda

Compared with the control group (the Pl ATCC 29,999 treatment alone), as the post-treatment time increased, the number of Pl ATCC 29,999 colonies counted was signifi-

cantly higher than that in the control for all mixing ratios (1:1, 1:3, and 1:5) ($F_{15,47}$ = 73.7, $p = 2 \times 10^{-16}$) (Figure 6). Among the different mixing ratios, the treatment with the ratio of 1:5 had the highest number of Pl ATCC 29,999 colonies. At 48–96 h post-treatment with the 1:5 treatment, the number of Pl ATCC 29,999 colonies in the hemolymph was significantly higher than that in the treatments with the other ratios, reaching 212,333 CFU/mL at 96 h. This suggests that as the proportion of Bt increased in the mixed solution, it enhanced the infection of Pl ATCC 29,999. We hypothesize that this is one of the reasons for the synergistic insecticidal effect of Pl and Bt.

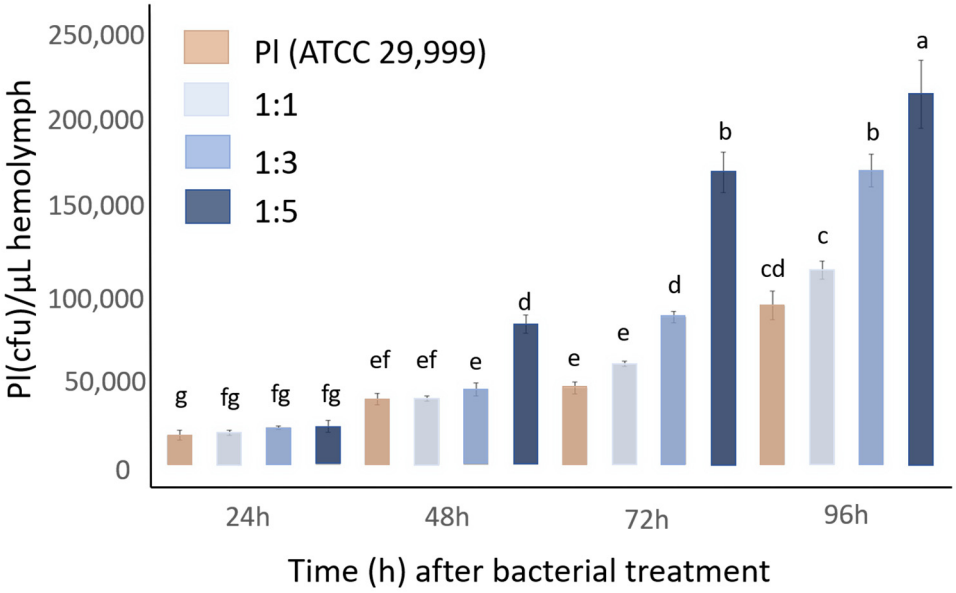

Figure 6. Infection of *Photorhabdus luminescens* (Pl) ATCC 29,999 into the hemocoel of fifth-instar *Spodoptera frugiperda* larvae with *Bacillus thuringiensis* subsp. *kurstaki* (Bt). After Pl was treated with or without Bt, the hemolymph of the treated larvae was collected every 24 h and cultured on NBTA medium for 48 h at 25 °C. Each measurement was replicated three times. The mixture of ATCC 29,999 and Bt enhanced the infection of ATCC 29,999 in the hemocoel of *S. frugiperda* ($F_{15,47}$ = 73.7, $p = 2 \times 10^{-16}$). Error bars indicate SE. Different letters (a, b, c, d, e, f, g) indicate significant differences ($p < 0.05$) as measured with Tukey's HSD test. Three plates of NBTA culture medium were prepared for the treatments at each mixing ratio, with five larvae per treatment.

3.3. Histopathological Sectioning of the Midgut Tissue of Spodoptera frugiperda

The treated *S. frugiperda* larvae were sectioned to observe the midgut tissue post-treatments. Compared with the healthy midgut of *S. frugiperda* in the control group (Figure 7a), the Bt treatment caused cracks in the midgut epithelial cells. It disrupted the integrity of the gut epithelium (Figure 7b,c), a typical pathology caused by Bt. In the treatment with Pl alone (Pl ATCC 29,999, Pl 2103-UV), vacuolation of the gut epithelial cells was observed, along with the appearance of vesicles and detached cell nuclei in the gut lumen (Figure 7d–g), which are pathologies caused by Pl infection. In the mixed treatments, more cracks were present in the gut epithelium as a result of Bt, and more vesicles appeared in the gut lumen. Additionally, the vacuolation phenomenon caused by the fixed dose of Pl also significantly increased, indicating the synergistic pathological effect of Bt on Pl infection (Figure 7h,i).

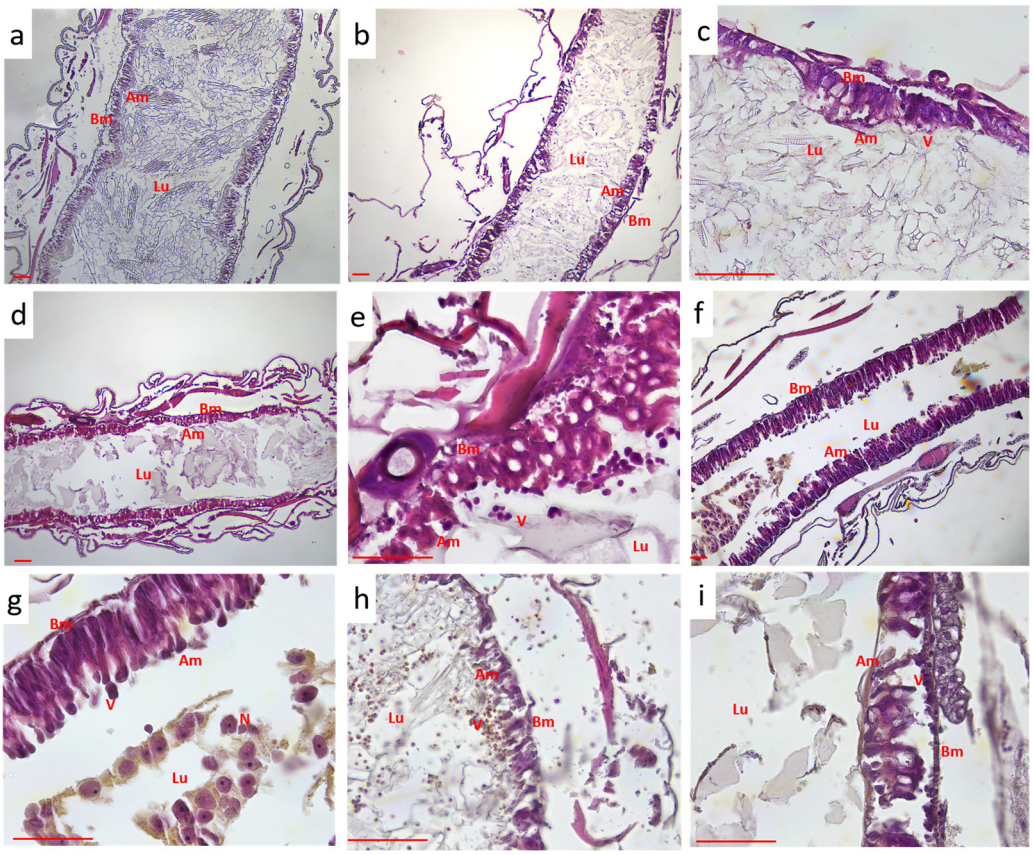

Figure 7. Histopathological effects on the midgut tissue of third-instar *S. frugiperda* larvae: (**a**) non-treatment, (**b**) *Bacillus thuringiensis* subsp. *kurstaki* (Bt), (**c**) detail of the section from the single treatment with Bt, (**d**) *Photorhabdus luminescens* (Pl) ATCC 29,999 in whole broth (after 120 h of incubation), (**e**) detail of the section from the single treatment with Pl ATCC 29,999, (**f**) Pl 2103-UV in whole broth (after 120 h incubation), (**g**) detail of the section from the single treatment with Pl 2103-UV, (**h**) combined treatment of Pl ATCC 29,999 and Bt (mixed at a ratio of 1:5), and (**i**) combined treatment of Pl 2103-UV and Bt (mixed at a ratio of 1:5). Scale bar = 90 µm. Am, apical membrane; Bm, basal membrane; Lu, lumen; N, nucleus; V, vesicle formation.

Compared with those in the control group and the Bt treatment alone, the number of vesicles produced in the Pl + Bt treatments was significantly higher ($F_{5,29}$ = 157.3, $p = 2 \times 10^{-16}$). Particularly in the Pl ATCC 29,999 + Bt treatment, the number of vesicles in the gut lumen dramatically increased. A significant increase in the number of vesicles is associated with increased cell membrane permeability and immunosuppression [33]. An excessive increase in cell membrane permeability may lead to cell damage and the entry of toxins into cells. It is hypothesized that Pl ATCC 29,999 caused more vesicles to form in the gut epithelial cells, exacerbating the pathogenesis of Bt. However, in the Pl 2103-UV + Bt treatment, the number of vesicles produced was not different from the treatments with Pl alone (Pl ATCC 29,999, Pl 2103-UV) (Figure 8).

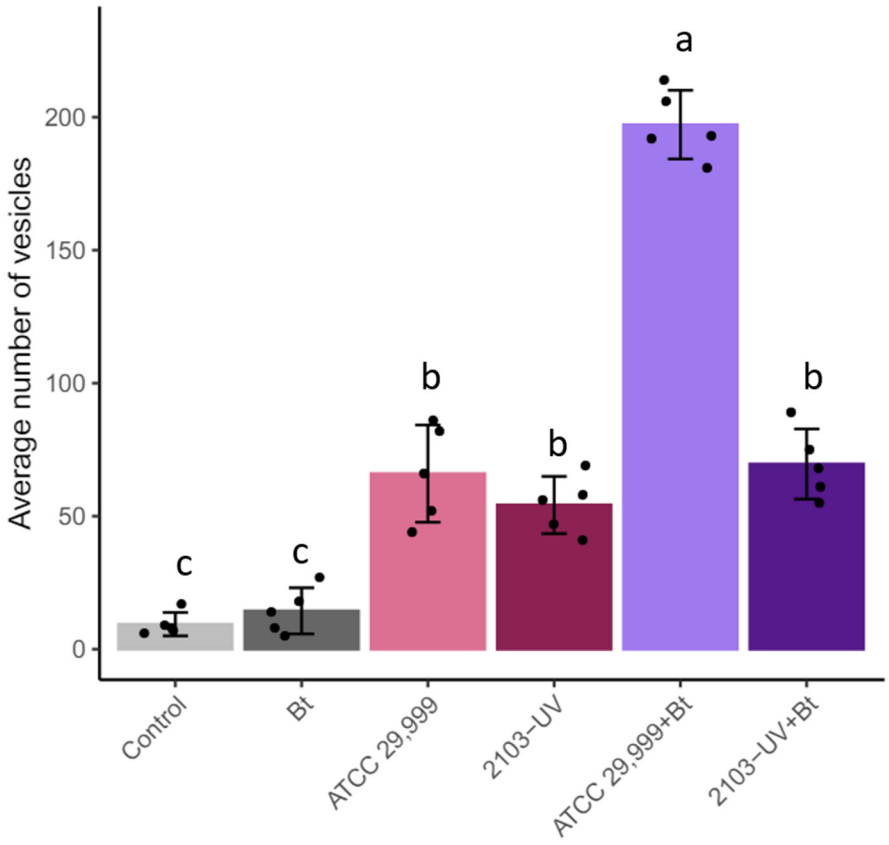

Figure 8. Histopathological effects on the midgut of third-instar *Spodoptera frugiperda* larvae due to combined treatments of the *Photorhabdus luminescens* (Pl) strains ATCC 29,999 and 2103-UV with *Bacillus thuringiensis* subsp. *kurstaki* (Bt). The *x*-axis represents the different treatments, and the *y*-axis represents average number of vesicles. Light gray bars represent the control, which represents the number of vesicles in healthy *S. frugiperda* midgut. Dark gray bars represent the number of vesicles in the midgut under Bt treatment. Light red bars represent the number of vesicles in the midgut under Pl ATCC 29,999 treatment. Dark red bars represent the number of vesicles in the midgut under the combined treatment of Pl ATCC 29,999 and Bt (mixed at a ratio of 1:5). Light purple bars represent the number of vesicles in the midgut after treatment with Pl 2103-UV alone. Dark purple bars represent the number of vesicles in the midgut after combined treatment with Pl 2103-UV and Bt (mixed at a ratio of 1:5). The combined treatments of Pl ATCC 29,999, Pl 2103-UV, and Bt increased the number of vesicles in the lumen of *S. frugiperda* ($F_{5,29} = 157.3$, $p = 2 \times 10^{-16}$). For each treatment, five photos of symptoms were taken in which the midgut cavity occupied 50% of the total image area, and the averages for each treatment were calculated using ImageJ (version 1.53a). Different letters (a, b, c) indicate significant differences ($p < 0.05$) as measured with Tukey's HSD test.

3.4. Phylogenetic Analysis and Porin Comparison of Photorhabdus luminescens ATCC 29,999 and Photorhabdus luminescens 2103-UV

To understand why Pl 2103-UV exhibited a better synergistic effect with Bt than Pl ATCC 29,999 did, we first compared the whole-genome sequencing results of Pl 2103-UV with those of other Pl strains in the NCBI database. In Figure 9, one can observe the phylogenetic relationship between Pl 2103-UV and Pl ATCC 29,999. We compiled the Pl strains that were closely related to Pl 2103-UV and Pl ATCC 29,999 and examined their differences in terms of their nematode hosts and geographical origins (Table 3) [34]. Pl 2103-UV is a native strain from Taiwan, while Pl ATCC 29,999 originated from Australia. Most other strains of Pl are from Mexico. We listed the porins produced by Pl 2103-UV and Pl ATCC 29,999 in Table 3. Porins have been reported in many studies to participate in the infection process of gram-negative bacteria. They are related to pathogenicity, bacterial colonization, and modulation of the host immune response [35]. We found that Pl 2103-UV

produces more types of porins than Pl ATCC 29,999 does. Among the porins produced by Pl 2103-UV, phosphoporin (PhoE) has been shown to induce the production of the cytokine tumor necrosis factor-α (TNF-α) by human leukocytes during acute inflammation, which is associated with cell apoptosis [36]. Through phylogenetic analysis of Pl 2103-UV and Pl ATCC 29,999, and by comparing the types of porins that they produce, we hypothesize that the diversity of porins may be one of the main reasons why Pl 2103-UV exhibited a better synergistic effect with Bt than that of Pl ATCC 29,999. We will further analyze the relationship between porins and insecticidal efficacy in the future.

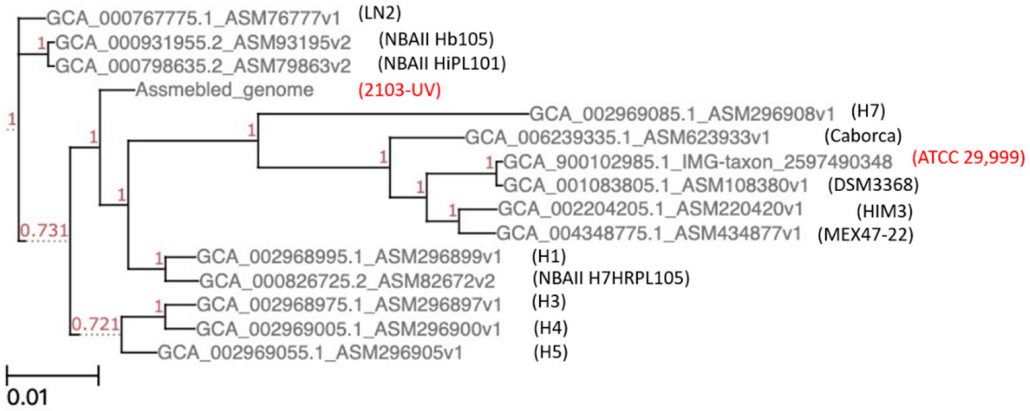

Figure 9. Phylogenetic analysis of the locally isolated *Photorhabdus luminescens* 2103-UV strain based on a comparison of 16S rDNA sequences. The 16S rDNA sequence of *P. luminescens* 2103-UV was compared with the 16S rDNA database for members of the *P. luminescens* species deposited in NCBI GenBank. The principle of constructing a phylogenetic tree is based on the similarity of DNA or protein sequences between organisms to infer their evolutionary relationships. First, the sequences of the organisms to be analyzed are aligned, and the sequence similarity between them is calculated to obtain a similarity matrix. Then, according to the similarity matrix, the evolutionary distances between organisms are computed using different evolutionary models. Next, based on the evolutionary distance matrix, clustering analysis algorithms are used to gradually cluster the microorganisms into a tree. Subsequently, various tree construction methods are adopted to construct a phylogenetic tree according to the clustering results, and the roots of the tree are determined by the outgroup method or other methods, distinguishing the outgroup and ingroup branches. Finally, the reliability of the constructed phylogenetic tree is evaluated using bootstrap confidence analysis, likelihood ratio tests, and other methods. The phylogenetic tree fully utilizes sequence alignment, evolutionary model calculations, clustering, and tree construction algorithms to recreate the evolutionary divergence process of organisms. The scale bar represents 0.01 nucleotide substitutions per site.

Table 3. The geographic origin of *Photorhabdus luminescens*, its nematode hosts, and the porins that is produces, respectively [34].

Strain	Nematode Host	Origin	Porin	Porin OmpA-F	Aquaporin	Glycoporin	Maltoporin	Aquaporin Z	Phosphoporin PhoE
P. luminescens	***Heterorhabditis* sp.**								
MEX47-22	*H. bacteriophora*	Mexico (Guanajuato)	+	A			+	+	
HIM3	*H. indica*	Mexico (Morelos)	+	A	+		+	+	
DSM 3368	*H. bacteriophora*	Australia (Victoria)	+				+		
ATCC 29,999	*H. bacteriophora*	Australia (Victoria)		A–F			+	+	
Caborca	*H. sonorensis*	Mexico (Caborca)	+	A, C				+	
H7	*H.* sp.*	India (Leh)	+	A	+				
2103-UV	*H.* sp.*	Taiwan (Kaohsiung)	+	A	+	+	+	+	+

* Species of *Heterorhabditis* that have not yet been identified.

4. Discussion

4.1. The Synergistic Effect of Photorhabdus luminescens and Bacillus thuringiensis on Lepidopteran Pests

A synergistic effect produced by *Photorhabdus luminescens* (Pl) and *Bacillus thuringiensis* subsp. *kurstaki* (Bt) has been previously documented in lepidopteran insects [37]. As symbiotic bacteria in entomopathogenic nematodes, Pl itself cannot breach the physical barriers of insects. Generally, it can only exert its pathogenicity after being introduced into the hemocoel with the assistance of nematodes. In this synergistic effect, Bt is similar to the nematode vector by assisting Pl in causing perforations in the insect midgut, enabling Pl to successfully infect the hemocoel. Once infected, Pl acts as a potentiating agent by secreting proteases and secondary metabolites that inhibit phenoloxidase synthesis in insects. This assists Bt in protoxin activation and overcoming the insect immune response, resulting in improved insecticidal activity [23,24,28,37,38].

In this study, in the mixed treatments of both Pl ATCC 29,999 and Pl 2103-UV with Bt, the LC_{50} values against *S. frugiperda* for all mixing ratios were lower than the LC_{50} of 164 ppm for Bt alone. This indicates that providing a mixture of Pl and Bt to *S. frugiperda* can enhance the insecticidal activity [37,39]. Among the different mixing ratios, the treatment with a ratio of 1:5 exhibited the most significant synergistic effect against *S. frugiperda* (ATCC 29,999 + Bt LC_{50} = 83 ppm, Pl 2103-UV + Bt LC_{50} = 31 ppm), resulting in approximately twofold and fivefold decreases in the LC_{50} compared with Bt alone, respectively. This means that the Pl ATCC 29,999 + Bt treatment reduced the lethal dose for 50% mortality of *S. frugiperda* by twofold, while the 2103-UV + Bt treatment at a ratio of 1:5 reduced it by fivefold (Table 2). However, in terms of the median lethal time, most LT_{50} values were around 36–48 h for the treatment at a ratio of 1:5 (Figure 5), indicating that there was no significant acceleration in the killing speed compared to that of Bt alone. In the context of *Plutella xylostella*, a synergistic effect between Pl ATCC 29,999 and *Bacillus thuringiensis* subsp. *aizawai* (Bta) was observed at a mixing ratio of 1:1, resulting in increased lethality. Antagonistic effects were observed at ratios of 1:4 and 4:1 [24]. The synergistic effects observed at different mixing ratios were likely due to variations in the insects tested or the specificities of different Pl and Bt strains. The *S. frugiperda* tested in this experiment is much larger than *Pl. xylostella*. In addition, the Bt strain used in this experiment was different from Bta, resulting in variations in toxins, so even if they shared the same toxin, their activity may have been different [40]. The combined treatment of Bt and *Photorhabdus* spp. was also reported to be effective against various lepidopteran pests, such as *Spodoptera litura*, *S. exigua*, and *Galleria mellonella*, demonstrating the synergistic insecticidal effects of Pl and Bt [28,39,40]. In the case of histopathological sections, in both *S. frugiperda* and *Pl. xylostella*, pathological features of both midgut epithelial cell disruption (a typical symptom caused by Bt) and cell vacuolation with increased vesicle formation (a typical symptom caused by Pl) were observed in the mixed treatments of Pl and Bt, confirming the occurrence of a synergistic effect [19,23,24,39,41–44].

The hemolymph assay showed that as the proportion of Bt increased in the mixed solution, the infection of Pl ATCC 29,999 in the hemocoel was enhanced (Figure 6), which was consistent with the results of [23]. From the histopathological perspective, it was also observed that as the Bt ratio increased, the pathological symptoms caused by Pl and Bt in the midgut were exacerbated (Figure 7). This indicates that Bt indeed facilitated the infection of Pl in the hemocoel, and the treatments with higher Bt mixing ratios also led to higher insecticidal activity. Therefore, this would be a key factor contributing to the synergistic effects between Pl and Bt.

4.2. Differences between Photorhabdus luminescens ATCC 29,999 and Photorhabdus luminescens 2103-UV

As Pl ATCC 29,999 and Pl 2103-UV were cultured in LB medium for up to 120 h, their insecticidal activity against *S. frugiperda* larvae significantly increased (Figure 1) because the highest protease activity was produced by Pl after 120 h of cultivation [24]. Compared

with Pl 2103-UV, Pl ATCC 29,999 reduced the leaf consumption of third-instar *S. frugiperda* larvae, similarly to what has been reported by Wu et al. [45]. The reason for this is unknown, but it may be related to the toxic proteins produced by Pl. The toxic *Tcs* proteins of Pl can damage the digestive system of insects. In contrast, toxic *Mcf* proteins induce abnormal behavior and reduce insect activity, which can decrease insect feeding behavior [46–51]. However, the particular mechanisms involved remain to be further investigated.

In the oral toxicity assay, the mixture of Pl 2103-UV and Bt was lethal at low concentrations, but increasing the concentration of Bt did not significantly increase the insecticidal activity, suggesting that the synergistic effect of Pl 2103-UV may be independent of the density of Bt. The insecticidal activity of Pl ATCC 29,999 increased with an increase in the Bt concentration, suggesting that the synergistic effect between Pl ATCC 29,999 and Bt had a greater correlation with the Bt concentration. This was also consistent with the results from the histopathological tissue sections, where the number of vacuoles in the midgut significantly increased when treated with the mixture of Pl ATCC 29,999 and Bt at a ratio of 1:5. However, there were no significant differences in the numbers of vacuoles between the mixed treatment with Pl 2103-UV and Bt and the single treatments with Pl ATCC 29,999 or Pl 2103-UV. This suggests that the modes of synergistic action among Pl ATCC 29,999, Pl 2103-UV, and Bt may be different. Compared with Pl ATCC 29,999, the Pl 2103-UV colonies had a darker color, appearing mustard yellow in the early stage of culture and turning into a blackish-brown color in the later stage (Table S2). Through phylogenetic analysis of the two *Photorhabdus* strains, we found that Pl 2103-UV produced more types of porins than Pl ATCC 29,999. Porins directly or indirectly affect the virulence, tolerance, and nutrient competition abilities of gram-negative bacteria. Porins are also involved in some key DNA repair molecules and can maintain cell membrane permeability and metabolic transport. The ability of Pl 2103-UV to withstand UV light may be related to its porins [52–57].

In the future, Pl porins can be further purified for bioassays to observe whether they have adverse effects that negatively affect the mortality or body weight of *S. frugiperda*. Additionally, it is worth exploring the inhibition of insects' immune response by the two strains of Pl. Then, by measuring the activity of melanization-related phenoloxidase in the hemolymph of *S. frugiperda* after treatment with Pl and by measuring the levels of reactive oxygen species (ROSs), which are important molecules in intracellular signaling and innate immune defense in insects, the immune inhibitory response induced by Pl can be assessed. This will help in understanding whether the two strains of Pl have different effects on inhibiting insects' immune responses [26,28,29].

4.3. The Potential of Photorhabdus luminescens as an Insecticide

Photorhabdus luminescens is capable of producing a variety of insecticidal toxins, which can be divided into four main categories. The first of these is the toxin complex (Tcs), a high-molecular-weight insecticidal protein complex consisting of Tca, Tcb, Tcc, and Tcd. Of these, Tca and Tcd have been reported as orally toxic to *Manduca sexta* larvae, *Leptinotarsa decemlineata* larvae, and *Bemisia tabaci* adults. After ingestion, they cause swelling and blebbing of columnar cells in the midgut, leading to the loss of nuclei, which has been shown to be a major cause of insect mortality [24,46,47,52,58]. PirAB is a binary toxin, and *pirA* and *pirB* encode the PirAB toxin via the flexible linker GL_4SER_3 in the DNA sequence. It is cytotoxic to insect midgut cells. When injected into the midgut of fourth-instar beet armyworm larvae, typical symptoms of apoptosis, such as cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation, have been observed. It has been reported to be injectable into *S. exigua* [59–61]. As for makes caterpillars floppy toxin (Mcf), it is injectable into the larvae of many lepidopteran pests, and causes damage to multiple cells after injection. The cell membrane is disrupted, leading to the apoptosis of midgut cells and plasmatocytes in the hemolymph. Apoptosis also causes the cells to lose their turgor, leaving the insect body flaccid and unsupported and, ultimately, leading to death [49–51]. *Photorhabdus virulence cassettes* (PVC) causes violent contraction of the insect host's actin, altering the arrangement of the cytoskeleton and disrupting hemocytes, resulting in a

decrease in the number of hemocytes in the hemolymph. It has insecticidal activity against *Galleria mellonella.* When the PVC plasmid from *Photorhabdus asymbiotica* ATCC 43,949 was expressed in *E. coli* K-12 and the culture or supernatant was injected into *Galleria mellonella* larvae, the mortality rate significantly increased (80–100%) [62,63]. In addition, several reports have shown that *P. luminescens* suppresses both cellular and humoral immune responses in insect hosts induced by external pathogen invasion [39,64]. Injection of the bacterium *P. luminescens* subsp. *laumondii* TT01 into *Drosophila melanogaster* modulated the immune *Tep4* while suppressing mRNA signaling of the toll-like receptor (toll pathway) and immune deficiency (Imd pathway) pathways, thereby reducing antimicrobial peptide (AMP) biosynthesis [65]. A culture of *P. temperata* subsp. *temperata* was used to inhibit the expression of two oxidases, Duox and Nox, involved in the synthesis of reactive oxygen species (ROSs), thereby reducing ROS production in *Pl. xylostella* [28]. Injection of a culture of *P. luminescens* H06 and the bacterial secondary metabolite benzylideneacetone (BZA) into *Octodonta nipae* inhibited the activity of phospholipase A2 (PLA2), which is necessary for the production of arachidonic acid (AA), an essential immune precursor of the eicosanoid class derived from AA. This also reduced the enzymatic activity of phenoloxidase (PO), effectively suppressing both cellular and humoral immune responses in insects [29]. However, potential barriers to the development of new microbial formulations include (1) difficulties in large-scale production, (2) high production costs, (3) environmental tolerance (temperature, humidity, UV light), and (4) short shelf life [66]. Previous research showed that culture broths of *Photorhabdus* spp. retained inhibitory activity against the root-knot nematode *Meloidogyne incognita* for approximately 2–5 months, reducing the egg mass numbers, female numbers, and the number of root galls [67]. The antifungal small molecule trans-cinnamic acid (TCA) produced by *Photorhabdus* spp. Has been shown to exhibit good thermal stability [68]. A 51.8 kDa metalloprotease isolated from *P. luminescens* 0805-P5G showed oral and injection toxicity against *Pl. xylostella*, retaining 100% of its enzyme activity after 30 min of treatment at 14–60 °C and 35% of its activity at 90 °C, demonstrating the heat stability of this protease [69]. When extracts from *P. luminescens* cultures were injected into *G. mellonella*, the extracts exhibited 100% insecticidal activity after 30 min of heat treatment at 70 °C. They retained 60% and 40% of their activity at 80 °C and 90 °C, respectively, indicating their high heat stability. Notably, treatment with proteinase K did not affect the insecticidal activity, suggesting that this insecticidal toxin may not be a simple protein toxin [70]. A similar situation was observed with extracts from *Photorhabdus temperata* J4 and J5, where culture supernatants retained 95% of their insecticidal activity after 30 min of heat treatment at 80 °C, demonstrating their heat stability. Meanwhile, treatment with proteinase K did not change the insecticidal activity of the culture supernatants from all screened strains [71]. The above information shows that *Photorhabdus* can produce various metabolites with insecticidal and antimicrobial properties that possess heat stability and UV resistance, highlighting its potential as a novel microbial formulation. However, information on its low-temperature tolerance, drought resistance, high humidity tolerance, and shelf life is still lacking, and further testing is needed to fully assess its viability.

5. Conclusions

Treatment using Pl ATCC 29,999 and 2103-UV in combination with Bt effectively reduced the required dosage of Bt by approximately 2–5 times, leading to higher insecticidal efficacy. Reducing Bt usage can slow down the development of resistance against Bt in *S. frugiperda*. This study used a hemolymph assay to reveal evidence of the quantity and infection status of Pl entering the hemocoel with the assistance of Bt, effectively enhancing the synergistic insecticidal efficiency. Furthermore, through histopathological tissue sections of the midgut, we discussed the synergistic pathological effects of Pl and Bt in depth. Pl caused the production of numerous vacuoles and exacerbated vacuolization symptoms in the gut lumen, indicating that the increased Bt concentration facilitated Pl's entry into the hemocoel of *S. frugiperda* and its speed of infection. Interestingly, the

treatment containing a mixture of Pl 2103-UV and Bt did not induce as many vacuoles in the gut lumen as the Pl ATCC 29,999 treatment. Additionally, while Pl ATCC 29,999 inhibited the feeding of *S. frugiperda*, Pl 2103-UV did not, suggesting that the modes of action and synergistic interactions with Bt may differ between these two strains. The results of the phylogenetic analysis provide a possible explanation for the differences in virulence among Pl strains, particularly in their porin profiles. For example, the phosphoporin (PhoE) of Pl 2103-UV may have the ability to induce apoptosis in insect cells. In addition, Pl ATCC 29,999 was collected in Australia, while Pl 2103-UV was collected in Taiwan. Due to the different collection locations, the characteristics of these two Pl strains may also differ. In this study, the synergistic lethal effects of Pl and Bt on the lepidopteran *S. frugiperda* were emphasized. The results of this study can serve as a reference case for using Pl as a synergistic agent in combination with other microbial insecticides to manage emerging lepidopteran pests, to continue to reduce pesticide usage, and to achieve sustainable pest management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14060864/s1, Table S1: The concentration of *Bacillus thuringiensis* subsp. *kurstaki*; oral toxicity test; Table S2: Colony morphology of *Photorhabdus luminescens* (Pl) ATCC 29,999 and 2103-UV after 48 h of cultivation on solid LA medium and the appearance of the bacterial cultures after 48 and 120 h of cultivation in liquid LB medium.

Author Contributions: L.-H.W.: Conceptualization, methodology, data curation, formal analysis, writing—review and editing, supervision, funding acquisition; T.-Y.C.: methodology, software, formal analysis, investigation, writing—original draft preparation, visualization; C.H.: resources, data curation. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science and Technology, Taiwan (MOST 111-2313-B-020-003-MY3) and by the Animal and Plant Health Inspection Agency (APHIA), Ministry of Agriculture (MOA) (112AS-5.5.3-BQ-B1).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: We are grateful to the scientists who conducted and published their primary studies, the three anonymous editors, and the reviewers for their constructive comments on the manuscript.

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

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