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Performance of *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) Strains on Eggs from Different Populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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Abstract

Tomato is the most widely cultivated fruit–vegetable worldwide, and the tomato leafminer (*Tuta absoluta*) is the primary pest of this crop. In this context, biological control using parasitoids belonging to the genus *Trichogramma* is crucial. This study aimed to evaluate the biological characteristics of *T. pretiosum* strains collected from different locations and exposed to eggs from various *T. absoluta* populations/generations, using parameters such as parasitism capacity, viability (percentage of emergence), sex ratio, and female longevity. The presence of endosymbionts in the *T. absoluta* populations was also assessed. The experiment followed a randomized design, with treatments consisting of eggs from *T. absoluta* populations collected in different years (2019, 2020, 2021, 2022, and 2023) and different strains of *T. pretiosum*. We used 20 replicates, with one female per replicate in each treatment, organized in a 5 × 4 factorial scheme (five populations of *T. absoluta* × four strains of *T. pretiosum*). The S2 strain of *T. pretiosum* was found to be the most efficient in terms of biological characteristics for parasitism of *T. absoluta* eggs, especially in *T. absoluta* populations collected in recent years (2022 and 2023). These results suggest that S2 is the preferred strain for future studies aimed at using this parasitoid as a control agent to combat *T. absoluta*. The endosymbionts *Arsenophonus* and *Serratia* were identified in *T. absoluta* populations collected in 2019–2020 and 2020–2021, respectively. These findings highlight the presence of these microorganisms in pest populations in different years.

Keywords: biological control; endosymbionts; *Solanum lycopersicum* L.; tomato leafminer; Trichogrammatid

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is the most widely consumed and cultivated fruit–vegetable worldwide [1]. Despite its high production, the crop is vulnerable to a variety of phytosanitary problems that require intensive management, from planting to harvest [2].

The main threat to this crop is the tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), which can result in significant losses, affecting up to 100% production. This pest causes substantial damage and represents a considerable challenge for control [3].

In Brazil, the control of *T. absoluta* is often carried out by multiple insecticide applications [3,4]. However, this approach is considered undesirable because of environmental and economic concerns. Repeated insecticide application can have adverse effects on the natural enemies of the pest, compromising the balance of the agroecosystem [5]. Moreover, the selection of insecticide-resistant *T. absoluta* populations is a significant risk, increasing the challenges in pest control [4].

The implementation of pest management techniques can provide significant benefits to production systems by reducing pesticide use by > 50%. Several tools are available for pest management in tomato cultivation, with biological control being promising [2]. Parasitoids belonging to the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) play a crucial role and are extensively employed in biological control programs worldwide. Their application is particularly effective in the management of lepidopteran pests and has been adopted in more than 15 million hectares across more than 40 countries [2,6].

Trichogramma pretiosum Riley is one of the most common species found in several countries, especially in disturbed environments, such as agroecosystems [2,4,7,8]. Several studies have shown that the choice of species or strain is a determining factor in the success of the mass release of these parasitoids [2]. Therefore, studies aimed at selecting species/strains are of great importance, especially for *Trichogramma* species, which exhibit considerable variation in foraging behavior (habitat and host location), host preference, recognition and acceptance of crops, and responses to environmental conditions [2,9].

The collection of *T. pretiosum* strains from the areas where they are released aims to eliminate adaptive costs, thus enabling pest management without adaptation costs for the parasitoid. However, each species or strain may exhibit distinct characteristics, such as parasitism rate and viability (emergence) of its offspring. This serves to identify the strain that is best adapted to the host within a biological control program [2,10].

Quality assessments of these strains are conducted over successive generations in the laboratory, focusing on key biological characteristics, such as parasitism rate and viability, which are expected to be at least 76% and 72%, respectively. Additionally, the sex ratio should be above 0.5 [7]. The implementation of quality control is essential, as maintaining these characteristics is a fundamental step in insect-rearing programs in the laboratory, ensuring that they resemble wild-type [2,7,11,12].

Changes observed in insect populations that are maintained in the laboratory are often linked to genetic control, involving selection effects on existing genotypes and random loss of genotypes due to genetic drift. However, when collecting wild species of natural enemies and/or hosts for laboratory rearing, genetic changes can be expected, owing to the domestication process of these species [2,13].

Beyond the intrinsic characteristics of the *T. pretiosum* strains, an increasingly relevant aspect in biological control studies is the role of endosymbiotic microorganisms associated with host insects, such as *T. absoluta*. These microorganisms, commonly vertically transmitted and widely distributed among various insect orders, have been shown to directly or indirectly influence several key aspects of host biology. These effects include changes in fecundity, modulation of immune responses, increased tolerance to environmental stressors, and impacts on tritrophic interactions, particularly with natural enemies, such as parasitoids and predators [14,15].

The objective of this study was to evaluate the biological characteristics of *T. pretiosum* strains collected from different locations and subjected to eggs from different *T. absoluta*

populations by assessing their parasitism capacity, viability (percentage of emergence), sex ratio, female longevity, and presence of endosymbionts in these *T. absoluta* populations.

2. Materials and Methods

2.1. Research Location

The assays were conducted in the laboratories of the Research Group on Integrated Pest Management in Agriculture (AGRIMIP), affiliated with the Department of Plant Protection of the School of Agricultural Sciences at São Paulo State University (UNESP), which is located on the Botucatu Campus, São Paulo, Brazil. The geographical coordinates of the site are 22°50′40.1″ S latitude and 48°26′02.5″ W longitude at an altitude of 804 m. The experiments were conducted in climate-controlled facilities, maintained at 25 ± 2 °C, a photoperiod of 12 h, and a relative humidity of 70 ± 10%.

2.2. Collection, Rearing, and Maintenance of T. absoluta in the Laboratory

Populations of *T. absoluta* were collected in the years 2019, 2020, 2021, 2022, and 2023 at the "Taquara Branca" farm, located in Sumaré, in the state of São Paulo, Brazil, where hybrids of indeterminate-growth tomatoes are cultivated for fresh fruit consumption. Infested compound leaves containing larvae were collected, rather than just leaflets, to maintain the turgor of the leaflets attached to the petiole. During collection, the leaves were stored in cardboard boxes arranged in a manner that did not exert pressure on the larvae and allowed aeration between layers. These boxes were then transported to the AGRIMIP laboratory.

The populations of *T. absoluta* used in this study were collected in 2019, 2020, 2021, 2022, and 2023 and were treated as distinct populations throughout the analyses. Each population was maintained under controlled laboratory conditions from its respective year of collection until the evaluations conducted in 2023, following the same rearing regime for all. The population collected in 2023 remained under laboratory conditions for a significantly shorter period and was, therefore, considered the most representative in terms of conserving the genetic characteristics of the original field population.

Populations of *T. absoluta* were maintained under controlled conditions inside rectangular cages measuring 0.4 m length × 0.4 m width (at the base) × 0.5 m height. Each population was housed in a separate cage to maintain its individual characteristics. These cages featured a lateral opening covered with a nylon mesh. Plastic pots with soil and tomato plants (30 days after transplanting and with six leaves) were placed inside the cages; no insecticides were applied to the plants. The egg-bearing plants were subsequently transferred to other cages of identical size to allow hatching. During the larval stage, plants damaged by the larvae were replaced every 3 days. When the larvae reached the pupal stage, they were maintained in cages with dried plants until adult emergence. Adults were fed a 10% honey solution (90% distilled water and 10% pure wild honey; 2 mL of the solution was soaked in cotton inside a Petri dish), while the larvae were fed tomato plants of the Santa Clara cultivar [16].

2.3. Collection, Rearing, and Maintenance of T. pretiosum Strains

The *T. pretiosum* strains used were collected from various host plants in different regions of the state of São Paulo, Brazil, and maintained in the AGRIMIP laboratory (Table 1). Four *T. pretiosum* strains were used in the present study.

To obtain the *T. pretiosum* strains S1, S2, and S4, eggs of *Ephestia kuehniella* were exposed in the field using sentinel traps. These traps consisted of egg cards fixed directly to host plants and protected with anti-aphid nets to prevent predation and external interference. For S3, natural ovipositions of *Spodoptera* spp. were used, collected opportunistically from

the field. After collection, all *T. pretiosum* strains were reared and multiplied on *E. kuehniella* eggs (Table 1).

Table 1. *Trichogramma pretiosum* strains with their respective natural host plants and collection origins.

Strain	Host Insect (Eggs)	Host Plants	Collection Origin
S1	*Ephestia kuehniella*	Brassicas	Pardinho, São Paulo, Brazil
S2	*Ephestia kuehniella*	Tomato	Mogi Mirim, São Paulo, Brazil
S3	*Spodoptera frugiperda*	Maize	Botucatu, São Paulo, Brazil
S4	*Ephestia kuehniella*	Atemoya	Pardinho, São Paulo, Brazil

S = strain.

All *T. pretiosum* strains were reared and multiplied by eggs of *E. kuehniella* (Zeller) (Lepidoptera: Pyralidae). This alternative host was reared on a diet of whole wheat flour (97%) and brewer's yeast (3%) [17,18]. Rearing was conducted in rectangular trays of 5 L (7 cm height × 29 cm width × 35 cm depth), each containing 1 kg of diet, into which 0.36 g of fresh *E. kuehniella* eggs were inoculated. The trays were then sealed with plastic adhesive tape and maintained in a climate-controlled room at 18 ± 2 °C and a photoperiod of 14 h for larval development. The trays remained in this room until the emergence of the first adults; thereafter, they were transferred to another room maintained at 25 ± 2 °C and a photoperiod of 14 h, where the adults were collected daily. For parasitoid rearing, the alternative host eggs were glued onto pieces of double-sided adhesive tape (8 cm length × 2 cm width, polyester, Dimencional®) and subsequently inactivated by exposure to ultraviolet light (45 min at 15 cm from the light source), thereby preventing cannibalism of parasitized eggs [18,19]. The eggs were then offered to the parasitoids over a 24 h period.

Adult parasitoids were kept in transparent plastic containers of 3.6 L (15 cm length × 15 cm width × 25.6 cm height), completely sealed with polyvinyl chloride (PVC) plastic film. For feeding, pure honey was provided and applied as thin streaks on the inner sides of the containers. The containers holding adults of the different *T. pretiosum* strains were maintained in a climate-controlled room at 25 ± 1 °C, a relative humidity of 70 ± 10%, and a photoperiod of 14 h. Newly emerged parasitoids were used for experiments or to maintain colonies. To prevent cross-contamination between strains, the colonies were kept in separate rooms.

2.4. Selection of T. pretiosum Strains for the Control of T. absoluta

The experiment was conducted in a completely randomized design, with treatments consisting of eggs from *T. absoluta* populations collected in different years (2019, 2020, 2021, 2022, and 2023) and different *T. pretiosum* strains, using 20 replicates, with one female per replication, organized in a 5 × 4 factorial scheme (five *T. absoluta* populations × four *T. pretiosum* strains).

Females (24 h old) from each strain were isolated in transparent flat-bottom glass tubes (30 mm width × 100 mm length), sealed with PVC plastic film (Magipack®), and fed with a droplet of pure honey. Twenty *T. absoluta* eggs were offered per replicate in each collection year, with 24 h of embryonic development per female (Figure 1). The eggs were collected using a fine-tipped brush (No. 10, Acrilex®) previously moistened with distilled water. Subsequently, they were transferred to a strip of Vergé-type paper (20 mm width × 70 mm length) moistened with small droplets of distilled water to facilitate adhesion of the eggs to the paper.

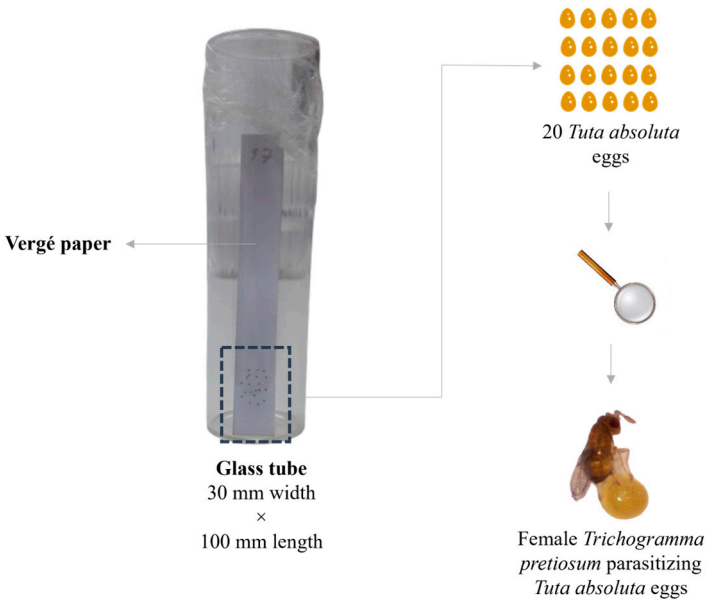

Figure 1. Schematic representation of the tube used and arrangement of *Tuta absoluta* eggs offered to each *Trichogramma pretiosum* female.

The estimation of longevity was assessed in F0 females, that is, the females used for egg parasitism. After 24 h, the strips with eggs were removed, and the females were evaluated daily to validate the longevity data. The following parameters were assessed: parasitism (%) (Equation (1)), emergence (%) (viability) (Equation (2)), female longevity after parasitism (days), number of adults emerging per egg, and sex ratio (Equation (3)).

$$\text{Parasitism } (\%) = \frac{\text{Number of parasitized eggs (black eggs)}}{\text{Total number of host eggs exposed}} \times 100 \quad (1)$$

$$\text{Viability } (\%) = \frac{\text{Total number of parasitized eggs with emergence hole}}{\text{Total number of parasitized eggs (black eggs)}} \times 100 \quad (2)$$

$$\text{Sex ratio} = \frac{\text{Total number of emerged females}}{\text{Total number of emerged females} + \text{total number of emerged males}} \quad (3)$$

The number of individuals per egg was calculated by dividing the total number of adults by the total number of emergence holes observed in the 20 *T. absoluta* eggs in each tube. The sex of the parasitoids was determined using a stereomicroscope based on the sexual dimorphism exhibited by their antennae [20].

All *T. pretiosum* strains used in the experimental assays were derived from populations maintained under laboratory conditions for an average of 25 generations prior to testing.

2.5. Statistical Analysis

The variables under study were subjected to residual normality analysis using the Lilliefors test, whereas the homogeneity of variances was assessed using Bartlett's test. For data showing disparities, a square root transformation $\sqrt{(x + 0.5)}$ was applied. Subsequently, analysis of variance (ANOVA) was performed, and in cases of significant differences, Tukey's multiple comparison test was applied at a 5% significance level ($p \leq 0.05$). All analyses were conducted using the GENES statistical software version 1990.2023.45 [21].

In addition, Pearson's correlation analysis was performed at a 5% significance level, along with multivariate analysis using the hierarchical clustering method based on standardized mean Euclidean distance (D) and Ward's minimum variance method, as well as principal component analysis (PCA) through biplot graphical analysis, to assess the degree of dependence and obtain an integrated evaluation of the biological characteristics

of the *T. pretiosum* strains evaluated in *T. absoluta* populations. Pearson's correlation and multivariate analyses were performed using OriginPro® 2021 software [22].

2.6. Sampling, DNA Extraction, and Detection of Endosymbionts via Polymerase Chain Reaction (PCR) in T. absoluta Populations

2.6.1. Genomic DNA Extraction

For each *T. absoluta* population (collected in the years 2019, 2020, 2021, 2022, and 2023), genomic DNA extraction was performed from 20 fourth-instar larvae. The individuals were macerated in a solution of 80 µL of 10% Chelex 100 resin (Bio-Rad Laboratories) and 8 µL of proteinase K (20 mg/mL) [23]. The samples were placed in a heat block at 95 °C for 20 min. Subsequently, the supernatant was collected for PCR.

The sample size of 20 larvae was defined based on established protocols in the literature for detecting endosymbionts in insects [24,25], which indicate that sampling between 15 and 30 individuals per biological group is sufficient to detect the presence and infection frequency of commonly occurring endosymbionts.

2.6.2. PCR Reaction

DNA amplification of the different endosymbionts of the genera *Arsenophonus*, *Cardinium*, *Carsonella*, *Hamiltonella*, *Regiella*, *Rickettsia*, *Serratia*, *Sodalis*, *Spiroplasma*, *Wolbachia*, and *Pantoea*, and for the microsporidium (*Nosema* sp.), was performed using specific primers for endosymbiotic bacteria and a universal primer for *Nosema* sp. PCR was carried out in an INFINIGEN thermocycler (model TC-96CG) under specific conditions for each set of primers targeting the endosymbionts and the microsporidium of interest (Tables 2 and 3).

Table 2. Primers used for the identification of endosymbionts in *Tuta absoluta*.

Endosymbiont/ Microsporidium	Target Gene	Primer Sequence 5′ > 3′	Size (bp) *	Reference
Arsenophonus	16S rRNA	F-CGTTTGATGAATTCATAGTCAAA R-GTCCTCCAGTTAGTGTTACCCAAC	600	[26]
Cardinium	16S rRNA	F-TACTGTAAGAATAAGCACCGGC R-GTGGATCACTTAACGCTTTCG	900	[27]
Hamiltonella	16S rRNA	F-TGAGTAAAGTCTGGAATCTGG R-AGTTCAAGACCGCAACCTC	700	[28]
Spiroplasma	16S rRNA	F-GCTTAACTCCAGTTCGCC R-CCTGTCTCAATGTTAACCTC	800	[29]
Rickettsia	16S rRNA	F-GCTCAGAACGAACGCTATC R-GAAGGAAAGCATCTCTGC	900	[30]
Serratia	16S rRNA	F-CGCAGGCGGTTTGTTAAGTC R-CTTCAAGGGCACAACCTCCA	268	[31]
Wolbachia	16S rRNA	F-CGGGGGAAAAATTTATTGCT R-AGCTGTAATACAGAAAGTAAA	700	[32]
Sodalis	16S rRNA	F-ACCGCATAACGTCGCAAGACCR- TAACCCAACATTTCTCAACACGAG	1000	[33]
Carsonela	16S rRNA	F-CACGTGCTACAATGAGTAAAACAA R-GGTTCCCCTACAGCTACCTTG	279	[34]
Pantoea	16S rRNA	F-ACGGAGGGTGCAAGCGTTAAT R-AGGTAAGGTTCTTCGCGTTGCA	630	[35]
Regiella	16S rRNA	F-ATCGGGGAGTAGCTTGTCAT R-TACGGYTACCTTGTTACGACTT	1000	[36]
Nosema	16S rRNA	F-CACCAGGTTGATTCTGCC R-TTATAGTCCTGCTAATGGTTC	222	[37]

* bp = base pairs.

Table 3. PCR cycles used for the identification of endosymbionts in *Tuta absoluta*.

Endosymbionts	PCR Cycle Conditions
Arsenophonus and *Hamiltonella*	Initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min.
Spiroplasma and *Regiella*	Initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 min.
Rickettsia and *Cardinium*	Initial denaturation at 95 °C for 2 min, followed by 30 cycles of 92 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min.
Serratia	Initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 1 min.
Wolbachia	Initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min.
Sodalis	Initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 min.
Carsonela	Initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.
Pantoea	Initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min.
Nosema	Initial denaturation at 95 °C for 4 min, followed by 45 cycles of 95 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 4 min.

The PCR mix consisted of 150 mM Tris-HCl pH 8.5, 40 mM $(NH_4)_2SO_4$, 4.0 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 U of Taq DNA polymerase (Neobio), 7.5 µL of nuclease-free water (Promega), 1 µL of each primer (10 mM), and 25 ng of genomic DNA per sample, totaling 25 µL of solution per tube and reaction. For *Nosema* sp., the mix was composed of 150 mM Tris-HCl pH 8.5, 40 mM $(NH_4)_2SO_4$, 4.0 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 U of Taq DNA polymerase (Neobio), 5 µL of nuclease-free water (Promega), 1.25 µL of each primer (10 mM), and 25 ng of genomic DNA.

PCR reactions included negative controls (reactions without DNA template) to monitor contamination as well as internal positive controls (DNA previously confirmed for each endosymbiont group tested) to ensure primer efficiency and specificity. The inclusion of these controls was essential to validate amplification results and to exclude false positives or negatives caused by technical errors or compromised reagents.

To visualize the PCR products, a 100 bp molecular marker (Norgen) and 1% agarose gel with Gel Red DNA staining were used, following the manufacturer's instructions (NeoBio). Agarose gels were visualized using an ultraviolet light transilluminator (Major Science). All DNA extraction procedures were performed at the Molecular Biology and Nematology Laboratory of the School of Agricultural Sciences at São Paulo State University (UNESP), in Botucatu, São Paulo, Brazil.

3. Results and Discussion

3.1. Performance and Comparative Analysis of Trichogramma pretiosum Strains Against Tuta absoluta Eggs

There were significant differences ($p \leq 0.05$) in the evaluated biological characteristics for the *T. absoluta* population factor (parasitism (P); longevity (L); number of adults per egg (NAE); and sex ratio (SR)), demonstrating that the treatments differed from each other, with no difference observed for viability. There were also significant differences ($p \leq 0.05$) in the evaluated biological characteristics for the *T. pretiosum* strain factor (parasitism (P); viability (V); longevity (L); and sex ratio (SR)), demonstrating that the treatments differed from each other, with no difference observed for the number of adults per egg. There was a significant interaction between *T. pretiosum* strains and different *T. absoluta* populations with respect to parasitism, number of adults per egg, and sex ratio, with no interaction between viability and longevity (Table 4).

Table 4. Summary of the analysis of variance for the biological characteristics evaluated in four *Trichogramma pretiosum* strains exposed to eggs from different *Tuta absoluta* populations.

Sources of Variation	Variables				
	P	**V**	**L**	**NAE**	**SR**
T. absoluta populations (A)	8.72 *	0.92 ^{ns}	8.66 *	3.09 *	3.72 *
T. pretiosum strains (B)	30.30 *	6.44 *	37.30 *	2.56 ^{ns}	5.94 *
A × B	2.32 *	1.47 ^{ns}	2.01 ^{ns}	4.30 *	8.81 *
Overall mean	47.30	97.50	12.40	1.01	0.68
CV (%)	27.70	6.11	6.97	0.35	0.49

* Significant at the 5% probability level. ns: not significant at the 5% probability level according to Tukey's test. CV = Coefficient of Variation; parasitism (P, in %); viability (V, in %); longevity (L, in days); number of adults per egg (NAE); sex ratio (SR).

These results highlight the influence of different *T. pretiosum* strains on the main biological parameters of eggs from various *T. absoluta* populations. This allowed the evaluation of the best *T. pretiosum* strain in terms of biological performance and parasitism against *T. absoluta* populations, as well as multivariate analyses of the relationships between these factors.

Parasitism differed among the studied *T. pretiosum* strains, with the highest parasitism rate observed for the S2 strain, which showed the greatest potential for use in *T. absoluta* control programs. It was also the only strain that achieved >80% parasitism of *T. absoluta* eggs from the population collected in 2023 (Table 5). Analysis of the effect of the populations on the strains revealed differences between strains S1, S2, and S4. The *T. absoluta* populations collected in 2023 (58.3%) and 2019 (32.2%) showed higher parasitism rates for S1, the 2021 population (58.3%) showed the lowest parasitism rate among the other populations for S2, and the 2022 (69.2%) and 2023 (56.6%) populations showed higher parasitism rates for S4 (Table 5).

Table 5. Mean values of the biological characteristics of four *Trichogramma pretiosum* strains exposed to eggs from different *Tuta absoluta* populations. Air temperature: 25 ± 2 °C, relative humidity: 70 ± 10%, and photoperiod: 12 h.

Biological Characteristics	*T. absoluta* Populations Collected in Different Years	*Trichogramma pretiosum* Strains			
		S1 [1,2]	S2 [1,2]	S3 [1,2]	S4 [1,2]
Parasitism (%)	2019	32.2 ± 9.07 abB	67.7 ± 4.59 abA	30.0 ± 9.03 aB	42.9 ± 6.84 bcB
	2020	16.6 ± 2.98 bB	66.3 ± 7.32 abA	37.7 ± 8.23 aB	22.0 ± 4.44 cB
	2021	30.6 ± 8.23 bB	58.3 ± 6.71 bA	47.8 ± 8.92 aAB	35.0 ± 6.23 bcAB
	2022	26.0 ± 5.57 bB	77.2 ± 5.35 abA	44.3 ± 9.42 aB	69.2 ± 4.08 aA
	2023	58.3 ± 6.92 aB	85.0 ± 3.51 aA	41.2 ± 5.51 aB	56.6 ± 7.45 abB
Viability (%)	2019	96.8 ± 0.63 aB	98.7 ± 0.62 aA	96.8 ± 1.20 aA	96.5 ± 2.58 aA
	2020	95.1 ± 2.12 aB	97.8 ± 1.14 aA	97.1 ± 1.66 aA	97.1 ± 1.31 aA
	2021	95.6 ± 2.44 aB	100 ± 0.00 aA	98.9 ± 0.41 aA	97.6 ± 1.27 aA
	2022	91.6 ± 1.98 aB	99.2 ± 0.45 aA	99.6 ± 0.26 aA	97.9 ± 0.75 aA
	2023	97.5 ± 1.18 aB	98.8 ± 0.69 aA	96.8 ± 1.39 aA	100 ± 0.00 aA
Longevity (days)	2019	15.1 ± 0.90 abA	10.7 ± 0.89 aB	16.4 ± 0.52 aA	10.8 ± 0.79 aB
	2020	17.7 ± 0.72 aA	10.9 ± 1.15 aB	16.7 ± 0.88 aA	11.5 ± 0.98 aB
	2021	14.5 ± 0.79 abA	9.65 ± 0.86 aC	13.1 ± 1.00 bAB	11.2 ± 0.89 aBC
	2022	13.2 ± 0.92 bA	9.75 ± 0.99 aB	14.0 ± 0.96 abA	9.15 ± 0.52 aB
	2023	12.5 ± 0.78 bA	10.6 ± 0.56 aA	11.3 ± 0.67 bA	10.6 ± 0.67 aA
Number of adults per egg (NAE)	2019	1.01 ± 0.00 aA	1.00 ± 0.00 aA	1.01 ± 0.00 bA	1.00 ± 0.00 aA
	2020	1.02 ± 0.02 aA	1.00 ± 0.00 aA	1.00 ± 0.00 bA	1.00 ± 0.00 aA
	2021	1.00 ± 0.00 aB	1.00 ± 0.00 aB	1.14 ± 0.11 aA	1.00 ± 0.01 aB
	2022	1.02 ± 0.01 aA	1.10 ± 0.03 aA	1.00 ± 0.00 bA	1.00 ± 0.00 aA
	2023	1.00 ± 0.00 aA	1.00 ± 0.01 aA	1.00 ± 0.00 bA	1.00 ± 0.00 aA
Sex Ratio	2019	0.54 ± 0.04 bB	0.76 ± 0.02 aA	0.52 ± 0.06 bB	0.76 ± 0.03 aA
	2020	0.76 ± 0.04 aAB	0.73 ± 0.03 aAB	0.79 ± 0.02 aA	0.65 ± 0.06 abB
	2021	0.77 ± 0.03 aA	0.78 ± 0.02 aA	0.53 ± 0.05 bB	0.69 ± 0.02 abA
	2022	0.70 ± 0.02 aAB	0.72 ± 0.02 aAB	0.75 ± 0.04 aA	0.59 ± 0.02 bB
	2023	0.52 ± 0.03 bB	0.71 ± 0.02 aA	0.71 ± 0.03 aA	0.72 ± 0.03 abA

[1] Means (± standard error of the mean) followed by the same lowercase letter in the column and uppercase letter in the row do not differ statistically according to Tukey's test at the 5% probability level ($p \leq 0.05$). [2] Data transformed using $\sqrt{(x + 0.5)}$.

Differences in parasitism potential among strains have been reported in several studies. For the selection of *T. pretiosum* species/strains on *Chrysodeixis includens* Walker (Lepidoptera: Noctuidae) eggs, parasitism ranged from 25.6% to 81.6%, leading to the identification of the best strains [18]. Similarly, parasitism provided a good indication for selecting *T. pretiosum* and *Trichogramma galloi* Zucchi species/strains for the control of *Duponchelia fovealis* (Lepidoptera: Crambidae) at different ages of the egg (24, 48, and 72 h) [38]. Additionally, strain selection trials have shown that five *T. pretiosum* strains tested on *T. absoluta* and *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) eggs exhibited the same parasitism potential [39]. In a study involving three *T. pretiosum* strains, it was observed that the "Goiânia" strain had a parasitism rate twice as high as the "Iguatu" strain on *E. kuehniella* eggs [40].

Viability is a crucial indicator for assessing whether a host is suitable for the complete development of the parasitoid. Regardless of the parasitism rate, low emergence rates can significantly compromise the effectiveness of biological control programs [18].

There was a significant exclusive effect of the *T. pretiosum* strain on viability (percentage emergence of parasitized eggs from *T. absoluta* populations). Strains S2 (98.9%), S3 (97.8%), and S4 (97.8%) showed higher mean viability than S1 (95.3%) (Table 5).

Viability varied among the tested strains but remained above 91% (Table 5). Similar results have been reported for *T. pretiosum* strains on *Neoleucinodes elegantalis* Guenée (Lepidoptera: Crambidae) eggs (ranging from 93.4% to 100%) [41] and *E. kuehniella* and *Helicov-*

erpa zea (Boddie) (Lepidoptera: Noctuidae) eggs (ranging from 86% to 95.0%) [42]. However, these results were higher than the emergence percentages of *Trichogramma* species/strains on *Gymnandrosoma aurantianum* Lima (Lepidoptera: Tortricidae) eggs, which ranged from 30.9% to 42.1%. This low parasitoid emergence rate is related to excessive moisture loss from *G. aurantianum* eggs [43].

High emergence percentages under laboratory conditions are favorable for the mass release of the parasitoid, especially when associated with high parasitism rates. In this study, there was no difference in the viability among the different host collection years. Conversely, in another study, the highest emergence percentage was found in the least accepted host (*Sitotroga cerealella*); this may be related to a selection process in which the surviving individuals are more capable of reaching the adult stage [44].

The viability of *T. pretiosum* strains obtained on different *T. absoluta* populations at 25 °C was similar to that reported by other researchers for this egg parasitoid species on different hosts [18,38,39,41,42]. For quality control of *Trichogramma* mass production, viability was considered satisfactory when the percentage of emerged adults exceeded 85% [2]; this was observed in all strains when exposed to *T. absoluta* eggs from different populations (Table 5).

Significance was observed independently for the factors "*T. pretiosum* strains" and "*T. absoluta* populations" in relation to the variable "longevity (days) of *T. pretiosum* females after parasitism." Among the strains, S1 (14.7 days) had the highest mean longevity, while S2 (10.3 days) recorded the lowest mean longevity. Analyzing the effect of populations in relation to the strains, there was a difference between strains S1 and S3, with the populations from 2019 (15.1 days), 2020 (17.7 days), and 2021 (14.5 days) providing greater longevity for S1. The populations from 2019 (16.4 days), 2020 (16.7 days), and 2022 (14.0 days) exhibited the highest mean longevity for S3 (Table 5).

It was observed that the higher the percentage of parasitism, the lower the longevity of the females (Table 5). The effect of parasitism on the longevity of *Trichogramma* females can vary depending on several factors, including parasitism intensity, environmental conditions, and the intrinsic capacity of the species. Generally, intense parasitism has negative effects on female longevity, not only because of the physical wear caused by the act of parasitizing but also because of metabolic stress, host quality, intrinsic species variation, and environmental conditions [45,46].

The survival and persistence of the parasitoid in an area depend on several factors, such as the continuous presence of the host, mortality caused by rainfall, agrochemical applications, and other climatic adversities [2]. According to the literature, *T. pretiosum* has the potential to remain active in the field for approximately 12 days, with parasitism remaining constant during the first 4 days. Therefore, parasitoid release should not be performed at intervals longer than 4 days. However, it is crucial to remember that the ideal time for applying any control method should be determined by the pest infestation level, which must be equal to or greater than the action threshold, to ensure economic viability of the control method [18,47].

When evaluating the number of *T. pretiosum* adults that emerged per egg (NAE) among *T. absoluta* populations in isolation, a difference was observed only in S3, with the 2021 population (1.14 adults per egg) showing the highest mean among all populations. Evaluating the interaction of *T. pretiosum* strains within *T. absoluta* populations, a difference was observed only for the year 2021, when S3 (1.14 adults per egg) demonstrated the highest mean among all strains (Table 5).

These values of NAE are similar to those found in a study evaluating the NAE from different *Trichogrammatidae* species/strains on *C. includens* eggs, which ranged from 1.0 to 1.2; these are lower than the number determined for other lepidopteran pest species, such

as *G. aurantianum*, which ranged from 1.4 to 1.8 [43]. The number of parasitoids per egg varied according to the volume and quality of the host egg. Host size not only influences the number of eggs laid by the female but also determines the adult size of *Trichogramma*. Adult size is directly related to the nutritional resources available for larval development [2]. Thus, there is an ideal number of parasitoids per egg depending on host size; for *T. absoluta* eggs, under the parasitism pressure studied, it ranged from 1.0 to 1.14 individuals per egg for the strains evaluated.

The sex ratio in *T. pretiosum* that emerged after parasitism differed among the studied *T. absoluta* populations. For S1, the highest mean values were observed in the 2020 (0.76), 2021 (0.77), and 2022 (0.70) populations. For S2, no differences were observed. S3 showed higher means for the 2020 (0.79), 2022 (0.75), and 2023 (0.71) populations. For S4, the lowest mean value was observed for the 2022 population (0.59). When evaluating the interactions of *T. pretiosum* strains within *T. absoluta* populations, differences were observed for all evaluated strains. For the 2019 population, S2 (0.76) and S4 (0.76) had the highest mean values. For 2020, S4 (0.65) had the lowest mean value. In 2021, S3 (0.53) showed the lowest mean. In 2022 and 2023, S4 (0.59) and S1 (0.52) had the lowest mean values, respectively (Table 5).

Sex ratio is an important biological characteristic in applied biological control programs, with a higher production of females being desirable because they are responsible for parasitism [2]. The sex ratio of the strains studied was lower than that recorded for *T. pretiosum* strains on *N. elegantalis* eggs (0.8 to 1.0) and *T. absoluta* (0.4 to 0.8) [39,41]. All four *T. pretiosum* strains achieved indices equal to or greater than 0.5 on all hosts, meeting the criteria established for the quality control of *Trichogramma* species in mass rearing. A high sex ratio can be beneficial in biological control programs because males, unlike females, do not contribute to pest reduction through parasitism [2,44].

Figure 2 shows Pearson's linear correlations among the different *T. pretiosum* strains for the main biological parameters when exposed to eggs from various *T. absoluta* populations.

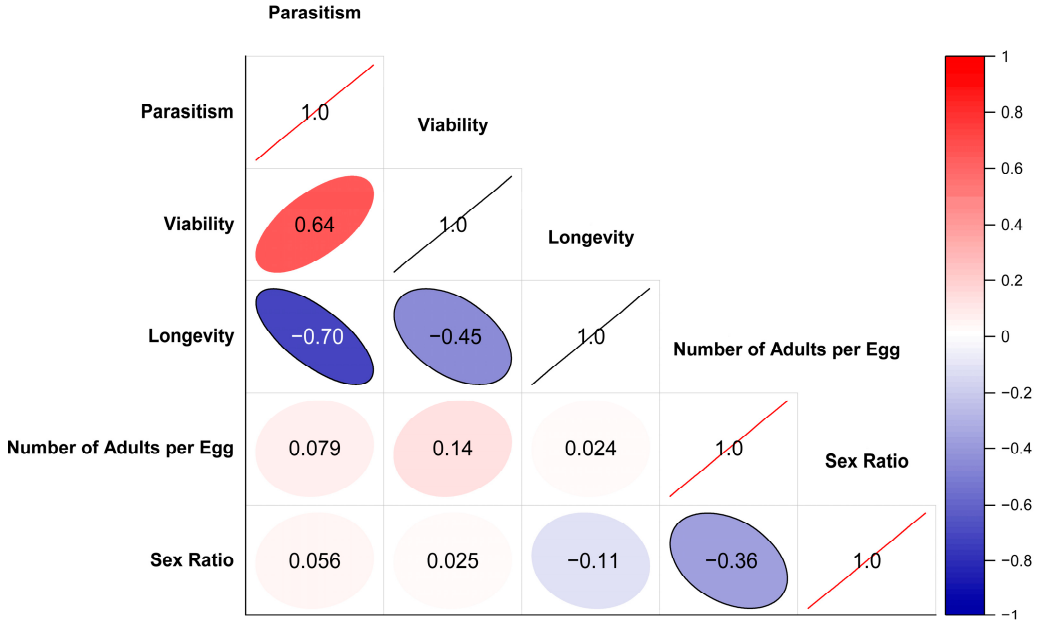

Figure 2. Pearson correlation matrix among the biological characteristics of four *Trichogramma pretiosum* strains (S1, S2, S3, and S4) exposed to eggs from different *Tuta absoluta* populations.

Linear correlation analysis was used to identify the nature and magnitude of the relationship between two variables, allowing the identification of indirect associations for traits of interest in the study subjects. The strength of correlations was classified as weak

(correlations between 0 and 0.30), moderate (correlations between 0.30 and 0.60), strong (correlations between 0.60 and 0.90), and very strong (0.90 to 1.00). Correlations equal to 0 indicated no relationship, whereas correlations equal to 1 indicated a perfect correlation between the two variables [41].

Correlations with strong or very strong magnitudes were considered relevant for interpretation purposes. A strong correlation was found between parasitism and viability (r = 0.64) (Figure 2). *T. pretiosum* strains exposed to *T. absoluta* eggs that achieved high percentages of parasitism also exhibited high adult emergence percentages (viability).

A strong inverse correlation was noted between parasitism and longevity (−0.70) (Figure 2). In other words, *T. pretiosum* females exposed to *T. absoluta* eggs that achieved high percentages of parasitism also exhibited reduced longevity, a factor reported in several studies [45,46].

Hierarchical clustering analysis (cluster analysis) distinguished different *T. pretiosum* strains based on biological parameters when exposed to *T. absoluta* eggs, grouping them according to their degree of similarity. This technique allowed the formation of groups that shared similar characteristics while presenting significant differences in relation to other groups (Figure 3).

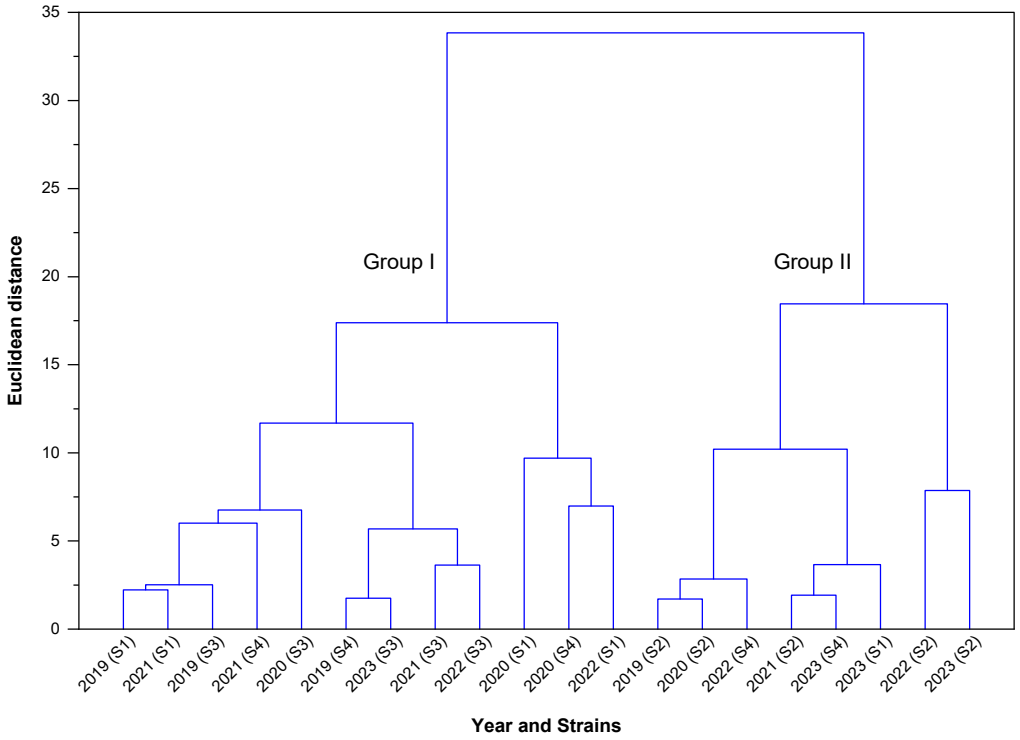

Figure 3. Dendrogram resulting from the clustering analysis based on biological parameters (parasitism, viability, female longevity, number of adults emerged per egg, and sex ratio) of four *Trichogramma pretiosum* strains (S1, S2, S3, and S4) exposed to eggs from different *Tuta absoluta* populations (2019, 2020, 2021, 2022, and 2023).

The analysis separated two distinct groups at a 30% distance. The first group (left) consisted of populations from 2019 (S1), 2021 (S1), 2019 (S3), 2021 (S4), 2020 (S3), 2019 (S4), 2023 (S3), 2021 (S3), 2022 (S3), 2020 (S1), 2020 (S4), and 2022 (S1). The second group (right) included populations from 2019 (S2), 2020 (S2), 2022 (S4), 2021 (S2), 2023 (S4), 2023 (S1), 2022 (S2), and 2023 (S2) (Figure 3). By reducing this distance to 15%, it was possible to form four distinct groups, with the fourth and last group consisting of strain S2 (collected from tomato crops in Mogi Mirim, SP) when exposed to eggs from populations collected in 2022 (S2) and 2023 (S2) (Figure 3). Thus, *T. pretiosum* S2 may be considered as the strain with

the greatest potential for use as an agent for *T. absoluta* control, as it was the most distinct among those studied and exhibited more favorable biological characteristics, indicating its biological potential. Furthermore, *T. absoluta* populations from more recent collection years have shown greater susceptibility to parasitism by this strain.

PCA was applied to establish a descriptive model for grouping the analyzed variables based on the different *T. pretiosum* strains exposed to *T. absoluta* eggs (Figure 4).

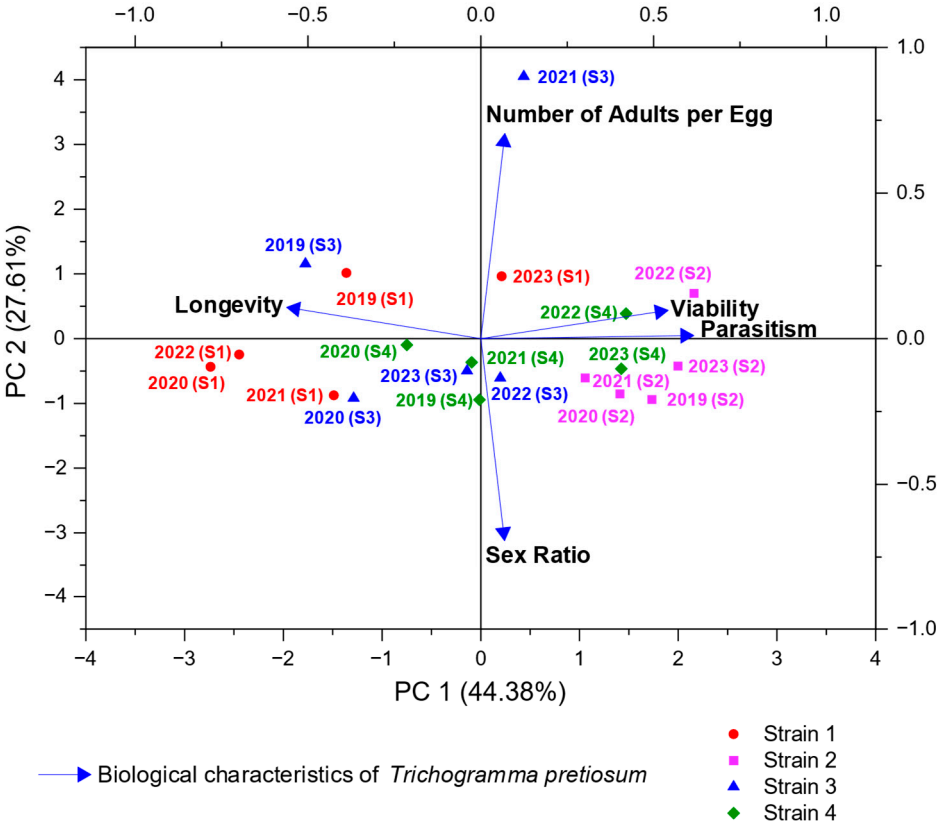

Figure 4. Bidimensional projection of the analyzed principal components (PC1 and PC2) based on biological parameters (parasitism, viability, female longevity, number of adults emerged per egg, and sex ratio) of four *Trichogramma pretiosum* strains (S1, S2, S3, and S4) exposed to eggs from different *Tuta absoluta* populations.

Principal component (PC)1 and PC2 explained 71.99% of the data variance.

PC1 explained the largest percentage of data variability (44.38%), with viability and parasitism most influencing this component. High correlations in PC1 were observed for all populations exposed to strain S2, as well as for most of strain S2, indicating that this group exhibited the most favorable biological characteristics for these strains (Figure 4).

For PC2, longevity showed the highest correlation, with strong correlations observed in most populations exposed to strains S1 and S3. This indicates that this group exhibited this biological characteristic as unfavorable for these strains since the fewer eggs parasitized, the greater the female longevity after the process (Figure 4).

The PCA results were consistent with those of the hierarchical clustering analysis in terms of the number of groups formed. Thus, both analytical techniques provided similar information regarding the structures of similarity and dissimilarity among the strains and populations studied (Figures 3 and 4).

For future studies aimed at the use of *T. pretiosum* as a control agent for *T. absoluta*, strain S2 is ideal because it presented the best biotic potential based on the analyzed parameters. This may also be attributed to it being collected from tomato crops, thereby making it more adaptable to parasitizing pests associated with this crop.

Although *T. pretiosum* is one of the most commonly used and widespread species worldwide for biological control, there is no standardization, and the strains are often described as cryptic species. Despite being morphologically identical, genomic-level differences may exist. Thus, biological differences may occur between species or strains, as predetermined by the genetic code [48,49].

The relationship between the number of host generations reared in the laboratory and the parasitism rate of *Trichogramma* may be influenced by several factors; in some cases, a reduction in parasitoid efficacy is observed over time. Factors that may contribute to this trend include host adaptation to laboratory conditions; that is, when host generations are reared in the laboratory for extended periods, specific adaptations to the controlled environment may occur. This can lead to genetic changes in the host, making it less susceptible to parasitism by *Trichogramma* [2,8,50,51].

Additionally, there is a loss of wild traits because hosts maintained in the laboratory for several generations may lose characteristics associated with their wild conditions. These changes can affect the natural interactions between the parasitoid and host. Controlled laboratory conditions may also affect these interactions since they may not completely reflect the natural conditions found in the field. This can lead to divergence in host–parasitoid interactions when compared to those that occur in nature [2,8,50,51].

Thus, the significant variability in the behavior of the four *Trichogramma* strains, through their biological characteristics, may be related to the habitat of origin, host acceptance, crop, and climatic conditions. These factors have been widely studied as quality traits in biological control programs [8,38,41,52,53].

3.2. Detection of Endosymbionts via PCR in T. absoluta Populations

Only two endosymbionts were detected in *T. absoluta* populations, bacteria belonging to the genera *Arsenophonus* (in the 2019 and 2020 populations) and *Serratia* (in the 2020 and 2021 populations), among the five populations studied (2019, 2020, 2021, 2022, and 2023) (Table 6).

Table 6. Endosymbionts identified in different *Tuta absoluta* populations.

Endosymbionts	*Tuta absoluta* Populations (Collection Years)				
	2019	**2020**	**2021**	**2022**	**2023**
Rickettsia	-	-	-	-	-
Hamiltonella	-	-	-	-	-
Wolbachia	-	-	-	-	-
Arsenophonus	+	+	-	-	-
Cardinium	-	-	-	-	-
Sodalis	-	-	-	-	-
Nosema	-	-	-	-	-
Carsonella	-	-	-	-	-
Spiroplasma	-	-	-	-	-
Serratia	-	+	+	-	-
Pantoea	-	-	-	-	-
Regiella	-	-	-	-	-

Endosymbionts play crucial roles in host reproduction, survival, detoxification, and nutrition. They enhance the ability of the host to survive on nutrient-poor diets and improve digestive efficiency by providing essential vitamins. Additionally, endosymbionts help prevent contamination by pathogenic bacteria, influence mating, and affect reproductive systems, among other aspects [15,54,55].

The relationship between insects and endosymbiotic bacteria can be either beneficial or harmful, particularly in cases involving pathogens. In beneficial relationships, endosymbionts perform functions, including nutrition—enhancing efficiency with poor diet, supporting fecundity, and protecting the host against infections. Although many of these relationships remain unknown, it is estimated that approximately 70% of insects live in symbiosis with intracellular microorganisms. The complexity of these interactions highlights the importance of endosymbionts in insect biology and ecology [14,24,56,57].

Some endosymbionts, such as *Baumannia*, *Blochmannia*, *Buchnera*, *Carsonella*, *Nardonella*, *Portiera*, *Sulcia*, *Tremblaya*, and *Wigglesworthia*, are obligate, requiring hosts to survive. In contrast, *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Regiella, Rickettsia*, *Serratia*, *Sodalis*, *Spiroplasma*, and *Wolbachia* are facultative and exist freely [58–60].

The endosymbionts *Serratia* and *Arsenophonus* are reported to be present in *T. absoluta* populations. The presence of these microorganisms influences various aspects of host biology, such as pathogen resistance, digestion, and interactions with parasitoids, although the specific impact of these endosymbionts on *T. absoluta* remains an area of ongoing research [24,25].

The endosymbiont *Serratia* was identified in *T. absoluta* populations collected in 2020 and 2021. This microorganism acts as a secondary endosymbiont and plays a beneficial role in insects, providing increased tolerance to high temperatures and pathogen resistance. Additionally, *Serratia* plays a significant nutritional role in amino acid synthesis. Despite these benefits, the presence of *Serratia* has side effects on both the insect host and its natural enemies. For example, *Serratia* infection decreases the longevity of predators feeding on the infected insects. This highlights the complexity of the interactions between endosymbionts and their hosts, with varied impacts on insect biology and ecology [61,62].

The endosymbiont *Arsenophonus* was identified in *T. absoluta* populations collected in 2019 and 2020. This microorganism acts as a secondary endosymbiont and has a symbiotic relationship with various insects; however, its exact function may vary among specific host species, potentially assisting in the nutrition of insect hosts. They can also synthesize essential nutrients that are absent or present in limited quantities in the insect diet. In some cases, they play a role in protecting the host against pathogens by competing with harmful microorganisms or producing antimicrobial substances [60,63]. In the type B hybridization of *Bemisia tabaci* (Hemiptera: Aleyrodidae) with the native biotype, the frequency of *Arsenophonus* in 24 hybrid carriers was significantly lower than expected, indicating either a loss of this endosymbiont in viable hybrids or a reduction in the viability of hybrids carrying it [64].

The analysis of endosymbiont identification in this study revealed that the populations maintained in the laboratory for several generations (collected in 2019, 2020, and 2021) were those that exhibited the presence of endosymbionts. In general, these populations demonstrated greater resistance to parasitism by the *T. pretiosum* strains studied. This suggests a correlation between the presence of endosymbionts and resistance to parasitism by *T. pretiosum* strains (Table 5).

The relationship between the endosymbionts *Serratia* and *Arsenophonus* in *T. absoluta* and parasitism by *T. pretiosum* involves complex interactions between the host, its endosymbionts, and the parasitoid. The presence of endosymbionts, such as *Serratia,* can influence various aspects of *T. absoluta* biology, including its reproduction, survival, and response to environmental stresses. These factors may indirectly affect *T. absoluta* parasitism [24].

Endosymbionts may modulate the host immune system; *Serratia* and *Arsenophonus* may influence the immune response of *T. absoluta*, affecting *Trichogramma* parasitism. Additionally, endosymbionts may affect host behaviors, such as oviposition site searching

and host selection. Some endosymbionts confer resistance to their hosts against parasitoids [63,65], affecting the ability of *Trichogramma* to parasitize *T. absoluta* eggs.

To date, there are no specific reports indicating the direct association of the endosymbionts *Serratia* and *Arsenophonus* with changes in oviposition-searching behavior and host selection in insects. These endosymbionts are known for their complex interactions with hosts, affecting aspects such as immunity and resistance to pathogens; however, there is no clear evidence of direct modification of these behaviors [24].

The relationship between endosymbionts and parasitism by *Trichogramma* may vary depending on the specific strains of *Serratia* and *Arsenophonus* and the environmental conditions and genetic characteristics of *T. absoluta* populations [65]. Endosymbionts often alter the chemical composition of their hosts, resulting in the production of volatile compounds or other chemical cues, as perceived by *Trichogramma*. The presence of *Serratia* and *Arsenophonus* may affect the quality of *T. absoluta* eggs as hosts, potentially affecting their susceptibility to parasitism by *Trichogramma* [66].

The relationship between endosymbionts, hosts, and parasitoids is highly contextual and dependent on various factors, including environmental conditions, specific strains of endosymbionts, and the genetic characteristics of populations. In several cases, symbiotic relationships with endosymbionts are maintained across insect generations because of the significant benefits these microorganisms confer to the host [67–69].

In the present study, the endosymbiont *Wolbachia* was not detected in any *T. absoluta* population. Notably, *Wolbachia* is one of the most frequently reported endosymbionts of *T. absoluta* [24,57,70]. Additionally, the endosymbiont *Pantoea* has been identified in some populations [24]. These findings highlight the diversity of endosymbionts coexisting in insect populations; the presence or absence of these microorganisms may vary among different studies and insect populations.

Endosymbionts can be transmitted from hosts to their parasitoids, and this transmission may occur during parasitoid development within the infected host. However, transmission effectiveness and the effects of endosymbionts on parasitoids may vary. Some endosymbionts may benefit the parasitoid, whereas others may have negative or no effects. Both horizontal transmission (from one organism to another) and vertical transmission (from one generation to the next) are possible but depend on specific interactions between the endosymbiont, host, and parasitoid [24,57,70].

Finally, high genetic homogeneity is present in the evaluated *T. absoluta* populations, which may be primarily related to genetic drift in this invasive species [24,71,72]. In reduced populations, genetic diversity can be lost due to strong genetic drift [73]. These genetic patterns underscore the importance of understanding the genetic structure of *T. absoluta* populations to implement more effective management strategies, particularly considering their invasive status and impact on agricultural crops.

4. Conclusions

Strain S2 of *T. pretiosum* had the most efficient biological characteristics for parasitizing *T. absoluta* eggs. This included high parasitism and good viability/emergence, particularly in *T. absoluta* populations collected in recent years (2022 and 2023). These results make S2 the preferred strain for future studies aimed at using biological control agents to combat *T. absoluta*. The endosymbionts *Arsenophonus* and *Serratia* were identified in *T. absoluta* populations collected in 2019–2020 and 2020–2021, respectively.

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data curation, A.B.D., D.M.S., D.d.L.A., N.N.P.d.S., D.F.D. and R.C.d.O.; writing—original draft preparation, A.B.D. and R.C.d.O.; writing—review and editing, A.B.D., D.M.S., D.d.L.A., N.N.P.d.S., D.F.D., D.P., S.S.J. and R.C.d.O.; supervision, A.B.D. and R.C.d.O.; project administration, A.B.D. and R.C.d.O.; funding acquisition, A.B.D. and R.C.d.O. All authors have read and agreed to the published version of the manuscript.

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