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Novel Strain *Bacillus velezensis* LAFUEL 03: Activity Against *Xanthomonas vasicola* pv. *vasculorum*, Control of Bacterial Leaf Streak of Corn and Genome Insights into Its Antagonistic Activity

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Abstract: The main objective of this study was to investigate the antimicrobial activity of three putative antagonist bacterial strains of *Bacillus* spp. against *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*) and their potential to control bacterial leaf streak (BLS) of corn. Additionally, the study included investigations on the genome of one of these antagonist bacteria, such as genome sequencing and mining of genes involved in biofilm formation, swarming motility, and synthesis of secondary metabolites. The growth of *Xvv* was inhibited by both cell suspensions and cell-free supernatants of the bacterial strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 in agar diffusion tests. All three antagonist strains significantly reduced the severity of BLS in the 3rd and 4th leaves of corn plants that were artificially inoculated at the V3 growth stage under greenhouse conditions. The 16S *rRNA* sequencing confirmed that the antagonistic bacterial strains belong to the genus *Bacillus*, with LAFUEL 03 having approximately 97% similarity to *B. velezensis*. *B. velezensis* LAFUEL 03 harbors genes related to the biosynthesis of secondary metabolites, biofilm formation/regulation, and swarming motility that enhances its potential for controlling BLS in corn and suggests a promising candidate for the development of a commercial biocontrol agent.

Keywords: antimicrobial activity; *Bacillus velezensis*; bacterial genomics; biological control; biosynthetic gene clusters; molecular identification

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1. Introduction

Bacterial leaf streak (BLS) of corn caused by *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*) is a disease reported for the first time in South Africa in 1949 [1]. Despite that, it was only after the introduction in the American continent [2–5] that major concerns for corn production arose, leading to several studies on pathogen and disease management [6–14].

Chemical control may reduce bacterial disease development in plants [15]. However, all bactericides available for agricultural use, which are mostly copper-based compounds, have contact activity and may require several applications to effectively control bacterial diseases [15,16]. Furthermore, copper oxychloride, which significantly reduces BLS severity, may cause severe phytotoxicity in corn plants [12]. In addition to the low effectiveness and phytotoxicity, a major concern is the selection pressure that these chemicals may pose on plant pathogens, favoring the emergence of resistant strains [16].

Biological control, defined as the use of a microorganism or its compounds to reduce the level of a disease [17], is an alternative to overcome chemical control problems. Further, biological control agents have many advantages regarding sustainability when compared to chemical pesticides, such as diverse mechanisms of action and low toxicity [18]. In addition,

they decompose more rapidly in the environment and are usually less toxic to non-target species [19]. Biological control agents may also help to suppress the development of resistant pathogens, as they usually exhibit different mechanisms of action compared to conventional pesticides [20,21].

Biological control agents may involve both bacteria and filamentous or yeast-like fungi [22]. Among the bacteria, the genus *Bacillus* has been studied extensively for this purpose due to its capability to form endospores, have different mechanisms of action, and produce metabolites with biotechnological applications [23]. These endospore-forming *Bacillus* strains are capable of suppressing and inhibiting plant pathogens [24]. The biocontrol potential of these bacteria is achieved through direct antibiosis, the induction of the host plant's immune system response (ISR), and competition for nutrients and space [25,26]. The antagonistic activities of *Bacillus* spp. are often associated with the production of secondary metabolites that have antibiotic properties. These antibiotics consist of low-molecular-weight peptides produced either ribosomally (such as bacteriocins) or non-ribosomally (including cyclic lipopeptides, small peptides, and polyketides). Currently, genome mining allows for the identification of gene clusters encoding bacteriocins, peptides, and polyketides. A total of 583 putative bacteriocin gene clusters were found in 328 strains of 57 Bacillales species. Additionally, 1231 putative non-ribosomal antimicrobial gene clusters were detected in 49 Bacillales species and grouped in 23 kinds of peptides and five kinds of polyketide compounds [27]. Forty-seven metabolites have been identified in antimicrobial *Bacillus* sp., and the mechanisms of action were documented for each one [28].

Effective measures for bacterial leaf streak of corn management have not yet been completely established [12]. However, biological control is a strategy that has been studied for the control of several bacterial plant diseases, including those caused by Xanthomonads [29]. Further, studies were carried out to explore the beneficial effects of *Bacillus* spp. as biocontrol agents for *Xanthomonas* spp., such as *X. citri* subsp. *citri* [30–32] *X. campestris* pv. *campestris* [33] and *X. euvesicatoria* [34,35]. Ref. [36] reported that *Pantoea ananatis* may have the potential to be a biocontrol agent against *Xvv*.

In view of the relatively limited efficiency of chemicals for the control of BLS and biocontrol as a more eco-friendly alternative for the management of bacterial diseases on plants, the objectives of this study were as follows: (i) to evaluate the in vitro activity of three putative antagonist bacterial strains against *Xvv*, the causal agent of BLS of corn; (ii) to determine their efficiency for the control of BLS in artificially inoculated corn plants; (iii) to raise genomic information of one of these antagonist bacteria, including genome sequencing and mining of genes involved in biofilm formation, swarming motility, and synthesis of secondary metabolites.

2. Materials and Methods

2.1. Source and Maintenance of the Bacterial Strains

RL1 *Xvv* strain [5] was obtained from the collection of plant pathogenic bacteria of the Laboratory of Bacteriology and Diagnosis in Plant Health of the Instituto de Desenvolvimento Rural do Paraná- IAPAR/Emater (IDR-Paraná), Londrina, PR, Brazil. The putative antagonist bacterial strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 were isolated as contaminants in antagonism studies and belong to the Laboratory of Plant Pathology of the Universidade Estadual de Londrina (UEL), Londrina, PR, Brazil. All the bacterial strains were grown on Nutrient Agar (NA) medium (g L^{-1}: 5 peptone, 3 beef extract/yeast extract, 20 agar) and incubated at 28 ± 2 °C for 48 h.

2.2. In Vitro Production of Metabolites and Cell-Free Supernatant

The inoculum was prepared with cell suspension of the strains LAFUEL 01, LAFUEL 02, and LAFUEL 03, adjusted to $O.D._{600nm}$ = 0.3. Aliquots of 40 µL of these suspensions were transferred to 40 mL of Luria Bertani (LB) broth (g L^{-1}: 10 tryptone; 5 yeast extract; 5 NaCl; pH 6.8–7.0) and kept under 200 rpm agitation at 28 °C for 24 h. After incubation, aliquots (1% *v*/*v*) of the inocula were transferred to 250 mL of medium 1 (LB broth) and medium

2 (International *Streptomyces* Project-2- ISP2—broth: g L^{-1}: 4 yeast extract; 10 malt extract; 4 dextrose) and kept under 200 rpm agitation at 28 °C for 72 h and 168 h for media 1 and 2, respectively. To obtain the cell-free supernatants (CFS), the fermented products (extract) were centrifuged at 10,000 rpm at 4 °C for 15 min and filtrated through 0.22 µm membranes.

2.3. Evaluation of the Activity of Three Putative Antagonist Bacterial Strains Against Xvv and Control of BLS of Corn

2.3.1. Antimicrobial Activity of the LAFUEL 01, LAFUEL 02, and LAFUEL 03 Bacterial Strains and Their Products

Experiments were performed using the agar diffusion method (pour plate) in an NA medium to evaluate the putative antimicrobial activity of LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains against the RL1 *Xvv* strain. A suspension of *Xvv* at 10^8 CFU mL^{-1} was incorporated in the still-melting NA medium (54 °C) for a final concentration of 0.25% (*v*/*v*). In the first experiment, 10 µL aliquots of the cell suspensions of the bacterial strains adjusted to $O.D._{600nm}$ = 0.3 were added to the medium. In the second experiment, 200 µL aliquots of the CFS of each strain were transferred to 8 mm diameter wells in the culture medium. The plates were maintained at 28 ± 2 °C for 72 h and then checked for bacterial growth in the presence of CFS. The antimicrobial activity was evaluated by measuring the growth inhibition zone of the RL1 *Xvv* strain. Both experiments were performed three times, and the results are the average of these experiments.

The experimental design was completely randomized with a two-factor factorial arrangement (2 culture media × 3 putative antagonist bacterial strains) and 8 replications. The results were submitted for analysis of variance, and the means were compared by the Tukey test at a 5% probability level. Data that did not meet the assumptions of the analysis of variance according to the Shapiro–Wilk and Bartlett tests were transformed by Box-Cox [37]. Data analysis was performed using the statistical software R Studio (2023.12.0) [38].

2.3.2. Minimum Inhibitory Concentration of the Cell-Free Supernatant

The concentration of the LAFUEL 01, LAFUEL 02, and LAFUEL 03 suspensions was 3×10^8 CFU mL^{-1} after fermentation and before the 0.22 µm membrane filtration. The CFSs obtained were subjected to a serial two-fold dilution to 1:64 in a 96-well polystyrene plate containing 100 µL of nutrient broth (NB) in order to determine the minimum inhibitory concentration (MIC) of the CFS of each strain against the RL1 *Xvv* bacterium. Then, 100 µL of the RL1 *Xvv* suspension in NB at the concentration of 10^8 CFU mL^{-1} was added to the wells and homogenized. Positive (RL1 *Xvv* + NB) and negative controls (CFS + NB) were included. The plates were maintained at 28 °C for 72 h. The evaluation of the RL1 *Xvv* growth was performed visually based on the absence or presence of turbidity. Each treatment had three replications, and the test was performed three times.

Inhibition curves were estimated using the non-parametric Kaplan–Meier method [39] to visually determine the activity of the CFS to inhibit bacterial growth as a function of the CFS dilution. The log-rank test was applied to compare the curves, as the observed and expected values of each treatment were compared under the hypothesis that the inhibition activity was the same for all treatments. This is equivalent to testing if the dilution for inhibition of bacterial growth is similar for each treatment. Data analysis was performed using the statistical software R Studio [38].

2.3.3. In Vitro Pellicle Formation and Swarming Motility Assays

The pellicle formation assay was carried out in a 24-well plate with 2 mL of tryptic soy broth (TSB) (Acumedia Manufacturers, Inc., Lansing, MI, USA), prepared and inoculated with 10 µL of an overnight culture adjusted to $O.D._{600nm}$ = 0.3. The pellicle formation was visually assessed 24 h after inoculation at 28 °C.

For the motility of the LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains, the evaluation was based on spreading each strain on 0.75% tryptic soy agar plates (TSA, Acumedia Manufacturers, Inc., Lansing, MI, USA). Overnight cultures were adjusted to $O.D._{600}$ = 0.3,

and 10 µL of the inoculum were plated in the center of a 60 mm Petri dish containing TSA medium. The ability to colonize the entire surface of the medium was evaluated 24 h after incubation at 28 °C.

2.4. Greenhouse Evaluation of the Putative Antagonist Bacterial Strains for the Control of Bacterial Leaf Streak of Corn

The study was carried out in a semi-climatized greenhouse at the Londrina Research Station of the Instituto de Desenvolvimento Rural do Paraná—IAPAR/Emater, Londrina, PR, Brazil. Seeds of the IPR 164 corn variety were sown in 5 L plastic pots containing a soil–sand–organic matter mixture (3:1:1 vol).

Products based on the putative antagonist bacterial strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 were prepared by fermentation in LB medium at a final concentration of 3×10^8 CFU mL^{-1}. Mancozeb and copper oxychloride were included as positive chemical controls and water as negative control. The products and doses included in the experiments are shown in Table 1. Doses of the positive chemical controls were those recommended by [12]. Fifteen days after sowing, corn plants at the V3 vegetative growth stage were sprayed with the treatments.

Table 1. Treatments and doses of LAFUEL 01, LAFUEL 02, and LAFUEL 03 products and chemicals included in the experiments for evaluation of the control of bacterial leaf streak of corn.

Treatment	Dose
Water	-
LAFUEL 01	3×10^8 CFU mL^{-1}
LAFUEL 02	3×10^8 CFU mL^{-1}
LAFUEL 03	3×10^8 CFU mL^{-1}
Mancozeb—750 g kg^{-1}	2 g c.p. [a] L^{-1}
Copper oxychloride—588 g L^{-1}	2 g c.p. L^{-1}

[a] c.p.—commercial product.

Three days after spraying the products, the corn plants were artificially inoculated with a suspension of the RL1 *Xvv* bacterium at a concentration of 10^8 CFU mL^{-1}. Approximately 10 mL of the bacterial suspension was sprayed on the leaves of each corn plant without wounding [40]. All plants were maintained in a moist chamber for 24 h before and 24 h after the bacterial inoculation. The disease severity was evaluated 21 days after inoculation on the 3rd and 4th leaves, based on the percentage of affected leaf area using the scale proposed by [40]. The reduction in the disease-affected leaf area was calculated as a percentage (%) of the disease in the negative control plants.

The experiments were carried out in a completely randomized design, with four replications. The experimental unit was a pot containing one plant. Two trials were performed. The analysis of the percentage of disease-affected leaf area was performed using non-parametric statistics. In the first trial, the ANOVA Kruskal–Wallis [41] was applied to the data, and the comparison of the treatments was performed by the Bonferroni test ($p < 0.05$). In the second trial, data were submitted for analysis of variance, and the means were compared by the Tukey test ($p \leq 0.05$). The statistical analyses were performed using the software R Studio [38].

2.5. Molecular Characterization of the Putative Antagonist Bacterial Strains

The LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains were subjected to molecular analysis of the 16S *rRNA* (ribosomal RNA) sequence for identification at the genus level. The strains were grown in NB medium at 28 °C and 200 rpm agitation for 16 h. Total DNA was extracted from a pellet of 1 mL of the bacterial suspension following the standard protocol of DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA quality was examined in agarose gel electrophoresis and quantified by using NanoDrop (Thermo Scientific, Waltham, MA, USA).

For the 16S *rRNA* sequence gene, the DNA was amplified using the universal primers fD1 CCGAATTCGTCGACAAC (forward) and rD1 AGAGTTGATCCTGGCTCAG (reverse) [42]. The 25 µL PCR reactions were performed including 1 µL of genomic DNA; 2.5 µL of 10X PCR buffer; 1 µL of each primer (10 µM); 0.8 µL of MgCl (50 mM) 2; 1 µL of dNTP (5 mM); 0.1 µL of GoTaq DNA polymerase (Promega Corp., Madison, WI, USA); and 17.6 µL of sterile miliQ water. The optimized cycling program was initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 50 s, annealing at 62.3 °C for 50 s, and 72 °C for 1 min and 45 s, ending with an extension cycle at 72 °C for 7 min.

Electrophoresis at 6 V/cm^2 in 1% (*w*/*v*) agarose gel in Tris-Acetate-EDTA buffer stained with Sybr Safe (ThermoFisher Scientific, Waltham, MA, USA) was performed to the amplified DNA and photographed using Gel Doc™ XR+, Bio-Rad, Inc, Hercules, CA, USA. PCR products were purified using the PureLink® Kit (Quick Gel Extraction Kit—Invitrogen, Carlsbad, CA, USA) and run in an automated sequencer by the Sanger method (Applied Biosystems, Foster City, CA, USA) at the Universidade Estadual Paulista (UNESP), Jaboticabal, SP, Brazil.

Initially, the sequences were subjected to nucleotide similarity comparison with sequences deposited in the GenBank, accessed through the NCBI (National Center for Biotechnology Information). For this query, we used the local Basic Local Alignment Search Tool (BLAST) [43]. The 16S *rRNA* gene sequences were also compared using the program RDP Classifier (version 2.2) [44].

2.6. Genome Sequencing and Mining of Genes of the Putative Antagonist Bacterial Strain LAFUEL 03

2.6.1. Genome Sequencing, Assembly, and Annotation

Based on previous antagonism tests against fungi and bacteria, the strain LAFUEL 03 was chosen for genome sequencing. The strain was cultivated in NB medium at 150 rpm and 28 °C for 16 h. DNA was extracted using the DNA extraction kit, DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Sequencing of the LAFUEL 03 strain was carried out in the Illumina MiSeq platform, using the MiSeq version 3 reagent kit (600-cycle, Illumina, San Diego, CA, USA) in the Soil Biotechnology Laboratory of Embrapa Soja, Londrina, PR, Brazil.

The sequence quality of the raw reads was analyzed using FastQC [45], then trimmed using Trim Galore! (v. 0.6.5) [46]. Clean reads were subjected to de novo assembly using SPAdes and incorporated in the Unicycler pipeline [47]. The genome G+C content was calculated using QUAST [48]. The completeness of the genome was calculated by analyzing the conserved marker genes using CheckM v.1.0.18 [49]. Genome annotation was carried out using the RAST platform [50], and the coding sequence (CDS) was predicted and classified into subsystems.

2.6.2. Secondary Metabolites Cluster Prediction

The web server antiSMASH 6.0, which combines different databases of genetic data, antimicrobial molecules, and biosynthetic gene clusters (BGCs), was used to predict the chemical structures, position, and possible function of the secondary metabolites clusters [51]. The analysis was carried out with the final FASTA file of the LAFUEL 03 strain.

2.6.3. Phylogenomic Comparison and Tree Reconstruction

For species identification of the LAFUEL 03 strain, ANI (Average Nucleotide Identity) with other *Bacillus* spp. was performed using orthoANI [52]. The program Roary [53] was used to carry out pan-genome analyses for species. All genomes used for Roary were annotated by Prokka [54]. The tree reconstruction was carried out using an alignment of the core genome sequences with 70% BLAST ID derived from Roary [53] based on the maximum likelihood method and bootstrapped 100 times using RaxML-NG [55]. All visualizations of the phylogenetic trees were produced with FigTree [56] version 1.4.4.

2.6.4. Mining of Biofilm Formation Related Genes

After annotating the genome, a local BLASTN was performed using a customized database that included the LAFUEL 03 strain genome. The query sequences consisted of a list of genes associated with biofilm formation and regulation, as identified on SubtWiki [57] and other studies [58–62] related to biofilm formation of *Bacillus subtilis*.

3. Results

3.1. Evaluation of the Activity of Three Putative Antagonist Bacterial Strains Against Xvv and Control of BLS of Corn

3.1.1. Antimicrobial Activity of the LAFUEL 01, LAFUEL 02 and LAFUEL 03, and Their Products

In the agar diffusion experiments with cell suspensions, the LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains were able to inhibit the growth of RL1 *Xvv* based on the presence of inhibition zones in the NA plates (Figure 1). The inhibition zones produced by the LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains were 31, 29, and 33 mm in diameter, respectively (Figure 1). However, no significant differences were observed in regard to the intensity of the antimicrobial activities of the putative antagonist bacterial strains against RL1 *Xvv* (Figure 1).

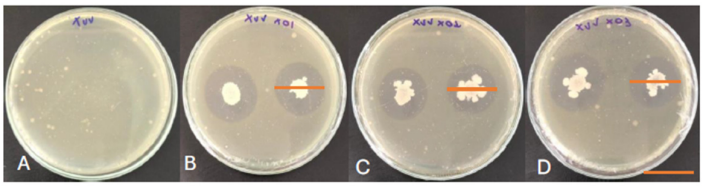

Figure 1. Agar diffusion test (pour plate) with cell suspensions of LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains against RL1 *Xanthomonas vasicola* pv. *vasculorum*—*Xvv*. The orange line indicates the diameter of the growth inhibition zone of RL1*Xvv*. (**A**), control; (**B**), LAFUEL 01; (**C**), LAFUEL 02; and (**D**), LAFUEL 03. Bar = 30 mm. Plates shown are representative of eight replicates of each treatment.

In the test with the CFSs of LAFUEL 01, LAFUEL 02, and LAFUEL 03, there was no effect on either the culture media or the putative antagonist bacterial strains. Furthermore, there was no significant interaction between the factors, culture medium, and putative antagonist bacterial strain in the two experiments (Table 2). For the CFSs of the bacterial strains grown in the LB medium, the diameter of the inhibition zones was 39, 40, and 38 mm, respectively (Figure 2A1–A4). Further, the diameter of the inhibition zones for the LAFUEL 01, LAFUEL 02, and LAFUEL 03 CFSs produced in the ISP2 medium were 37, 39, and 38 mm, respectively (Figure 2B1–B4).

Table 2. Growth inhibition zones (mm) of RL1 *Xanthomonas vasicola* pv. *vasculorum*—*Xvv* strain in agar well diffusion test (pour plate) by cell-free cell supernatants (CFSs) of the LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains produced in Luria Bertani (LB) and International Streptomyces Project-2 (ISP2) media.

CFS	Culture Medium		Mean [a]
	LB	ISP2	
LAFUEL 01CFS	39.00	37.00	38.00 [n.s.]
LAFUEL 02 CFS	40.25	39.25	39.75
LAFUEL 03 CFS	38.25	38.50	38.37
Mean	39.17 [n.s.]	38.25 [n.s.]	

[a] data represent average of eight replicates; [n.s.]: not significative ($p < 0.05$).

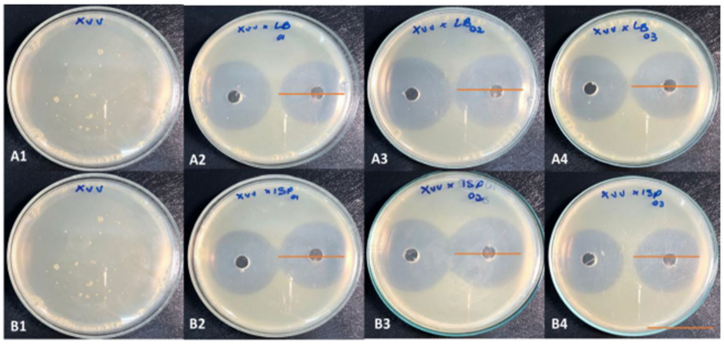

Figure 2. Agar well diffusion tests (pour plate) of cell-free supernatant of LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains produced in LB (**A**) and ISP2 (**B**) media against RL1 *Xanthomonas vasicola* pv. *vasculorum*—*Xvv*. (**A1**,**B1**), control; (**A2**,**B2**), LAFUEL 01 CFS; (**A3**,**B3**), LAFUEL 02 CFS; (**A4**,**B4**), LAFUEL 03 CFS. The orange bar indicates the diameter of the growth inhibition zones of the RL1 *Xvv* by the cell-free supernatant of the putative antagonist bacterial strains. Bar = 40 mm. The plates shown are representative of the eight replicates of each treatment.

3.1.2. Minimum Inhibitory Concentration of the Cell-Free Supernatants

RL1 *Xvv* strain growth was suppressed at the concentration of 1:8 of the LAFUEL 01, LAFUEL 02, and LAFUEL 03 CFSs obtained from cultures grown in LB medium. Further, RL1 *Xvv* bacterium growth was also suppressed by all the CFSs at a concentration of 1:16 of cultures grown in the ISP2 medium. However, there was no significant difference regarding the strains and the culture media, according to the non-parametric analysis of Kaplan–Meier (log-rank test) (Supplementary Figure S1). Therefore, the CFSs of the LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains produced in both media have the same potential to inhibit the growth of RL1 *Xvv*.

3.1.3. In Vitro Assays for Pellicle Formation and Swarming Motility

The three LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains were able to colonize the whole surface of the TSB medium in both assays for pellicle formation and swarming motility. Pellicle formation on the air–liquid interface was visible, covering the whole surface of the wells, where thick layers were formed, resulting from cell growth (Figure 3A). In the motility test performed on the TSA 0.75% medium, we observed that the cell inocula of the three putative antagonist bacterial strains, starting in the middle of the Petri dishes, were also able to grow over the whole surface (Figure 3B).

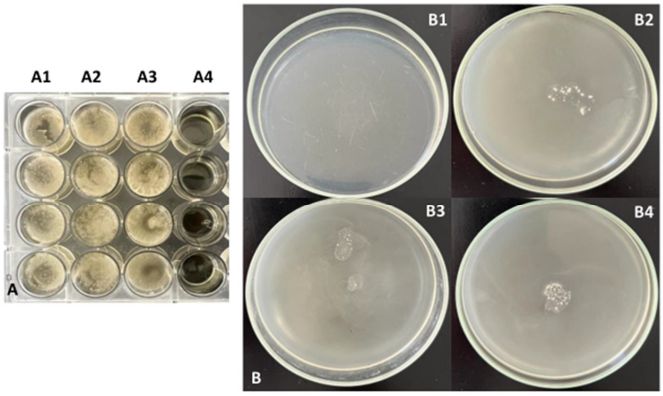

Figure 3. In vitro assays for pellicle formation (**A**) and swarming motility (**B**) by the LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains. Pellicle formation on TSB medium: (**A1**), LAFUEL 01; (**A2**), LAFUEL 02; (**A3**), LAFUEL 03; (**A4**), negative control. Representative plates of motility test on TSA 0.75% medium: (**B1**), negative control, (**B2**), LAFUEL 01, (**B3**), LAFUEL 02, and (**B4**), LAFUEL 03.

3.2. Greenhouse Evaluation of the Putative Antagonist Bacterial Strains for the Control of Bacterial Leaf Streak of Corn

Based on the disease severity assessed in the 3rd and 4th leaves, the LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains significantly reduced BLS on the corn plants and showed even better control efficiency of the disease than the mancozeb and copper oxychloride treatments in both experiments (Table 3). The disease severity in experiment A was low, but the results had a similar tendency as in experiment B. For experiment B, the treatments with the LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains provided a disease control efficiency of 65, 33, and 52%, respectively, based on the disease severity in the third leaf. In this experiment, the mancozeb and copper oxychloride treatments had a disease control efficiency of 56 and 60%, respectively. On the fourth leaf, the bacterial strains treatments had a disease control efficiency above 75%, while mancozeb and copper oxychloride had only 47% and 43% control efficiency, respectively (Table 3).

Table 3. Severity and efficiency of control of bacterial leaf streak in plants of IPR 164 corn variety treated with LAFUEL 01, LAFUEL 02, and LAFUEL 03 putative antagonist bacterial strains, and mancozeb and copper oxychloride fungicides. Severity was assessed in the 3rd and 4th leaves 21 days after inoculation with RL1 *Xanthomonas vasicola* pv. *vasculorum* strain.

Treatment [a]	Experiment A			
	Disease Severity [a] (%)		Efficiency of Control [b] (%)	
	3rd Leaf	4th Leaf	3rd Leaf	4th Leaf
Water	1.4 a[c]	4.4 a	-	-
Mancozeb	0.5 ab	0.5 abc	64	89
Copper oxychloride	0.5 ab	1.5 ab	64	65
LAFUEL 01	0 b	0.2 bc	100	95
LAFUEL 02	0 b	0 c	100	100
LAFUEL 03	0 b	0.2 bc	100	95
	Experiment B			
Water	22.5 a	37.5 a	-	-
Mancozeb	10.0 b	20.0 b	56	47
Copper oxychloride	9.0 b	16.3 b	60	57
LAFUEL 01	8.0 b	8.0 b	65	79
LAFUEL 02	15.0 b	8.8 b	33	77
LAFUEL 03	10.8 b	8.3 b	52	78

[a] Percentage of affected leaf area based on the diagrammatic scale proposed by Robaina et al. (2020) [40]; [b] Efficiency of disease control based on the disease severity in the water treatment; [c] For experiment A, values followed by the same letter in the column do not differ according to the Bonferroni test ($p < 0.05$); for experiment B, values followed by the same letter in the column do not differ according to the Tukey test ($p < 0.05$). Data represent the average of four replicates per treatment.

3.3. Molecular Characterization of the Putative Antagonist Bacterial Strains

3.3.1. Molecular Identification of the Bacterial Strains and Phylogenetic Analysis

The molecular analysis of the 16S *rRNA* gene sequences based on the RDP Classifier [44] and the search for similarity using BLAST [43] revealed that the LAFUEL 01, LAFUEL 02, and LAFUEL 03 strains belong to the genus *Bacillus*. However, the analyses of the 16S *rRNA* gene sequence did not allow the identification of all three *Bacillus* sp. strains at the species level. However, the strains have 99% of similarity between them based on the 16S *rRNA*.

When compared to various strains, including the type strains of the three primary species within the B. subtilis group, the strain LAFUEL 03 showed the highest ANI similarity (97.76%) with the *Bacillus velezensis* LABIM22 (Table 4, Figure 4). Further, the strain LAFUEL 03 also showed high ANI similarity with *B. velezensis* JS25R (97.69%), *B. velezensis*

YAU B9601-Y2-1 (97.62%), and *B. velezensis* type strain FZB42 (97.56%), above the threshold for species delineation.

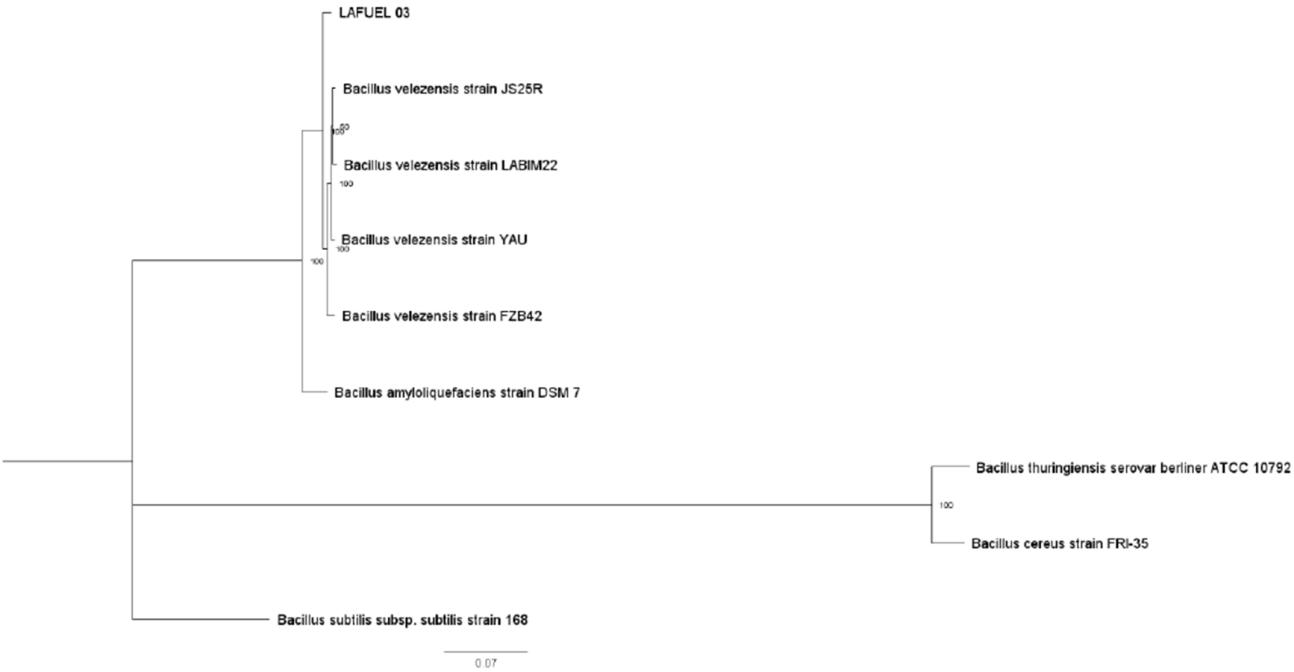

Figure 4. Phylogenomic tree representing the relationship of the strain LAFUEL 03 with other strains of *Bacillus* spp. The matrix was generated by RaxML and exported to FigTree. The bootstrap value with 100 replications is shown on the nodes.

When compared to the type strain DSM7(T) of *B. amyloliquefaciens*, an average nucleotide identity (ANI) of 93.99% was observed, suggesting that the LAFUEL 03 strain and DSM7(T) are distinct species. This classification is significant for this bacterial group due to the inconsistent delimitation that has occurred over the years [63–65]. A lower similarity of LAFUEL 03 was shown with *B. subtilis* subsp. *subtilis* type strain 168, 76.91%. Based on the comparisons using RaxML, LAFUEL 03 was placed within a cluster that included most of the *B. velezensis* strains (Figure 4).

Table 4. Comparison of the genome of the strain LAFUEL 03 with different *Bacillus* spp. genomes.

Strain	GenBank	ANI (%)	GC (%)	Bp (Gb)	Source/Reference
LAFUEL 03	JAUHJT000000000	100	46.3	3.97	This study
B. velezensis					
LABIM 22	CP045993	97.76	46.4	3.99	Soil [66]
JS25R	CP009679.1	97.69	46.4	4.01	Not specified/Unpublished
YAU B9601-Y2	NC_017912.1	97.62	45.9	4.24	Wheat rhizosphere [67]
FZB42	CP000560.2	97.56	46.5	3.91	Soil [68]
B. amyloliquefaciens					
DSM7(T)	NC_014551.1	93.99	46.1	3.98	Not specified [69]
B. subtilis					
168	NC_000964.3	76.91	43.6	4.21	Type strain [70]
B. thuringiensis					
ATCC	NZ_CM000753.1	66.29	34.7	6.26	Type strain [71]
B. cereus					
FRI-35	CP003747.1	66.45	35.4	5.38	Not specified/Unpublished

3.3.2. Genome Assembly and Annotation of the LAFUEL 03 Strain Sequence

The genome of the strain LAFUEL 03 was assembled in 39 contigs, with an *N50* of 985,989 and an *L50* of 2. The LAFUEL 03 genome was estimated to have 3,974,042 bp, and the annotation showed that the genome has 4103 CDS, with 83 of them related to RNA sequences (Table 5).

Table 5. Summary of the characteristics of the genome of the LAFUEL 03 strain.

Attribute	Value
Genome size (bp)	3,974,042
Coverage (x)	300
GC content (%)	46.3
Contigs	39
N50	985,989
L50	2
Total predicted CDS	4103
RNAs	83
Completeness (%)	99.81
GenBank accession no.	JAUHJT000000000

3.3.3. antiSMASH Analysis of Secondary Metabolism for the Strain LAFUEL 03

The webserver antiSMASH found 17 biosynthetic gene clusters (BGCs) in the LAFUEL 03 genome (Table 6). Among the 17 clusters, 13 showed similarities with BGCs already described in the MiBiG [72] that are linked to the synthesis of surfactin, butirosin A/B, macrolactin H, bacillaene, fengycin, difficidin, rhizocticin A bacillibactin, and bacilysin. Four of the 17 BGCs did not show any similarity with clusters already present in the database.

Table 6. Biosynthetic gene clusters (BGCs) found in the LAFUEL 03 strain genome using the webserver antiSMASH6.0.

Cluster	Type [a]	From (bp)	To (bp)	Most Similar Known Cluster		Similarity
1	PKS-like	63,368	104,612	Butirosin A/B	Saccharide	7%
2	Terpene	186,633	207,373			
3	TransAT-PKS	543,564	634,934	Macrolaction H	Polyketide	100%
4	NRPS, transAT-PKS, T3PKS	859,722	962,368	Bacillaene	Polyketide + NRP	100%
5	NRPS, RiPP-like	73,713	125,505	Bacillicabtin	NRP	100%
6	Lantipeptide class II	289,489	315,857			
7	Other	640,076	681,494	Bacilysin	Other	100%
8	Terpene	84,718	106,601			
9	T3PKS	186,289	227,389			
10	TransAT-PKS	355,477	429,810	Difficidin	Polyketide + NRP	100%
11	NRPS	1	25,225	Surfactin	NRP: Lipopeptide	39%
12	NRPS, transAT-PKS	30,803	107,548	Rhizocticin A	Other	16%
13	NRPS	179,136	206,929	Surfactin	NRP: Lipopeptide	47%
14	NRPS, betalactone, transAT-PKS	1	87,594	Fengycin	NRP	80%
15	NRPS	1	12,861	Fengycin	NRP	20%
16	NRPS	1	9388	Fengycin	NRP	18%
17	NRPS	1	9172	Surfactin	NRP: Lipopeptide	8%

[a] NRPS, non-ribosomal peptide synthetase; NRP, non-ribosomal peptide. PKS, polyketide synthetase. AT, acetyltransferase; T3PKS, type 3 Pks.

3.3.4. Genes in the Strain LAFUEL 03 Related to Biofilm Formation/Regulation

A collection of genes directly or indirectly associated with biofilm production and root colonization was identified in the genome of the strain LAFUEL 03 (Table 7). Key genes such as *abrB*, *galE1*, *degU*, *degQ*, and *remA* obtained from the SubtWiki database had 91, 98, 86, 87, and 98% of similarity, respectively, with genes found within the LAFUEL 03 genome, using BLASTN. Important operons, such as *epsA—epsO* (97–99%), *sfrAA-AC* (80%, 75%, 87%), and *yqxX-sipW-tasA* (98%, 98%, 98%) were also found in the LAFUEL 03 sequence. On the other hand, sequences with similarities to the genes *bslA*, *comA*, *kinB—kinD*, *lytS*, *slrA*, and *tapA* were not found.

Table 7. Genes related to biofilm formation/regulation in the LAFUEL 03 strain.

Gene	Position in the LAFUEL 03 Strain Genome (bp)	Identity with Reference (%)	Described Function on Subtwiki
abrB	45,187 to 45,476	91	Repressor of genes that induce sporulation
degQ	71,346 to 71,211	87	Stimulates the production of degradative enzymes and extracellular poli-gama-glutamate
degU	448,510 to 449,199	86	Capsule biosynthesis (along with SwrA), DegU non-phosphorylated is necessary for motility on swarming
epsA	353,065 to 352,358	98	Synthesis of extracellular polysaccharides
epsB	352,352 to 351,672	99	
epsC	351,427 to 349,634	96	
epsD	349,618 to 348,479	97	
epsE	348,482 to 347,640	98	Motility and glycosyltransferase inhibitor needed for EPS biosynthesis
epsF	347,647 to 346,511	98	
epsG	346,507 to 345,404	98	
epsH	345,385 to 344,348	98	
epsI	344,343 to 343,267	98	Synthesis of poli-N-acetyl glucosamine
epsJ	343,270 to 342,236	97	
epsK	340,722 to 342,239	98	
epsL	340,117 to 340,725	97	
epsM	339,473 to 340,120	97	
epsN	338,296 to 339,468	98	
epsO	337,352 to 338,317	97	
galE1	344,050 to 345,042	98	Galactose utilization
kinA	517,186 to 519,006	78	
motA	487,638 to 486,823	79	
motB	486,851 to 486,109	76	
remA	733,387 to 733,656	98	Transcriptional regulator of genes of the extracellular matrix acts along with SinR, AbrB, and DegU
sigD	808,233 to 808,997	87	
sigH	15,391 to 16,046	87	
sigW	21,892 to 22,455	83	
sinI	474,811 to 474,638	80	Biofilm formation control
sinR	474,604 to 474,269	97	Transcriptional regulator of post-exponential-phase responses genes
sipW	472,788 to 473,372	98	Bifunctional signal peptidase that controls surface-adhered biofilm formation and processes TasA and TapA
spo0A	507,969 to 508,769	99	Regulator of the sporulation initiation
srfAA	8 to 1141	80	Surfactin synthesis
srfAB	1361 to 6174	75	
srfAC	1389 to 5185	87	
swrA	424,288 to 424,624	85	Important activator of the flagellar biosynthesis, controls the number of flagellar basal bodies, controls degU activity
swrB	809,025 to 809,225	80	Controls SigD activity. Activates type 3 flagellar secretion by the protein membrane FliP
swrC	217,287 to 220,430	80	
tasA	473,436 to 474,221	98	Major component of the biofilm matrix, forms amyloid fibers
yhxB	58,091 to 59,833	99	
yqxM	472,145 to 472,816	98	

4. Discussion

We tested the antagonistic activity of the bacterial strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 against *Xvv*. These strains were grown in two different media with the objective of establishing the best conditions for the growth and production of bioactive metabolites. We evaluated the biofilm formation and swarming motility in vitro. We also tested the potential of these three strains for in planta control of bacterial leaf streaks of corn. Furthermore, we performed molecular characterization of the three strains based on 16S *rRNA* sequencing. The LAFUEL 03 strain was also chosen for complete genome sequencing and phylogenetic comparisons. Genes involved in the secondary metabolites production and those related to biofilm regulation/formation were further investigated in regard to their potential for the biocontrol of *Xvv*.

The double culture and CFS antagonism experiments carried out in vitro showed the antagonism of LAFUEL 01, LAFUEL 02, and LAFUEL 03 against *Xvv*. In planta experiments also demonstrated their potential to control the BLS disease of corn. Furthermore, the results of the in vitro experiments indicated that metabolites produced by the bacterial strains would be responsible for the control of BLS in corn plants, which means a control based on compounds with antimicrobial activity, although plant resistance induction cannot be ruled out.

Several studies have reported in vitro bacterial antagonism against *Xanthomonas* spp. and the control of diseases caused by these bacteria through agents such as *Bacillus* spp.,

Pantoea spp., and *Pseudomonas* spp. [32,73,74]. However, to our knowledge, there is only one study on the biological control of *Xvv* [36]. In this study, [36] performed antagonism experiments with *Pantoea ananatis* against *Xvv*. Although no antagonistic activity of *P. ananatis* was observed in vitro, reduced BLS severity was observed in the plants treated with the biocontrol agent [36]. Co-infiltrations of *Xvv* and *P. ananatis* also resulted in significantly smaller lesions compared to those in the plants inoculated with *Xvv* alone [36]. While 40% of the leaves were diseased in the plants inoculated with *Xvv* alone, only 19% of the leaves had BLS lesions when the plants were co-inoculated with *Xvv* + *P. ananatis* [36].

In a study carried out by [75], eight bacilli-type bacterial strains were plate diffusion tested against *X. campestris* pv. *campestris* (*Xcc*). All the strains formed inhibition halos with an average of 20 mm. Strains of *B. subtilis* and *Pseudomonas fluorescens* had an antagonistic activity against *Xcc* [76]. Inoculations performed in cauliflower plants revealed that the combination of both bacteria applied one week before *Xcc* inoculation reduced disease incidence to levels lower than treatments with streptomycin. However, both antagonistic bacteria could not inhibit the in vitro growth of *Xcc* at concentrations below 10^6 CFU mL^{-1}. These findings pointed to the potential of both *B. subtilis* and *Pseudomonas fluorescens* as biocontrol protective agents against *Xcc*.

In our study, the inhibition zones in double culture and in CFS experiments were ~30 and ~40 mm, respectively, for both media tested. These values were comparable to the ones obtained with rhizospheric *Bacillus thuringiensis* and *Pseudomonas aeruginosa* for *Xcc* by [33]. They evaluated the antagonism of *P. aeruginosa* and *B. thuringiensis* against *Xcc* in vitro and in vivo. The combined use of the strains produced an average inhibition zone of 18.1 $\pm$ 1.4 mm radius ($p < 0.05$). Both bacteria were able to control the black rot disease through foliar sprays or seed soak and soil drench techniques, with reductions in disease ranging from 32 to 60%. However, the strains applied in a combined manner were significantly more effective, with 75% in disease reduction.

Several studies report biocontrol on diseases caused by *Xanthomonas* spp., including citrus canker [31,77] bacterial blight of cotton [78], and tomato bacterial spot [34,73] with strains of *Bacillus* sp.

The sequencing of the gene 16S *rRNA* allowed the taxonomic identification of the strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 as belonging to the genus *Bacillus*. Genomic analysis has revealed that the strain LAFUEL 03 has ~97% similarity with several strains of *B. velezensis*. Further, the ANI of $\geq$95% is a threshold level to classify organisms in the same species [79,80], whereas genomes of organisms with ANI values < 94% indicate that these organisms may belong to different species [80]. In this study, an ANI of ~97% confirmed that LAFUEL 03 belongs to *B. velezensis* species.

Bacteria display several different population behaviors to colonize an ecological niche, i.e., to cope with adverse environments and to adapt to competitive or collaborative interactions with other microbial species. Population behaviors may range from the formation of sessile biofilms to several forms of cellular motility [81]. Bacterial biofilms are clusters of bacteria that are attached to a surface and/or to each other and embedded in a self-secreting matrix [82]. Bacteria gain several advantages from living in biofilms, including protection from predation, desiccation, exposure to antibacterial substances, and better uptake of nutrients released in the plant environment. Biofilms provide survival sites for pathogenic and beneficial bacteria, including protection and increasing the bacteria's potential to survive and evolve in the plant environment [83]. Depending on the kind of surface occupied, biofilms can be categorized as submerged (solid–liquid interface), colony (solid–air interface), or pellicles (liquid–air interface) [84]. *Bacillus* species have an important ability to form biofilms, which increase their biocontrol potential [85–88].

One form of motility is swarming, which is the rapid movement of groups of flagellated cells across surfaces [89]. This behavior is driven by flagella in a thin-liquid film on semi-solid surfaces. For most bacterial species, surface motility is facilitated by the production of surfactants, which also enable them to successfully colonize the host environments [90].

The genome of the strain LAFUEL 03 has specific clusters of genes related to the biosynthesis of secondary metabolites and genes involved in biofilm formation and swarming motility, which may play a significant role in pathogen antagonism. Other studies also evaluated the potential of *B. velezensis* strains as biocontrol agents. For instance, the CMRP 4490 strain has almost the same genes related to biofilm formation and swarming motility found in the LAFUEL 03 strain [66]. Besides pathogen antagonism, other studies demonstrated the activity of plant growth promotion of *B. velezensis* strains, mainly for soybeans [68,91]. The high genomic similarity of the LAFUEL 03 and the reference strain *B. velezensis* FZB42 suggests that the LAFUEL 03 strain could also present activity of plant growth promotion.

Research involving *B. velezensis* strain FZB42 linked its antagonistic activity against *Erwinia amylovora*, the pathogen responsible for fire blight in apples, pears, and other members of the Rosaceae family [92], to the production of difficidin and bacilysin [93]. The results indicated that two gene clusters responsible for synthesizing these molecules were identified in the genome of strain LAFUEL 03, both showing 100% similarity in the MiBiG database. Additionally, the LAFUEL 03 strain exhibited 100% similarity with a gene cluster involved in bacillibactin production, a key siderophore [94,95].

Genomic analysis has shown that LAFUEL 03 possesses specific gene clusters involved in the biosynthesis of secondary metabolites, as well as genes related to biofilm formation and regulation and swarming motility, all of which are important for antagonizing pathogens. In addition to the findings from in silico analysis, LAFUEL 03 demonstrated strong in vitro antagonism against *Xvv* and significantly reduced BLS severity in corn plants. The results of our study indicated that the *Bacillus* sp. strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 are promising biological control agents for *Xvv*, the causal agent of bacterial leaf streak of corn.

5. Conclusions

The bacterial strains LAFUEL 01, LAFUEL 02, and LAFUEL 03, as well as their metabolites, have antagonistic activity against RL1 *Xanthomonas vasicola* pv. *vasculorum* strain and are able to control BLS in corn plants. Further, the sequencing of the gene 16S *rRNA* allowed the identification of the strains LAFUEL 01, LAFUEL 02, and LAFUEL 03 as belonging to the genus *Bacillus*, and the genomic analysis revealed that the strain LAFUEL 03 belongs to the *Bacillus velezensis* species. *B. velezensis* LAFUEL 03 harbors genes related to the biosynthesis of secondary metabolites, biofilm formation/regulation, and swarming motility that strengthens its capability to control BLS of corn and indicates a major potential for developing a commercial biocontrol agent.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14112495/s1. Figure S1. Probability of growth inhibition of *Xanthomonas vasicola* pv. *vasculorum* strain RL1 by the strains LAFUEL 01, LAFUEL 02 and LAFUEL 03 of *Bacillus* spp. grown in two culture media, LB and ISP2, according to the nonparametric Kaplan-Meier analysis (log-rank test).

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