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Evaluation of Raw Cheese as a Novel Source of Biofertilizer with a High Level of Biosecurity for Blueberry

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Abstract: Today's agriculture requires the search for new and modern tools in order to improve and expand the use of its crops and to increase their sustainability. The use of plant growth-promoting PGP bacteria (PGPB) is the target of much research and seems to be an ideal strategy as long as the strains are properly selected for this purpose. Among the bacteria, lactic acid bacteria (LAB) are considered a suitable alternative due to their high biosafety and mechanisms for promoting plant growth. In view of this, in this work we decided to isolate LAB with PGP capacity from raw milk cheese of the PDO "Serra da Estrela". A total of 88 strains with a high diversity and remarkable capacity to control food-borne and pathogenic microorganisms were isolated. In addition, most of them showed excellent capacities for phosphate solubilization and the production of indole-3-acetic acid and siderophores. Subsequently, we also studied their inoculation in blueberry seedlings. Among the isolates, strains QSE20, QSE62 and QSE79 showed the most remarkable ability to efficiently colonize the rhizosphere of this plant, improving root development and increasing the number of secondary roots.

Keywords: lactic bacteria; PGPB; blueberry cultures; root colonization; raw milk cheese

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1. Introduction

The study of lactic acid bacteria (LAB) associated with the fermentation processes of dairy products, such as cheese, has shown that these populations play a crucial role in the organoleptic properties of fermented dairy products [1]. Industrial cheese manufacturing processes are based on the elimination or maximum reduction of the native populations of microorganisms present in milk through the pasteurization process and the addition of starters to control the fermentation process [2]. Nevertheless, there are a large number of cheeses that belong to traditional varieties and their production requires the use of raw milk [3]. Therefore, these cheeses are produced according to traditional methods [3] and in compliance with Protected Designation of Origin (PDO) regulations [4,5]. As far as we know, they contain a high diversity of LAB, both at the specific and infraspecific levels [6–8], the origin of which is related to horizontal and vertical contaminants in the environment of the milk production and extraction [9]. On the Iberian Peninsula, especially in the inner-western region, there is a particular variety made from raw milk and curdled with vegetable rennet from *Cynara cardunculus* [10], resulting in soft cheeses with numerous

regional variants in which vertical transmission is enhanced, among the well-known are Torta del Casar cheese, Azeitão cheese and Serra da Estrela cheese [4,8,11,12].

Considering that the use of bacterial genera poses some biological risk, several efforts have been made in recent years to search for new microorganisms that have the potential to be used as biofertilizers and biostimulants with no, or fewer, toxic effects. Among the most studied microorganisms in the last few years, *Serratia* or *Pantoea* stand out, but several species and strains from these genera have health implications due to their pathogen potential [13,14]. Since LAB are able to produce bacteriocins and control pathogenic bacteria in food to prevent outbreaks associated with the consumption of fresh fruits and vegetables, it is not surprising that they are also a target of many studies [15]. As far as we know, the use of LAB as plant growth-promoting bacteria (PGPB), especially from the plant environment, is supported by their ability to exert plant growth-promoting mechanisms, highlighting the production of siderophores, phytohormones and/or phosphate solubilization [16]. In addition, they can produce secondary metabolites that have the ability to control bacterial and fungal phytopathogens by releasing lytic enzymes, competing for space and/or producing bacteriocins [17,18]. In this way, LAB have optimal properties to positively interact with plants and humans and can be considered probiotics that play a dual role as probiotic agents [19].

However, LAB are not the most abundant microorganisms in agricultural microbiomes [1,17,20–22], and their isolation from the rhizosphere, phyllosphere and endophytic environments of various plants can be difficult due to their metabolic needs and biological properties, as well as their growth requirements, which may be limited by the presence of other, more abundant taxa in these environments [23].

For this reason, the present work proposes to use cheeses made from raw milk from the PDO "Serra da Estrela", the diversity of LAB of which is practically unknown, focusing on the potential ability to interact with plants. Second, we also propose the application of this bacteria to promote plant growth for the improvement of blueberry cultivation, a booming crop in the Beira interior region, where the PDO "Serra da Estrela" is embedded. Since this crop must be developed under acidic conditions, the use of LAB as biofertilizer could present advantages over other bacterial biostimulants due to the acidophilic nature of these bacteria, combined with its PGP mechanism and high biosecurity characteristics.

2. Materials and Methods

2.1. Strains Isolation

Putative biofertilizer strains were isolated from raw cheeses from "Serra da Estrela" PDO due to the absence of a pasteurization process in milk employed in their preparation. A total of three different commercial cheeses from Lactiser Ltd. Limited (Seia, Portugal) were selected for the strain isolation. Three replicates for each cheese were analyzed in a total of 9 samples. The methodology used for strains isolation was described by Sanchez-Juanes et al. [8], using the MRS agar (Sigma Co., St. Louis, MO, USA), and the inoculated plates were incubated at 28 °C for 48 h.

2.2. MALDI-TOF MS Performing and Data Analysis

The sample preparation and the MALDI-TOF MS analysis were carried out as previously published [8], using a saturated matrix solution of α-HCCA (Bruker Daltonics, Bremen, Germany) dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid. Amounts of biomass between 5 and 100 mg were used to obtain the spectra indicated by the manufacturer. The calibration mass was done based on the Bruker Bacterial Test Standards (BTS), using masses as averages: RL36, 4365.3 Da; RS22, 5096.8 Da; RL34, 5381.4 Da; RL33meth, 6255.4 Da; RL29, 7274.5 Da; RS19, 10,300.1 Da; RNase A, 13,683.2 Da and myoglobin, 16,952.3 Da.

The score values proposed by the manufacturer are the following: a score value between 2.3 and 3.00 indicates highly probable species identification. A score value between 2.0 and 2.299 indicates secure genus identification and probable species identification. A

score value between 1.7 and 1.999 indicates probable genus identification, and a score value < 1.7 indicates no reliable identification.

Cluster analysis was performed based on a comparison of strain-specific main spectra created as described above. The dendrogram was constructed by the statistical toolbox of Matlab 7.1 (MathWorks Inc., Natick, MA, USA) integrated with the MALDI Biotyper 3.0 software. The parameter settings were: 'Distance Measure = Correlation average' and 'Linkage = Complete'. The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

2.3. Phylogenetic Analysis of pheS Gene

The amplification and sequencing of the *pheS* gene were carried out as indicated by Doan et al. [24] using the primers *pheS*-21-F (5′-CAYCCNGCHCGYGAYATGC-3′) and *pheS*-23-R (5′-GGRTGRACCATVCCNGCHCC-3′). The sequences obtained were compared with those from GenBank using the BLASTN program [25]. The obtained sequences and those of related bacteria retrieved from GenBank were aligned using the Clustal W program [26]. The phylogenetic distances were calculated according to Kimura's two-parameter model [27]. The phylogenetic trees were inferred using the Neighbor-joining model [28], and MEGA 7.09 software [29] was used for all the phylogenetic analyses.

2.4. Evaluation of Antimicrobial Activity

The evaluation of the antimicrobial activity was performed based on the method of agar solid diffusion by perforation of the cylindrical cavities [30]. Briefly, pathogenic/foodborne bacteria were further cultivated on Mueller–Hinton (MH) at 37 °C for 24 h. The technique was conducted using a suspension with a standard McFarland turbidity of 0.5. Each LAB strain was grown on MRS broth for 72 h at 28 °C and 125 rpm. Next, the medium was centrifuged at 6.000× *g* for 4 min, and each well (4 mm high × 5 mm diameter), was dug into the Mueller–Hilton (MH) agar plates (55 mm) and filled with 10 µL of each sample. The supernatants were prepared by growing LAB bacteria on MRS broth at 28 °C for 120 h at 125 rpm. Subsequently, they were firstly centrifuged for 2 min at 12.000 rpm and then filtered (0.22 µm). Negative control (MRS broth) and two positive controls. penicillin (0.05 mg/mL) and gentamicin A (10 mg/mL), were also tested. The Petri dishes were incubated at 37 °C for 24 h. The antimicrobial effect was measured in triplicate and determined in terms of inhibitory zone diameter (IZD) in mm.

2.5. Analysis of Plant Growth Promotion Potential of Isolated Strains

The solubilization of insoluble phosphate was analysed on Pikovskaya plates containing 2% $CaHPO_4$, $Ca_3(HPO_4)_2$, or hydroxyapatite and incubated for 15 days at 28 °C [31]. Standardized measurements were conducted employing a phosphate solubilization index (PSI), which was calculated as the ratio between the halos around the colony and the colony size. Siderophore production was evaluated in M9-CAS-AGAR [32] modified with the addition of a cationic solvent, HDTMA, which stabilizes the Fe–CAS complex, and hence allows the detection of siderophore production due to the appearance of an orange halo around colonies. On the other hand, indole acetic acid production was evaluated in JMM medium [32] supplemented with 167 mg/L of tryptophan. After 7 days of incubation, the supernatants were recovered by centrifugation at 5000× *g* and filtered using 0.22 µm Millipore filters (Millipore Co., Burlington, MA, USA). Afterwards, 1 mL of Salkowsky reagent was added to 2 mL of supernatant, and the red color formed was measured by spectrophotometry at 550 nm using an ATI Unicam 8625 Spectrometer (Mattson, Madison, WI, USA).

2.6. In-Plant Evaluation of Plant Growth Promotion Ability

For plant colonization assays, 30 seeds of each *Vaccinium myrtillus* plant were surface sterilized by immersion in 70% ethanol for 30 s followed by soaking in an aqueous 5% sodium hypochlorite solution for 2 min. The seeds were then washed six times with

sterile water and germinated in water–agar plates overlaid with Whatman number 1 sterile paper wetted with sterile water. Subsequently, seeds were transferred to plates with Rigaud & Puppo Agar plates overlaid with Whatman number 1 sterile paper. Each seed was inoculated with 300 µL of a suspension of selected strains with a concentration of 10^7 UFC/mL. The plates were then placed in darkness in a growth chamber at 24 °C until the seedling roots emerged. Data were collected on days 7 and 14 after inoculation.

2.7. Colonization Ability

Plant colonization assays was conducted employing seedlings grown in parallel for PGP ability assays under the same conditions. The evaluation of colonization abilities of isolated strains was developed by immunofluorescence location of the cells of the inoculated strains. Inmunolocation was performed following whole-mount preparation. Roots were excised from the plant and fixed in formaldehyde (4%, *v*/*v*) overnight. Then, roots were blocked on a solution of powdered milk (3%, *w*/*v*) and Triton-X (0.03%, *v*/*v*) in PBS (pH 7.4) for 1 h at room temperature. Afterwards, roots were washed three times with PBS and incubated with a primary antibody (Gram-Positive Marker Antibody, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C. Next, roots were washed three times with PBS and incubated with a secondary antibody (m-IgGkappa BP-CFL 488, Santa Cruz Biotechnology, Dallas, TX, USA). Finally, they were washed with PBS and stored submerged in PBS at 4 °C and protected from light until their use.

Uninoculated roots of blueberries were included in the experiment as negative controls. Fluorescence microscopy was carried out with a Zeiss Axio Imager A1, and the excitation of green fluorescent proteins and Alexa-488 fluorophore linked to secondary antibody was accomplished using a mercury lamp. Root cells were stained with 10 µM of calcofluor white (Sigma, St. Louis, MO, USA).

Colonization density was performed by qPCR analysis. Total DNA was extracted employing NZY Plant/Fungi gDNA Isolation kit (NZYTech, Lisbon, Portugal) from inoculated roots, measuring root section length to reference the final values. The qPCR condition was performed following Pontonio et al. [33]. Primers *SKfw* (5-GGGGATAACAYYTGGAAACAG-3) and *SKrw* (5-CTCGGCTACGTATCATTGTCTTG-3) were employed to detect the concentration of lactic bacteria. Each qPCR reaction was performed in triplicate, using NZYSupreme qPCR Green Master Mix (NZYTech, Lisbon, Portugal) on a CFX96 Biorad real-time Thermocycler (Bio-Rad, Hercules, CA, USA). The PCR core program was as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 7 min. Melt curve analysis of PCR amplicons was initiated at 60 °C, increasing by 1 °C until the final temperature of 95 °C was reached. A calibration curve for qPCR was generated using lactic bacteria suspension with known cell densities (from 3.2 ± 0.2 to 7.5 ± 0.1 Log CFU) of a pure culture of strain *Lactiplantibacillus plantarum* QSE79 isolated in the present study.

3. Results

3.1. Identification of Isolated Strains

A total of 88 strains of LAB were isolated from the cheeses of the PDO "Serra da Estrela". These cheeses are produced in the traditional way from raw milk and coagulated with thistle rennet and display a fermentation period of two months. For this purpose, three different cheeses were used, and three samples were taken from each cheese, yielding counts of $11.6 \pm 2.3 \times 10^8$, $9.4 \pm 0.4 \times 10^8$ and $12.7 \pm 1.4 \times 10^8$ CFU/g of cheese, respectively.

The results of strains identification using MALDI-TOF showed that the isolates were distributed among 6 genera and 8 different species, as follows: *Lactiplantibacillus (L.) plantarum*, *Lacticaseibacillus (La.) paracasei*, *La. rhamnosus*, *Lactococcus (Lc.) lactis*, *Levilactobacillus (Lv.) brevis*, *Latilactobacillus (Lt.) curvatus*, *Leuconostoc (Le.) mesenteroides*, *Le. citreum*. Of the 88 strains, 77 matched and had score values greater than 2.0 as their species were available in the Biotyper 3.0 database. On the other hand, 11 strains that matched showed a score of less than 2.0 but greater than 1.5 (Table 1). Since these strains were classified using

a grouping based on the correlation mean, a total of 15 different groups with matching distribution between the obtained species and similarity scores was obtained. Group I included strains with a similarity score greater than 2.0–2.5; in particular, two subgroups were observed for *L. plantarum* and one which represented the majority and showed great homogeneity (Figure 1).

Table 1. Results of identification of isolated strains by MALDI-TOF MS and *pheS* gene sequencing. In bold, selected strain for each group to sequence the *pheS* gene.

Strains	Group	MALDI TOF Identity		*pheS* Identity		
		Species	Scores	Close Type Strain	Identity %	Accession Number
QSE21, QSE41, QSE43, QSE45, QSE49, QSE50, QSE52, QSE56, QSE58, QSE59, **QSE60**, QSE66, QSE71, QSE73, QSE75, QSE76, QSE77, QSE78, QSE81, QSE84, QSE86, QSE87, QSE92	I	*Lactiplantibacillus plantarum*	2566–2046	*Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917^T	99.75	OM802174
QSE44, QSE48, QSE54, QSE61, **QSE79**, QSE83	II	*Lactiplantibacillus plantarum*	2464–2076	*Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917^T	99.85	OM802180
QSE64	III	*Lactiplantibacillus plantarum*	1969	*Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917^T	100	OM802177
QSE20, QSE51, QSE67A, QSE74	IV	*Lacticaseibacillus paracasei*	2426–2085	*Lacticaseibacillus paracasei subsp. tolerans DSM 20258T*	98.80	OM802167
QSE62	V	*Lacticaseibacillus paracasei*	2343	*Lacticaseibacillus paracasei* subsp. *paracasei* ATCC 25302^T	98.59	OM802175
QSE67B	VI	*Lacticaseibacillus paracasei*	2388	*Lacticaseibacillus paracasei* subsp. *paracasei* ATCC 25302^T	98.60	OM802178
QSE01, QSE04, QSE14, QSE23, QSE33, QSE36, **QSE38**, QSE40, QSE47, QSE55, QSE57, QSE69, QSE72, QSE82	VII	*Lactococcus lactis*	2540–2319	*Lactococcus lactis* subsp. *lactis* LMG 6890^T	99.24	OM802173
QSE68	VIII	*Levilactobacillus brevis*	2301	*Levilactobacillus brevis* LMG 6906 ^{T}	99.76	OM802179
QSE02, QSE03, QSE05, QSE06, QSE12, QSE13, QSE15, QSE16, QSE19, QSE24, QSE27, QSE30, **QSE32**, QSE39	IX	*Latilactobacillus curvatus*	2447–2001	*Latilactobacillus curvatus* JCM 1096 ^{T}	99.75	OM802171

Table 1. *Cont.*

Strains	Group	MALDI TOF Identity		*pheS* Identity		
		Species	Scores	Close Type Strain	Identity %	Accession Number
QSE18, QSE29, QSE31, QSE35, QSE42, QSE46, **QSE63**, QSE65, QSE88, QSE89, QSE91	X	*Leuconostoc mesenteroides*	2332–1800	*Leuconostoc mesenteroides* subsp. *cremoris* LMG 6909^T	99.15	OM802176
QSE11, QSE22, QSE34, QSE53, QSE70, QSE80, QSE90	XI	*Leuconostoc mesenteroides*	1924–1579	*Leuconostoc mesenteroides* subsp. *cremoris* LMG 6909^T	99.44	OM802165
QSE17A, QSE17B	XII	*Leuconostoc mesenteroides*	1825–1665	*Leuconostoc mesenteroides* subsp. *cremoris* LMG 6909^T	99.74	OM802166
QSE37	XIII	*Leuconostoc citreum*	1753	*Leuconostoc citreum* LMG 9849^T	98.89	OM802172
QSE26	XIV	*Lacticaseibacillus rhamnosus*	2447	*Lacticaseibacillus rhamnosus* ATCC 7469 ^{T}	98.30	OM802169
QSE28	XV	*Leuconostoc mesenteroides*	1970	*Leuconostoc mesenteroides* subsp. *cremoris* LMG 6909^T	99.74	OM802170

Group II consisted of strains with a similar similarity score to group I (between 2.0 and 2.4) with *L. plantarum*. Group III also showed identification with *L. plantarum* but had a similarity score of 1.925. Strains belonging to these groups showed similar similarity scores for *L. plantarum* subspecies (variables ranging from 2.2 and 2.0), with group I most closely matching *L. plantarum* ssp. *argentoratensis* DSM 16365^T. In addition, groups I, II and III had higher scores compared to *L. plantarum* ssp. *plantarum* DSM 20174^T.

Group IV included strains with a similarity score between 2.0 and 2.5 for *L. paracasei* (Table 1) and a higher score compared to *L. paracasei* ssp. *tolerans* DSM 20258^T than *L. paracasei* ssp. *paracasei* DSM 5622^T (score value of 2.005). Groups V and VI consisted of strains with score values ranging from 2.388 to 2.344 in *L. paracasei* and had lower score values for *L. paracasei* ssp. *paracasi* DSM 5622^T than group IV. In these three groups, the score value in relation to the type strain of the nearest subspecies, *L. paracasei* ssp. *paracasei* DSM 5622^T, was always below 2.0.

Strains of *Lc. lactis* were included in group VII and had a score value between 2.540 and 2.319 (Table 1). These strains showed high homogeneity in their dendrogram distribution, and all of them had a similarity score of about 2.200 for *Lc. lactis* ssp. *lactis* DSM 20481^T.

Group VIII included only a single strain that is consistent with *Lv. brevis*, showing a score value of 2.301 and greater similarity to the type strain *Lv. brevis* DSM 20054^T.

Group IX included 14 strains with score values ranging from 2.447 to 2.001 in terms of *curvature* similarity to *Latilactibacillus curvatus*, with the higher similarity score for *Latilactobacillus curvatus* DSM 20019^T (scores from 1.900 to 1.700) standing out. Group X, consisting of 11 strains, had scores ranging from 2.332 to 1.800 in relation to different strains of the species *Leuconostoc mesenteroides*, especially for the subspecies *mesenteroides* or *dextranicum*. Group XI included strains with score values from 1.924 to 1.579, giving an identification at the genus level within *Leuconostoc* but likely for the species *Le. mesenteroides*. Group XII included two different strains that were also identified in the genus *Leuconostoc*, but with score values (1.825 and 1.665) that did not assign to a species. Finally, in agreement with the score values obtained, groups XIII, XIV and XV had only one strain in each group and were assigned to the genus *Leuconostoc* (groups XIII and XV) and the species *Lacticaseibacillus rhamnosus* (Table 1).

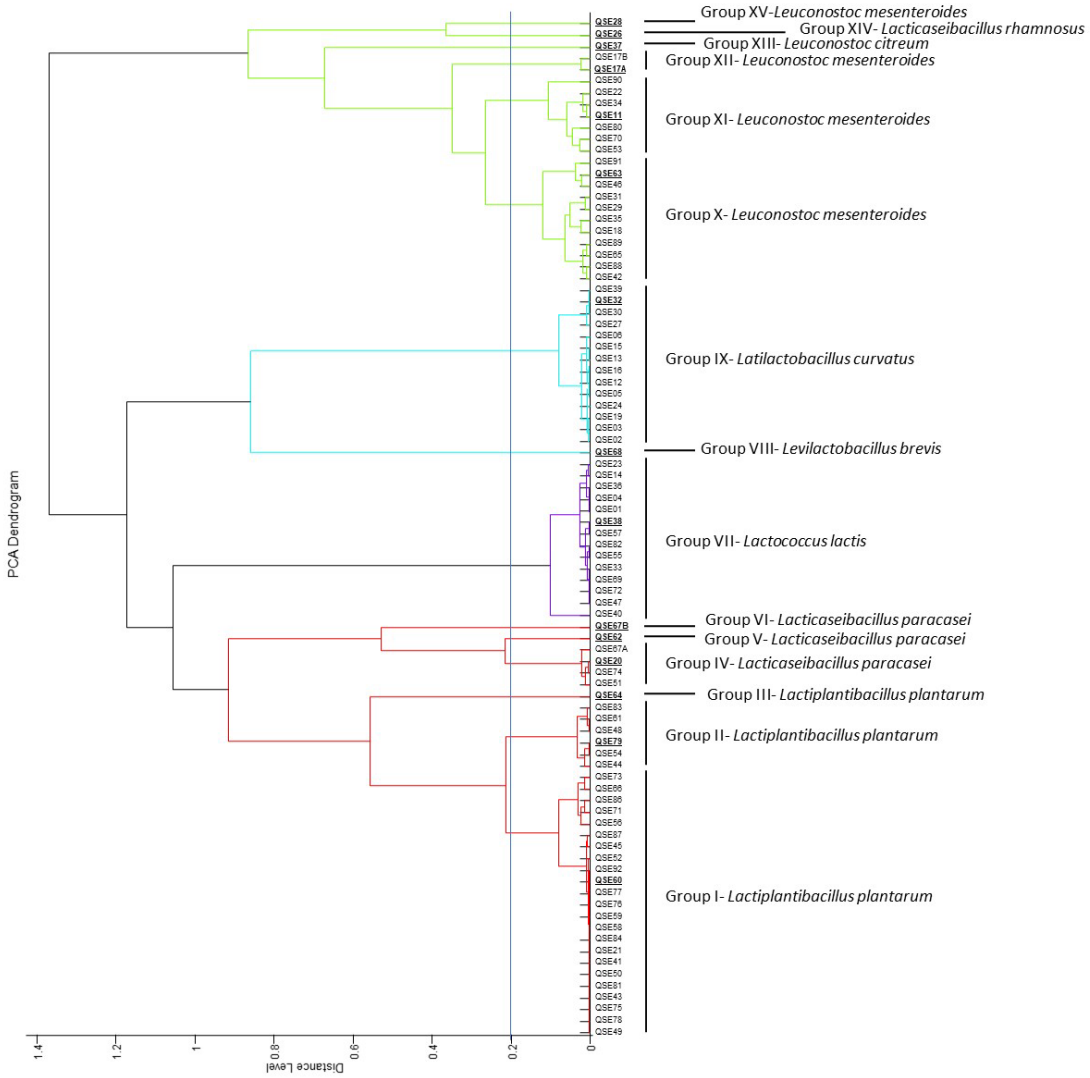

Figure 1. Cluster analysis of MALDI-TOF MS spectra of strains isolated in this study. Distance is displayed in relative units. Representative strains of each group selected for *pheS* gene analysis are marked in bold.

In this way, and based on the results for some groups of the dendrogram, sequencing of the housekeeping gene *pheS* was performed to complete the identification of each strain, mainly from groups XIII, XIV and XV, and to clarify the position of the remaining ones relative to the different subspecies. The obtained data are presented in Table 1 and Figure 2. Thus, QSE60, QSE79 and QSE64, representatives of groups I, II and III, showed percentage similarities in the *pheS* gene of 99.75%, 99.85% and 100%, respectively, for *L. plantarum* subsp. *plantarum* ATCC 14917^{T}, the type strain of this subspecies. Moreover, there was no doubt about its identification because the next subspecies, *argentoratensis*, appeared with percentages of similarity of 90% in the *pheS* gene (Figure 2). The representative strain of group IV, QSE20, showed a percentage similarity in the *pheS* gene of 98.80% for the type strain *La. paracasei* subsp. *tolerans* DSM 20258^{T}, while strains QSE62 and QSE67B, representatives of groups V and VI, showed a similarities in the *pheS* gene of 98.60% and 98.59%, respectively, with respect to the type strain *La. paracasei* subsp. *paracasei* ATCC 25302^{T}. Thus, we can conclude that these three groups belong to *La. paracasei*, while the first group belongs to the subsp. *tolerance* and the other two groups belong to the subsp. *paracasei*, confirming their phylogenetic position (Figure 2). The group XIV belonging to this genus, which consists of strain QSE26, was identified as *La. rhamnosus* because it has

a percentage similarity of 98.30% for the type strain of this species and shows a clearly defined phylogenetic position (Figure 2).

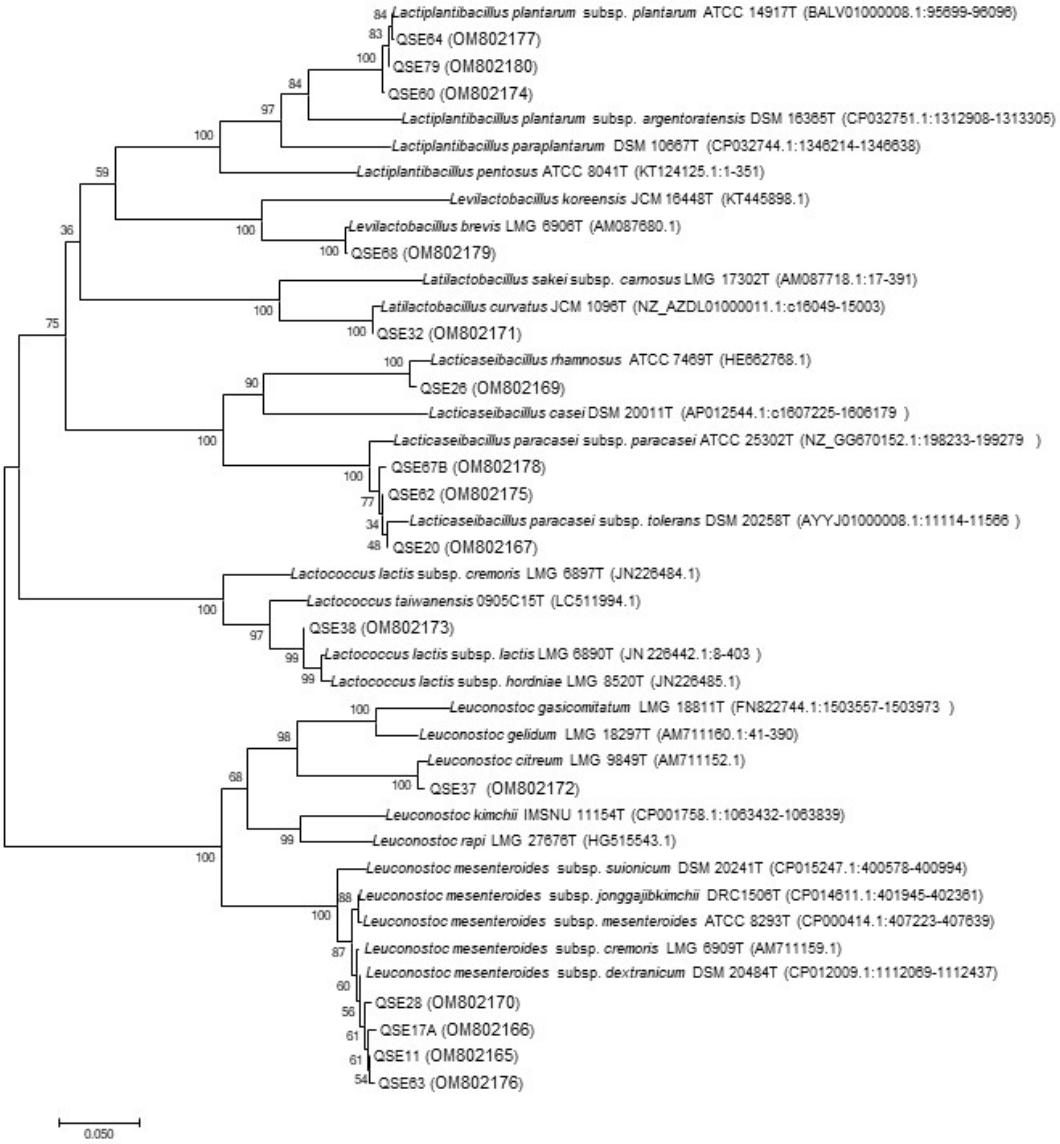

Figure 2. Neighbor-joining phylogenetic unrooted tree based on *pheS* gene partial sequences (400 nt) showing the taxonomic location of 15 representative strains from different groups of MALDI-TOF MS within the closely related type strains.

The identification of group VII was confirmed by the identification of strain QSE38, whose *pheS* gene had a percentage of 99.24% with respect to the sequence of *Lc. lactis* subsp. *lactis* LMG 6890^T, which is the type strain of this species. The strain QSE68, which belongs to group VIII, confirmed the identification obtained via MALDI-TOF, and showed a percentage similarity in the *pheS* gene of 99.76% for *Lv. brevis* LMG 6906^T. Group IX was represented by strain QSE32, which was consistent with the results obtained in MALDI-TOF, and had a percentage similarity in the *pheS* gene of 99.75% for the sequence of the type strain *Lt. curvatus* JCM 1096^T (Figure 2).

Groups X, XI, XII and XV, represented by strains QSE63, QSE11, QSE17A and QSE28, respectively, were identified as *Le. mesenteroides* by MALDI-TOF, and their specific affiliation was confirmed by sequencing of the *pheS* gene (Table 1 and Figure 2). However, the data from MALDI-TOF showed uncertain affiliation with respect to the subspecies, while the *pheS* gene of these strains showed percentage similarities of 99.15%, 99.44%, 99.74% and

99.74%, respectively, with respect to *Le mesenteroides* subsp. *cremoris* LMG 6909^T. The strains QSE63, QSE17A and QSE28 showed 98.90% similarity and strain QSE11 showed 99.14% in relation to *Leuconostoc mesenteroides* subsp. *dextranicum* DSM 20484^T. As such, it is difficult to assign the representative strains of groups X, XI, XII and XV, and thus to assign these groups themselves to a particular subspecies based on the percentage similarity achieved (Table 1 and Figure 2). Group XIII, consisting only of strain QSE37, showed a percentage similarity of the *pheS* gene of 98.89% for the sequence of the type strain of *Le. citreum*, which occupies a unique position in the phylogenetic tree (Figure 2).

3.2. Screening for Antimicrobial Activity

Analysis of the capacity of LAB to produce antimicrobial substances is a proven fact and can be a considerable advantage in their competition for space, e.g., in the rhizosphere. The study of the capacity of the representative strains of each MALDI-TOF MS group was carried out by the method of agar solid diffusion by perforation of the cylindrical cavities. Strains QSE20, QSE62, QSE63 and QSE79 showed the highest activities and the highest number of inhibited microorganisms (Figure 3). Of these, strain QSE20 stands out as the only strain that showed activity against *Salmonella typhimurinum* and, together with QSE79, against *Enterococcus faecalis*. The latter strain also showed activity against eight strains studied, some of which were of notable importance, such as *Bacillus cereus*, *E. faecalis*, *Acinetobacter baumanii* and *Staphylococcus aureus*. Although most strains showed activity against *Proteus mirabilis* and *S. aureus*, *Serratia marcenscens* also proved to be slightly sensitive to many of the selected isolates. Isolates QSE11, QSE17A and QSE38 showed no activity against almost all studied microorganisms (Figure 3).

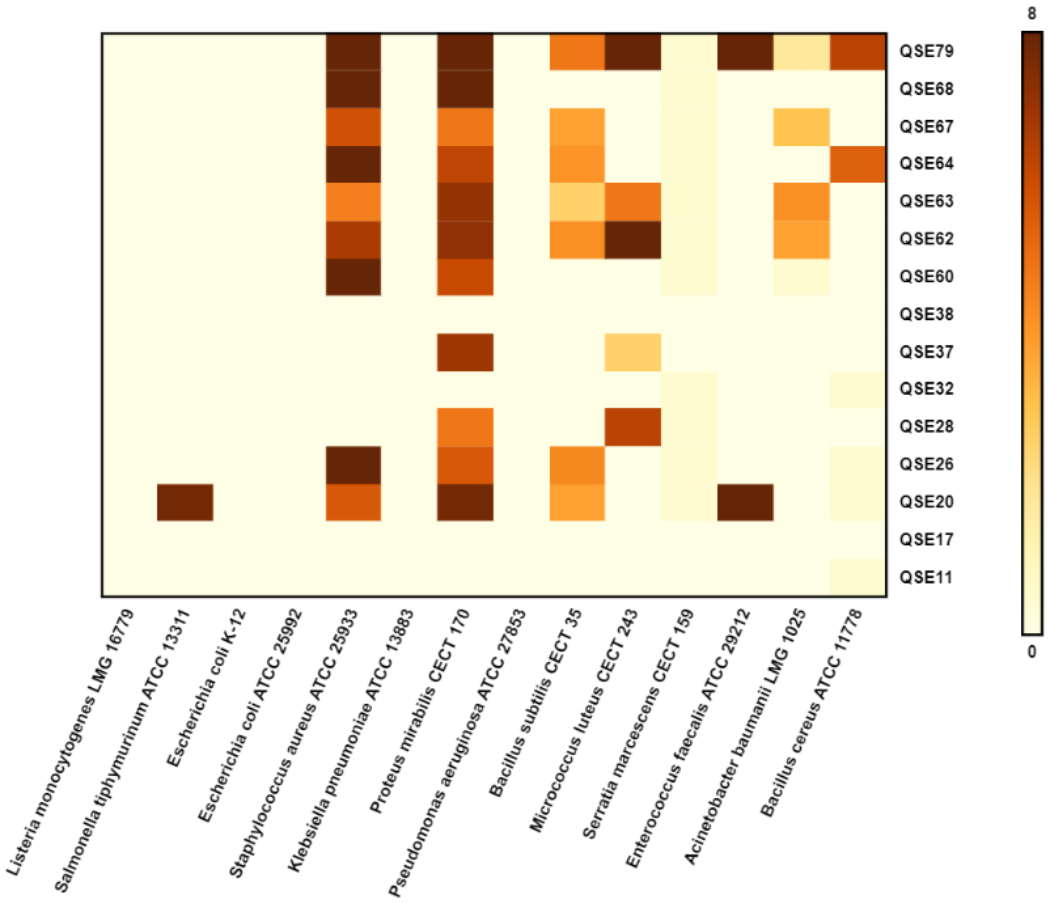

Figure 3. Inhibition activity of each selected strain against different food-borne bacterial pathogens. Units are in centimeters of radius from the margin of the cavity.

3.3. Plant Growth-Promotion Mechanisms

Evaluation of the capacity for plant growth promotion of the selected bacteria showed that, in general, all group-selected strains presented some PGP mechanisms (Figure 4). In fact, all isolates, except strain QSE37, showed the ability to produce lower levels of indoleacetic acid (values ranging from 9 to 68 µg/mL) (Table 2). The second most common mechanism was the production of siderophores, which was observed in all isolates, except for strains QSE28 and QSE37. At this point, it is necessary to highlight the activity of some strains, such as QSE63, QSE64, QSE11 and QSE79, the PSI production of which ranged from 1 to 0.85. On the other hand, the solubilization of a phosphate source was observed in all isolates, except strains QSE28 and QSE38. However, the strains showed a greater ability to solubilize tricalcium phosphate than dicalcium phosphate, while solubilization of hydroxyapatite was the least common mechanism, occurring only in strains already able to solubilize other inorganic phosphate sources. According to these data, most of the selected strains seem to be able to promote plant growth through the presence of several associated mechanisms (the solubilization of phosphate from several sources, production of siderophores and indole-acetic acid).

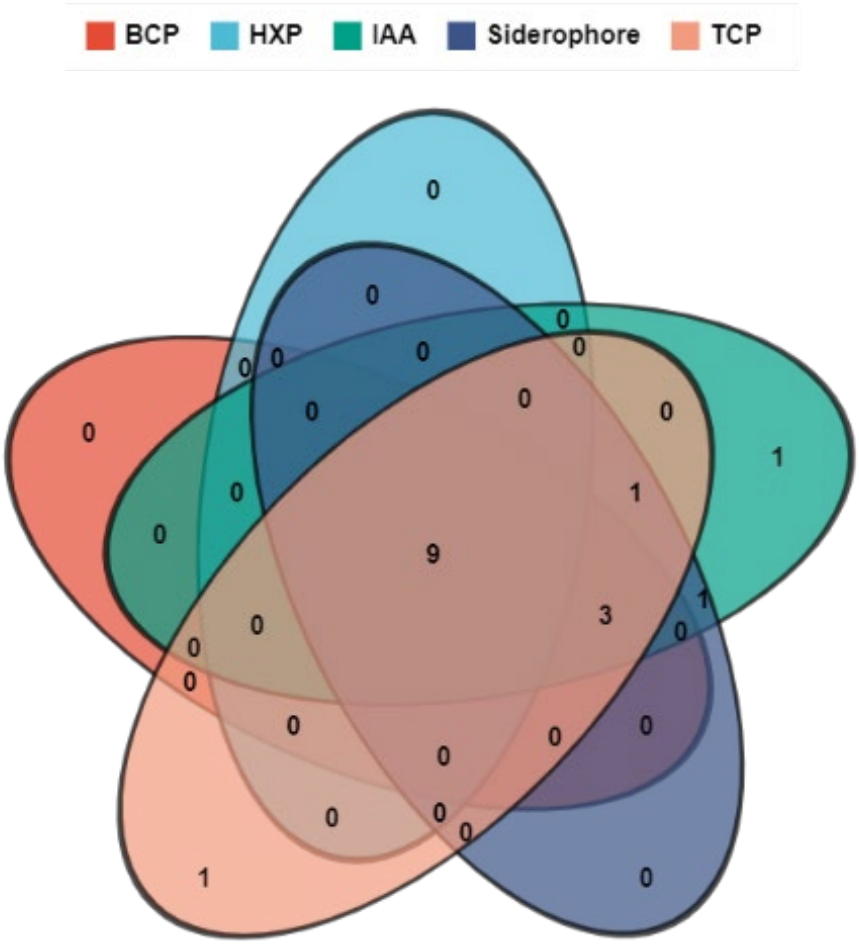

Figure 4. Venn diagram of PGP mechanisms detected in the selected strains. BCP: bicalcium phosphate; TCP: tricalcium phosphate; HXP: hydroxyapatite; IAA: indole-3-acetic acid.

Table 2. Summary of PGP mechanisms detected in each selected strain.

Strain	BCP	TCP	HXP	Siderophore	IAA
QSE11	0.14	0.46	n.d.	0.85	11.0
QSE17A	0.07	0.45	n.d.	0.27	12.0
QSE20	0.52	2.69	1.09	0.42	42.0
QSE26	0.56	1.85	0.88	0.55	24.0
QSE28	n.d.	n.d.	n.d.	n.d.	32.0
QSE32	n.d.	0.26	n.d.	0.08	17.0
QSE37	n.d.	0.09	n.d.	n.d.	n.d.
QSE38	n.d.	n.d.	n.d.	0.09	26.0
QSE60	0.29	1.87	0.77	0.50	28.0
QSE62	0.22	1.00	0.67	0.58	68.0
QSE63	0.11	1.19	0.92	1.00	2.0
QSE64	0.50	2.41	0.91	0.87	9.0
QSE67B	0.67	1.27	0.83	0.28	26.0
QSE68	0.13	0.39	n.d.	0.50	28.0
QSE79	0.32	2.23	0.73	0.85	52.0

BCP: bicalcium phosphate; TCP: tricalcium phosphate; HXP: hydroxyapatite; IAA: indole-3-acetic acid, expressed in μg/mL; n.d.: not detected.

3.4. Plant Growth-Promotion Assays on Blueberry Seedlings

Once the potential to promote plant growth was analyzed, the evaluation of the promotion of growth in blueberry plants was carried out using seeds. For this purpose, the representative strains for each MALDI-TOF MS group were inoculated into blueberry seedlings. After 7 days of inoculation, the QSE20, QSE62 and QSE79 strains were able to increase root development in a statistically significant way ($p < 0.0001$), revealing percentages of 42.75%, 59% and 66.9%, respectively (Table 3), when compared with control. An increase in the number of secondary roots was also observed in these strains, together with QSE28, QSE60, QSE62, QSE67A and QSE68 strains ($p < 0.0001$), QSE38 ($p < 0.001$) and QSE32 ($p < 0.05$) (Figure 4). In the case of aerial length, no differences were found between the treatments and the control (Figure 5C). After 14 days' inoculation, the strains QSE20, QSE26, QSE28, QSE60, QSE62, QSE64, QSE67A, QSE68 and QSE79 produced an increase in root development ($p < 0.0001$) and, together with the strain QSE38, an increase in the number of secondary roots (Table 3 and Figure 5E). As with the results of day 7, on day 14 after inoculation, no differences were observed in the length of the aerial parts (Table 3 and Figure 5F).

Table 3. Growth parameters evaluated for blueberry seedlings inoculated with selected strains.

Treatment	7 dpi			14 dpi		
	Root Length	Secondary Roots	Aerial Length	Root Length	Secondary Roots	Aerial Length
Control	1.45 ± 0.21	1.40 ± 0.57	0.83 ± 0.09	2.61 ± 0.73	1.96 ± 0.95	1.09 ± 0.15
QSE11	1.52 ± 0.31	1.67 ± 0.79	0.92 ± 0.07	3.04 ± 0.74	2.57 ± 1.44	0.95 ± 0.13
QSE17A	1.57 ± 0.34	1.77 ± 0.35	0.88 ± 0.08	3.14 ± 0.74	2.23 ± 0.49	1.26 ± 0.11
QSE20	**2.07 ± 0.36** ***	**2.40 ± 0.91** ****	0.97 ± 0.13	**4.14 ± 0.86** ****	**4.36 ± 1.27** ****	1.33 ± 0.19
QSE26	**1.96 ± 0.41** *	1.85 ± 0.66	1.05 ± 0.07	**4.31 ± 0.73** ****	**3.11 ± 0.83** ****	1.09 ± 0.07
QSE28	1.76 ± 0.17	**2.12 ± 1.02** ****	0.79 ± 0.11	**4.22 ± 0.44** ****	**3.56 ± 1.86** ****	1.23 ± 0.13
QSE32	1.51 ± 0.24	**1.90 ± 0.81** *	0.91 ± 0.11	3.32 ± 0.48	2.66 ± 1.36	0.94 ± 0.11
QSE37	1.41 ± 0.37	1.52 ± 0.42	1.08 ± 0.07	2.82 ± 0.66	1.91 ± 0.76	1.36 ± 0.08
QSE38	1.67 ± 0.28	**2.01 ± 0.61** ***	0.86 ± 0.12	3.01 ± 0.56	**3.56 ± 0.85** ****	1.23 ± 0.12
QSE60	1.78 ± 0.33	**2.15 ± 0.68** ****	0.83 ± 0.06	**3.92 ± 0.85** ****	**3.31 ± 1.05** ****	1.30 ± 0.09
QSE62	**2.31 ± 0.45** ****	**2.68 ± 1.03** ****	0.73 ± 0.09	**4.16 ± 0.90** ****	**3.77 ± 1.87** ****	1.35 ± 0.15
QSE63	1.52 ± 0.37	1.55 ± 0.76	0.96 ± 0.14	2.74 ± 0.74	2.38 ± 1.28	1.12 ± 0.14
QSE64	1.67 ± 0.44	1.62 ± 0.61	0.79 ± 0.07	**3.67 ± 0.94** ****	**2.72 ± 0.94** *	0.92 ± 0.10
QSE67A	1.86 ± 0.27	**2.41 ± 0.95** ****	1.00 ± 0.10	**3.72 ± 0.48** ****	**4.38 ± 1.19** ****	1.26 ± 0.10
QSE68	1.74 ± 0.24	**2.18 ± 0.84** ****	0.82 ± 0.08	**4.18 ± 0.57** ****	**2.74 ± 1.41** *	1.06 ± 0.09
QSE79	**2.42 ± 0.44** ****	**2.36 ± 0.67** ****	0.92 ± 0.07	**4.84 ± 0.88** ****	**3.90 ± 1.13** ****	1.45 ± 0.13

Means (n = 30) are shown. Treatment mean values in bold type were higher than those for the uninoculated control treatment, according to Tukey comparison tests at $p \leq 0.05$ (*), $p \leq 0.0001$ (***), $p < 0.0001$ (****).

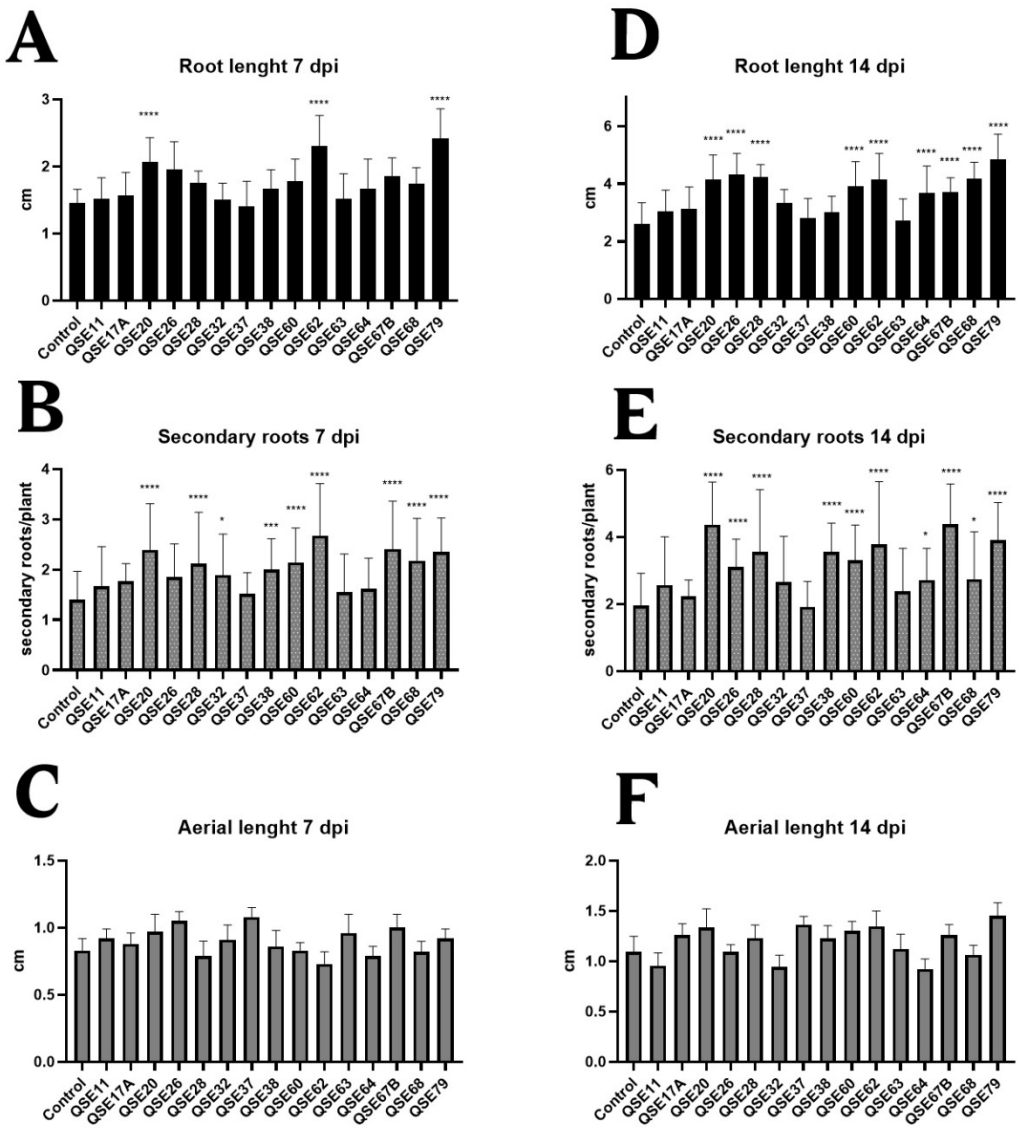

Figure 5. Results of plant growth promotion assay at 7 dpi (**A**–**C**) and 14 dpi (**D**–**F**). Statistical differences for each treatment with the uninoculated control treatment, according to Tukey comparison tests at $p \leq 0.05$ (*), $p \leq 0.0001$ (***), $p < 0.0001$ (****).

3.5. Colonization Ability

Root colonization ability was studied in the strains that gave the best results in promoting plant growth in the plant assays by direct immunolocalization. For this reason, strains QSE20, QSE62 and QSE79 were selected. As can be seen in Figure 6, after 14 days of inoculation, colonization of the root surface occurred in all three cases, although differences in density were observed. Among the strains, QSE20 exhibited the least root colonization, as evidenced by local accumulations and large root zones (Figure 6A,B). These data were verified by quantification, and colonization was found to be 3 Log–4 Log CFU/cm throughout the root, with a higher concentrations in the upper regions, decreasing toward the elongation zone, and a slight increase in the apical region. Strains QSE62 and QSE79 showed more competent colonization of the root surface, as shown in Figure 6C,E, occupying large areas. This colonization appeared to be evident even in strain QSE79 (Figure 6E). On the other hand, strain QSE62 showed colonization with a more three-dimensional arrangement developing between the root hairs of the root, possibly through the formation of biofilms (Figure 6D). In strain QSE79, strong development was observed on the root surface, with high colonization density and homogeneity throughout the root (Figure 6F).

An increase in concentration per cm of root was observed in both strains compared to QSE20, with a concentration of 3 Log–7 Log CFU/cm, depending on the root region, and a similar arrangement. The lowest densities were observed in root elongation regions, while the highest densities were found in the upper root regions, which is to be expected since there is a greater abundance of root hairs in this area. These data show competent colonization of the selected isolates on the root surface of blueberry.

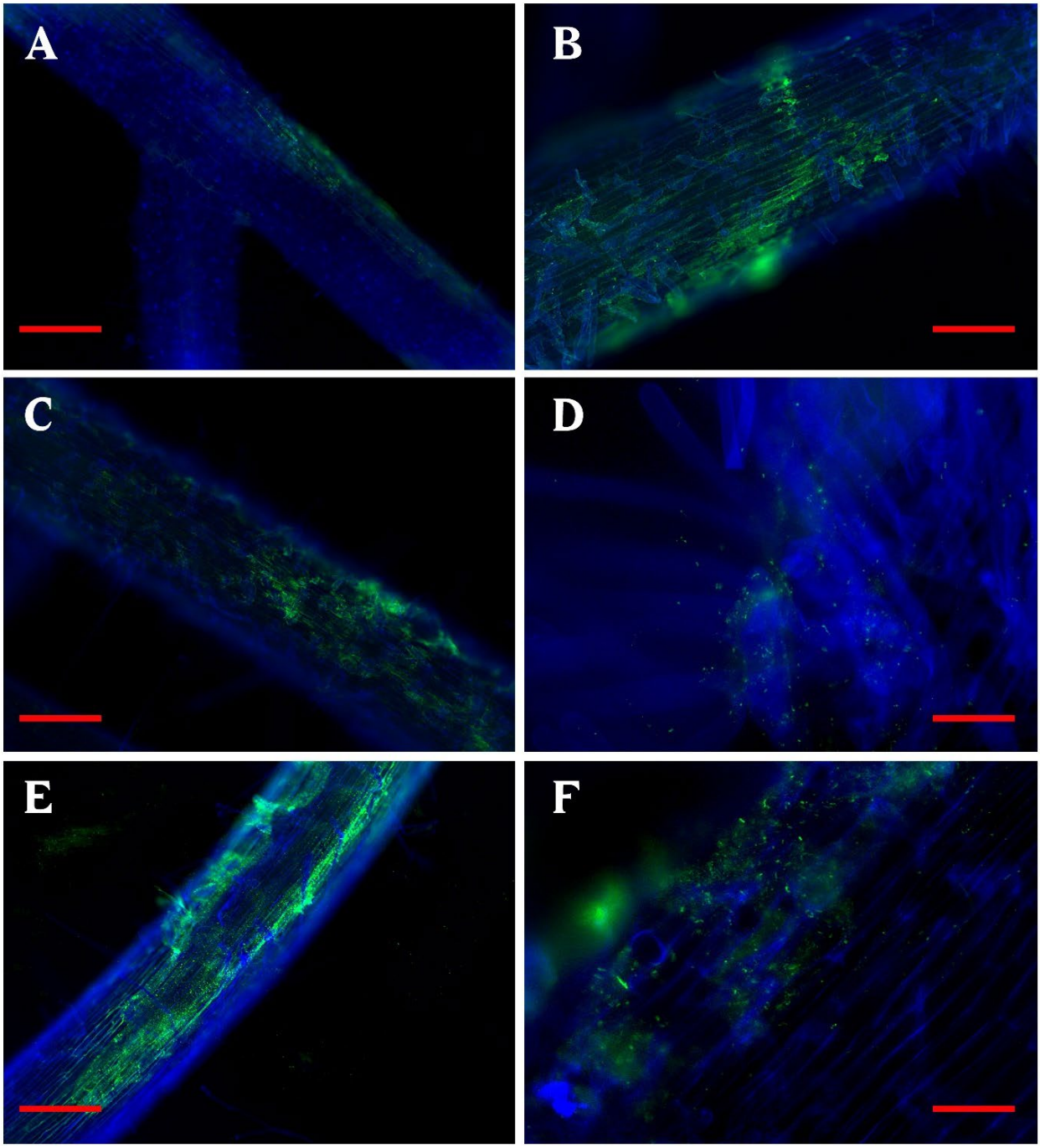

Figure 6. Fluorescence optical micrographs of roots of blueberry seedlings colonized by QSE20 (**A**) 500 µm, (**B**) 200 µm, QSE62 (**C**) 500 µm, (**D**) 75 µm and QSE79 (**E**) 500 µm, (**F**) 75 µm. In green, inmunolocated cells of studied strains, and in blue, root cells stained with calcofluor white.

4. Discussion

Raw milk cheeses are common in the Mediterranean region, and most of them are manufactured in a traditional way; they are included, in several varieties, within the Denominations of Origin classification, ensuring genuineness in their manufacture and maturation as well as in the products employed [9]. On the Iberian Peninsula, most precisely in the center region of Portugal, we found an important group of cheeses curdled by the use of thistle rennet from *C. cardunculus* and which has been intensively studied given the high diversity of LAB [8]. In the present work, we proposed the use of LAB as biofertilizers in agricultural crops because some studies had already shown their usefulness in promoting plant growth [16,34]. Its usefulness lies mainly in the principles of biosafety that, due to its probiotic nature, ensures the use of inoculants innocuous to humans, animals and plants [35]. LAB can be isolated from plant samples, mainly from environments where fermentative processes were predominant. In addition, and considering the saprophytic nature of this group of bacteria, they can also be isolated from agricultural samples [34,36]. However, it is important to take into account that agricultural environments are dominated by other groups of bacteria that frequently hinder the possibilities of isolating these groups, such as the presence of *Proteobacteria* and *Firmicutes*, which are fast-growing and highly competitive bacteria in synthetic media [37]. Therefore, it is beneficial to obtain LAB from other environments capable of ensuring a possible vertical exchange from the environment of populations, such as cheeses made with raw milk, as proposed in this study [38].

In this work, cheeses made with raw milk were shown to contain diverse populations of LAB with a marked probiotic character and the ability to control the populations of pathogens present in milk [2,7,39,40]. Among them, the soft cheeses from PDO "Serra da Estrela" cheese has a significant LAB diversity, as detected in this study with the identification of six different genera and eight species. Similar results have been obtained from Spanish PDO "Torta del Casar" cheeses, where seven different species were detected using similar identification techniques [8], and in Portuguese PDO Azeitão cheese, where six different species of LAB were identified [4]. Studies of the microbiota of PDO "Serra da Estrela" cheeses are scarce as it is impossible to make comparisons between them; they only allow for recognizing the presence of the major groups, which are, *Lactococci, Lactobacilli* and *Enterococci* [12]. Thus, studies using metagenomics of the populations of LAB present in "Serra da Estrela" cheese showed the presence of *Lactococcus*, *Lactobacillus* and *Leuconostoc* but also revealed a great variability between populations [41]. In these cases, the presence of *L. plantarum* and *Le. mesenteroides* was common in all these cases [4,8], with other variable populations of genera previously classified in the genus *Lactobacillus* [42]. In addition, MALDI-TOF MS is a rapid method for the characterization of LAB populations from food sources; the identification conducted by this technique was confirmed by sequencing, and in those cases of scores below 2, a highly accurate identification was observed, which, together with other studies, confirms its usefulness [8,24,43].

Some of these strains have shown an important capacity to inhibit the growth of human pathogens and food-borne species, namely *Staphyllococcus aureus* and *Serratia marcenscens*; the capacity to inhibit this last one was also detected in other soft cheeses [4]. In a general way, this property is associated with their capacity to produce bacteriocins, molecules with a peptide nature that are widely distributed among LAB [35,44]. The capacity to produce bacteriocins and thereby control and eliminate populations of food-borne pathogens in dairy products has been studied in other raw milk cheeses, with results indicating that populations from the prepared environment represent a rich and safe source of competent LAB in the manufacture of cheese [3]. In addition, this property can also be relevant in other biotechnological aspects, such as in the design of biofertilizers as proposed herein, considering their ability to direct competition for space with rhizospheric microorganisms [36,45].

In the last few years, knowledge about different species with the capacity to promote plant growth has diversified, with most showing how new genera can have a positive effect beyond the most common ones such as *Rhizobium*, *Azotobacter* or *Bacillus* [46]. How-

ever, one of the main problems is the appearance of outbreaks of food-borne pathogens associated with the consumption of vegetables [47], which can be enhanced by the use of strains belonging to, for example, the *Enterobacteria* class, which has a potential to be used as biofertilizer that has been well described [13,48]. This aspect can be a remarkable problem because, in recent years, the need to find highly efficient biofertilizers has led to the search for bacterial genera that, despite presenting excellent mechanisms for promoting plant growth, have characteristic pathogenicity such as that observed with *Burkholderia, Enterobacter, Ochrobactrum, Ralstonia, Staphylococcus, Stenotrophomonas, Serratia* or *Klebsiella* [49]. This aspect has been frequently considered when inputs with a high microbial load are used, such as sewage sludge, compost tea or compost, in which the population composition of microorganisms found in the product is not controlled and which often incorporates pathogenic taxa with the negative implications that they present for the health of the applicator and, mainly, for the final consumer; this is especially relevant when referring to a product for fresh consumption [50,51]. In addition, in recent years, research seems to have focused on genera such as Serratia or Pantoea because they have excellent mechanisms for promoting plant growth, such as phosphate solubilization, phytohormone production and nitrogen fixation, in some cases [13,52]; however, their use is subject to their pathogenic nature, as some authors have mentioned [53], being aware that the benefits of their use are fewer than the negative implications they present with respect to human health. Therefore, we must increase the biosafety levels in the selection of microorganisms used in the design of biofertilizers [54]. For this reason, the use of LAB as competent a biofertilizer with a high degree of biosafety has been proposed to improve crop production without causing health risks to farmers and consumers [16]. Thus, it was observed that the strains belonging to the *Leuconostoc* genus presented less or absent activity. In this way, the populations of LAB isolated from cheese seems to have an important capacity in the control of food-borne pathogens, modulating the populations of cheese through the elimination of pathogens [35,55], which can be translated to other foods [56]. Therefore, their use in agriculture can act as the initial source in horticultural and fruit crops, such as blueberries. The obtained data show a consonance with those described by other authors, where the strains belonging to *Lactobacillus* (and other related genera) present higher activity and greater robustness due to the production of various types of bacteriocins [57].

In addition, in recent years, a series of experiences have suggested the possibility of using LAB as crop bioinoculants, mainly associated with the control of diseases and pests, due to their ability to produce several secondary metabolites against some fungi, such as *Botrytis cinerea* [17]; phytopathogenic bacteria, such as *Erwinia* [18,21]; and nematodes [20]. However, they have also shown to have aptitudes related to the promotion of plant growth, as observed in this work, underlining the capacity of some isolated strains to solubilize phosphate from different inorganic sources. This evidence is in agreement with other previous works [21,34,58]. Regarding the production of siderophores, we can find a controversy once some authors indicate that some genera, e.g., *Lactobacillus,* do not require iron in their metabolism [59], and therefore, the production of siderophores is not an expected ability; on the other hand, some interaction due to LAB presence was also reported. In this study, isolates of the genus *Lactobacillus* and related genus showed a considerable capacity, as did strains belonging to the genus *Leuconostoc* (Table 2). These data enhance the necessity for further and more detailed studies regarding plant-associated LAB in order to gain an in-depth understanding of their potential.

Another relevant aspect is the production of phytohormones, which has been confirmed in some of our isolates but was significantly lower compared to the values observed in other PGP bacteria [32,60]. This could not be considered a disadvantage, since some authors point out that the production of phytohormones must be balanced to avoid adverse effects, with high values being detected more frequently in phytopathogenic strains [61,62]. According to Patten and collaborators [63], the most probable route of biosynthesis in LAB is the indole–pyruvate route, and this evidence is relatively common in dairy products [64] and plants [65], where indole catabolism affects their odors. So, the effective

concentration in plant–microorganism interactions depends on the export and/or diffusion mechanisms. The presence of these mechanisms turns LAB into an interesting tool since they can counteract desirable characteristics and be considered an ideal biofertilizer [66].

Until now, few works have analyzed the growth-promoting capacity of LAB in plants. Tverdokhlib et al. [67] observed an increase of 8% in the size of wheat roots after their incubation with *L. plantarum* UN12. These data are much lower than those observed in the present work, where the increase was greater than 50% in most of the experiments. In the same way, the inoculation with *Lactobacillus* sp. KLF01, an indole-acetic acid-producing and phosphate-solubilizing strain, produces an increase in root and aerial lengths and chlorophyll content in tomato and pepper [68]. It has also been described that the inoculation of IAA-producing *Lactobacillus* is capable of improving germination and root development under normal conditions, such as saline conditions, in lettuce and radish [69]. The use of the LCP-1 strain belonging to the genus *Lactococcus* showed an increase in the germination rate of tomatoes and an improvement in their development [70]. In our case, the strains belonging to the genus *Lactococcus* revealed great uniformity (Figure 1) and only showed an increase in the number of secondary roots. That is probably related more to the dairy industry itself than to vertical transmission from the environment. Although the use of *Leuconostoc* has not been studied until now, in the present study, remarkable results, such as those observed in *Lactiplantibacillus* and *Lacticaseibacillus*, have not been observed.

A fundamental aspect of the plant–microorganism interaction involves the colonization of the root plant. In this study, it was clear which strains produced a greater increase in the studied parameters and more efficient colonization of the root surface (Figure 6). As far as we know, these root colonization patterns had already been observed in *Rhizobium* [71], *Bacillus* [72] and *Phyllobacterium* [73] genera, and thus they were considered efficient biofertilizers. This potential is considered a fundamental requirement in the selection of a competent biofertilizer, regardless of the genus studied herein because, although the rhizospheric or endophytic location is not necessary, its arrangement ensures a more remarkable interaction [72]. In turn, the studied strains showed similar colonization patterns as other competent strains, such as *Bacillus amyloliquefaciens* FZB42, where a more intense colonization is reported in the upper root zones of different plant species [74]. This fact indicates similar behavior between different species, such as that of Phylum Firmicutes.

5. Conclusions

The PDO "Serra da Estrela" cheeses present a high diversity of LAB, with isolation of six genera and eight different species, namely *Lactiplantibacillus (L.) plantarum, Lacticaseibacillus (La.) paracasei, La. rhamnosus, Lactococcus (Lc.) lactis, Levilactobacillus (Lv.) brevis, Latilactobacillus (Lt.) curvatus, Leuconostoc (Le.) mesenteroides* and *Le. citreum*. Some species, such as *Lc. lactis*, showed a low infraspecific diversity, while the remaining ones displayed a notorious degree of heterogeneity among them. In addition, the MALDI-TOF MS analysis proved, one more time, that it is a versatile and useful tool for the characterization of food microbial populations. In a general way, the main selected isolates exhibited different mechanisms able to promote plant growth and control undesirable bacteria in food in variable ways. Among the identified isolates, the QSE20, QSE62 and QSE79 strains exerted the most remarkable root colonization and also improvement of the root development and the number of secondary roots. The obtained data indicate that raw milk cheeses can be considered a reliable source for the isolation of highly efficient LAB, which can be applied to the development of biofertilizers given their high biosafety level. The use of these bacteria can open a new horizon in the design of biofertilizers for the application of bacterial biostimulants that can be transmitted to the consumer by vertical transfer and can act as probiotics.

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editing, F.S.-J., G.A., L.R.S. and J.D.F.-F.; supervision, J.D.F.-F.; project administration, G.A.; funding acquisition, G.A. All authors have read and agreed to the published version of the manuscript.

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