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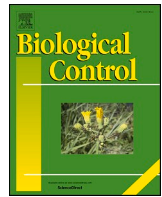

Probiotic *Bacillus subtilis* SB8 and edible coatings for sustainable fungal disease management in strawberry

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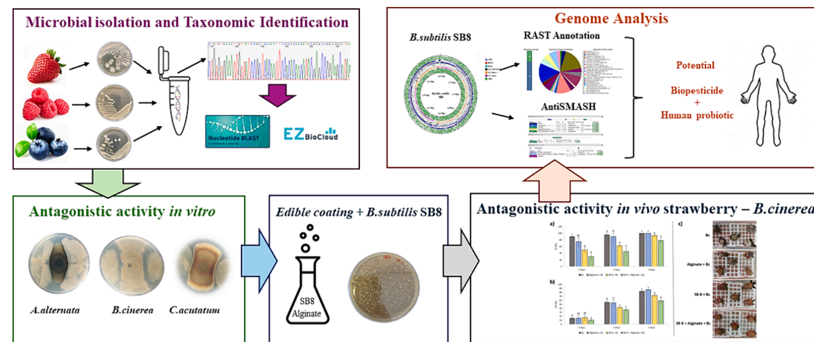
HIGHLIGHTS

- Berries: a potential source of probiotics and MBCAs belong to *Bacillus*.
- Strawberry isolates excel at antagonizing fungal phytopathogens.
- Edible coating-SB8 reduces *B. cinerea* infection in strawberries, enhancing safe.
- SB8 genome reveals dual potential as BCA and human probiotic.

GRAPHICAL ABSTRACT



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ABSTRACT

Agriculture faces the critical challenge of providing safe food while reducing post-harvest phytopathogens losses, exacerbated by climate change. Berries, prized for their taste and nutrition, confront economic hurdles due to fungal diseases, notably strawberries. Exploring ecological alternatives, like biopesticides with probiotic properties in synergic with edible coatings, has emerged as a novel strategy to address this challenge. Our aim was to isolate bacteria capable of serving dual roles: combating fungal diseases while also enhancing food safety for consumers potentially applicable in conjunction with edible coatings. Strawberries, blueberries, and raspberries were surface disinfected, obtaining 19 isolates on MRS medium. Among these, we predominantly isolated *Bacillus* species, along with lactic acid bacteria (LAB) and potential pathogens. Selected bacterial strains were tested against three major phytopathogens: *Botrytis cinerea*, *Alternaria alternata*, and *Colletotrichum acutatum*. *Bacillus subtilis* SB8, *B. tequilensis* SB4.3.1, and *B. cabrialesii* SB4.3 showed promising results. *B. subtilis* was particularly notable for its antagonistic effects and it's recognized as Generally Recognized As Safe (GRAS). An *in vivo* assay using the SB8 strain, combined with an alginate-based edible coating, demonstrated a reduction in *B. cinerea* infection. Sequencing the SB8 genome (approximately 4.0 Mb) revealed genes responsible for antimicrobial

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compound production and probiotic traits. Our study highlights the potential of these strategies to enhance the safety and sustainability of strawberry production, providing a novel approach to combat fungal diseases and ensure the safety of these fruits.

1. Introduction

Safe agricultural production faces a significant challenge in meeting the demands of a growing global population while ensuring the safety of food for human consumption (Garcia et al., 2020). Berries, such as strawberries, blueberries, raspberries, and blackberries, have garnered increasing interest worldwide due to their organoleptic characteristics and nutritional properties, serving as rich sources of vitamins, minerals, fiber, and antioxidant compounds (Kumar et al., 2018; Tong et al., 2023). Despite their benefits, berries are highly sensitive fruits due to their lack of protective coverings, making them exceptionally vulnerable to water loss, mechanical damage, and pathogen infestation (Romero et al., 2022). Indeed, fungal phytopathogens pose significant challenges during berry harvesting, impacting both the quality and quantity of the crop, and can result in economic losses of 25–50 % of total production post-harvest (Nunes, 2012).

One of the primaries phytopathogens affecting berries is *Botrytis cinerea*, but *Alternaria* spp. and *Colletotrichum* spp. are also significant fungal pathogens for berry crop (Bell et al., 2021). The ascomycete *B. cinerea*, responsible for gray mold, is considered the main fungal pathogen, causing the major post-harvest disease in strawberries (Kozhar and Peever, 2018). This widespread filamentous fungus affects a wide range of plants and is responsible for the loss of over 1400 known hosts (Poveda et al., 2020). It accounts for up to 70–80 % of plant diseases in major crops worldwide, causing devastating economic losses for agricultural producers (Hernández et al., 2022). *B. cinerea* can infect both fruits and vegetative strawberry tissues at any developmental stage, but it is particularly damaging during storage and transport (Petrasch et al., 2019). This pathogen significantly reduces the shelf life of fresh strawberries, which is only five days at 0–4 °C post-harvest (Chen et al., 2018), with an estimated 30 % loss during this period (Massoud et al., 2021).

Strawberries are cultivated worldwide due to their distinctive flavour and nutritional content, including bioactive compounds such as vitamins C and E, phenolic compounds, anthocyanins, and β-carotene (Saeed et al., 2021). Spain ranks sixth globally in strawberry production and has been the major strawberry exporter since 1988, exporting 23.8 % of the world's strawberries in 2019, primarily to European countries (Aoki and Akai, 2023).

Over the years, the control of fungal pathogens has relied heavily on pesticide treatments, which remain a popular management strategy. If plants are not treated with fungicides, more than 80 % of strawberry blooms and fruits may not survive (Petrasch et al., 2019). However, the use of chemical methods can lead to serious environmental pollution and increase pathogen resistance, posing threats to human health and food safety (Jawad et al., 2020). In response to these challenges, innovative approaches are being explored to enhance the sustainability and safety of berry production, including the development of microbial-based biological control agents (MBCAs) as environmentally friendly alternatives to chemical pesticides.

MBCAs can act through various mechanisms, including competition for nutrients and space, production of hydrolytic enzymes, biofilm formation on fruit surfaces, secretion of antibiotics, and induction of host defence mechanisms (Carmona-Hernandez et al., 2019). The use of probiotic human bacteria as biological control agents against post-harvest pathogens in berries is a promising approach (Lu et al., 2021). Probiotics are microorganisms that provide health benefits to their host when consumed in appropriate amounts and have been extensively used as natural bio-preservation agents to reduce pathogen proliferation in food (Chen et al., 2020). Notably, lactic acid bacteria (LAB), which have Generally Recognized As Safe (GRAS) status, are particularly effective in this regard (Nasrollahzadeh et al., 2022).

Considering the increasing consumer demand for healthy and safe diets, probiotics offer a sustainable alternative for controlling post-harvest pathogens in berries (Massoud et al., 2021). Research and development on probiotic films and edible coatings as active packaging to reduce post-harvest diseases continue to grow (Sáez-Orviz et al., 2023). Edible coatings have emerged as an effective to prolong shelf life and maintain fruit quality (Massoud et al., 2021). These coatings can help maintain the product's quality by reducing water loss, maintaining firmness, and lowering microbial load on the fruit surfaces (Romero et al., 2022).

Therefore, the objective of this study was to isolate and identify MBCAs with probiotic and biopesticide potential from the microbiota of various berries. Additionally, we aimed to investigate their compatibility with edible coatings to reduce fungal diseases caused by *B. cinerea* in strawberries. Furthermore, we conducted genetic analyses of the most promising strain to elucidate their potential in enhancing the shelf life of strawberries and their probiotic potential.

2. Materials and methods

2.1. 1 Sample and microbial isolation

Strawberry (*Fragaria x ananassa*) variety Portola, raspberry (*Rubus idaeus*) variety Versalles, and blueberry (*Vaccinium corymbosum*) variety Legacy, were kindly supplied by the nursery "Viveros Campiñas" in Segovia (Spain). First, eight strawberries and ten raspberries and blueberries each were disinfected using ethanol 70 % solution (w/v) for 1 min, followed by five washes with sterilized water. This disinfection procedure was performed individually for each type of berry to ensure cleanliness. Subsequently, the berries were crushed in sterilized mortar under aseptic conditions and plated 100μl onto on Man, Rogosa, and Sharpe agar (MRS) purchased from Condalab®. All plates were then incubated at 37 °C for 72 h. Colonies were selected based on cell morphology and the characteristic white coloration of LAB until pure cultures were obtained.

2.2. DNA extraction and taxonomic identification of isolates

To identify the isolates, firstly DNA was extracted from pure colonies individually using the Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich®) following the manufacturer's instructions. The DNA obtained was stored at −20 °C. For bacterial taxonomic identification, the sequence of the 16S rRNA was performed using the primers 27F (5′AGAGTTTGA TCCTGGCTCAG) and 1522R (5′AAGGAGGTGATCCANCCRCA) for the PCR reaction under the following conditions: preheating at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 1 min 30 s. The final extension in the last cycle was performed at 72 °C for 10 min. In the case of fungi isolates, amplification of the ITS region was performed using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTG ATATGC-3′) for the PCR reaction under the following conditions: preheating at 95 °C for 9 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. The final extension in the last cycle was performed at 72 °C for 10 min. Bacterial amplicons (approximately 1500 bp) and fungal amplicons (approximately 500 bp) were checked on a 1 % (w/v) agarose gel (NYZTECH®) using the GeneRuler 1 kilobase and GeneRuler 100 bp DNA Ladder (Thermo Scientific TM), respectively, as size markers. For purification of

PCR products, the kit NZYGelpure was used according to the manufacturer's instructions (NYZTECH ®). Then, PCR products were sequenced using the Macrogen sequencing service (Macrogen Inc., Seoul, South Korea). The sequences obtained were assembled using BioEdit software 9 (Hall et al., 2011).The bacterial isolates were identified using EZBioCloud (https://www.ezbiocloud.net/identify), and the fungal isolates were identified using NCBI's Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE = BlastSearch).

2.3. In vitro antagonistic activity

Based on the results, only bacterial strains identified as both safe and possessing biotechnological potential were selected. The phytopathogenic fungi used were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain): *Botrytis cinerea* (CECT 20973), *Alternaria alternata* (CECT 2997) and *Colletotrichum acutatum* (CECT 21009).

As a preliminary screening, all bacterial isolates were individually tested against the selected phytopathogens to elucidate their potential antagonistic activity. Fungal disks (approximately 6 mm) were excised from fully grown 7-day- old cultures and placed at the center of the PDA medium. Bacterial inoculum was adjusted to a OD 600nm = 0.5, and 5 µl was inoculated on both sides of the fungal disks. The plates were incubated at 25 °C for five days. Plates were observed routinely. The experiment was repeated twice with a total of four plates for each bacterial and fungal species.

Based on the results, three different strains were selected due to their superior performance in the preliminary antagonistic assay to quantify the antagonist activity based on the methodology described by Poveda et al. (2022), with some modifications. A 6 mm diameter mycelium disk of each pathogenic fungus, obtained from the periphery of the colonies grown on PDA medium, were placed at the centre of a PDA plate. On two equally spaced sides, 30 mm away from each pathogenic fungus, 5 µl suspensions of bacteria with an OD 600nm = 0.5 were inoculated. Controls were performed with and without the application of 5 µl of H_2O solution, and no difference was found between them. Starting on day 3 of a 12d incubation period at room temperature, the antagonistic effect on each fungal pathogen growth was recorded. For isolate, the entire treatment was replicated on eight plates, and the entire experiment was repeated twice.

Fungal areas were measured using ImageJ photographic analysis software, at three, five- and seven-days post-inoculation. To determine which strains are the most effective antagonists, the inhibition rate (IR) was calculated according to the following formula: IR = (AC − AT) /AC x 100, where AC represent the colony area of the pathogenic fungus in the control treatment, and AT means the colony area of the pathogenic fungus in bacterial treatments.

2.4. Behaviour of the selected strain in edible coating

Among the isolates, the bacterial strain SB8 was selected due to its potential as a GRAS bacterium. Subsequently, its compatibility with edible coating was evaluated. The coating was prepared as follows: 2 %(w/v) of sodium alginate (Manuel Riesgo, Madrid) and 0.25 % (v/v) of glycerol (Fisher Scientific) were mixed with pure water in a flask. Subsequently, a burette was attached to each flask, and they were covered with cotton and gauze, and with aluminium foil before being placed on a shaker for 10mins at 300 rpm. Once the emulsion was homogenized, it was sterilised in an autoclave. The emulsion was left to cool at room temperature before strains were inoculated. For each flask, isolates exhibiting the best antagonist activity were inoculated at OD600nm = 0,1.

To evaluate the behaviour of the selected strain on the edible coating over time, serial dilutions were carried out. Then, 100µl was inoculated into MRS Petri plates and incubated at 37°C for 48h. The dilution process was repeated every 24h, for a total of four days, and the CFU (Colony Forming Units) were counted. The experiment was performed in triplicated.

2.5. Control of B. cinerea in strawberries with edible coating and bacterial strain

Based on the observed results, an *in vivo* assay was conducted to study the reduction of fungal diseases. The study comprised five distinct treatments: 1.untreated strawberries, 2.strawberries with an alginate edible coating as a control, 3. strawberries inoculated with a plug of *B. cinerea*; 4. strawberries with SB8 (*B. subtilis*) in a water solution with a plug of *B. cinerea*, and 5° strawberries with SB8 (*B. subtilis*), used in synergy with the alginate edible coating solution and a plug of *B. cinerea*. Each treatment was performed with 10 healthy strawberries and by triplicate, superficially sterilizing strawberries with 70% ethanol for 30s (Chen et al., 2020). As Chen et al. (2020), it was followed by a 2min treatment with food-grade sodium hydroxide. Finally, the strawberries were rinsed with sterile purified water three times. Similarly, alginate coating samples were prepared as described in the preceding section. The strawberries for treatments 4 and 5 were introduced into both the water and coating isolate solutions, each with a final OD 600nm = 0.1. After each treatment, the strawberries were dried and placed in groups of five in sterilized food containers with damp paper to maintain high relative humidity. The containers were stored in a dark, closed place at room temperature for five days, where daily records and observations were made.

The measurement of the percentage of infected strawberries was conducted as disease incidence (DI), as defined by Fernandez-San Millan et al. (2021). DI was calculated as follows: DI % = (number of infected strawberries /total number of strawberries) × 100.

In addition, disease severity (DS) was evaluated using an empirical 0 to 4 scale: 0 = no symptoms; 1 = 1–25 % of the surface with fungal damage; 2 = 26–50 %; 3 = 51–75 %; 4 = 76–100 %.The DS index was determined as follows: DS % = [$\sum$ (number of infected fruits in each scale × disease scale)/(total fruits examined × highest disease scale)] × 100.

2.6. Draft genome sequence, annotation, and genotypic analysis of the strain SB8

Strain SB8 was cultivated on MRS plates and harvested after 48 h at 28°C. Genomic DNA extraction was conducted using the ZR Fungal/Bacterial DNA MiniPrep kit according to the manufacturer's instructions (Zymo Research). Subsequently, the draft genome sequence was generated through shotgun sequencing on an Illumina MiSeq platform via a paired-end run (2 × 251 bp). Assembly of sequence data was performed using Velvet 1.2.10. Gene calling and annotation were conducted utilizing RAST 2.0 (Rapid Annotation using Subsystem Technology) along with The SEED viewer framework. Additionally, the Carbohydrate Active Enzyme (CAZy) database was employed to identify protein families within the SB8 strain and secondary metabolites were analysed using antSMASH 7.0.

2.7. Data/statistical analysis

Statistix 8.0 software was used for statistical analysis of *in vitro* antagonism inhibition ratios and *in vivo* DI and DS. For pairwise comparisons, one-way ANOVA with Tukey's multiple range test at $P < 0.05$ was used; different letters indicate significant differences.

SB8 behaviour in edible coating was analysed by one-way ANOVA. Fisher LSD (Least Significant Differences) test was applied for determining group differences at 95% significant level. Statgraphics Centurion XVL was used for carrying out the statistical analysis.

3. Results

3.1. Isolation and identification of bacterial strains in berries

A total of eight strawberries, along with ten blueberries and ten raspberries, were collected under aseptic conditions. After three days of inoculation on MRS agar plates, a total of nineteen microorganisms were

Table 1
Analysis and identification of isolates from strawberries (SB), blueberries (BB) and raspberries (RB) based on 16S and ITS rRNA gene sequences. Table includes the closest match using EZBioCloud for 16S and NCBI databases for ITS.

Source of isolation	Phylogenetic group	Strain	Top match	Nucleotide identity (%)
SB	Ascomycota	SB 1.A	*Pichia terricola AUMC 10796* (T)	100
	Ascomycota	SB 2	*Metschnikowia chrysoperlae CBS:9803* (T)	100
	Ascomycota	SB 3	*Pichia terricola AUMC 10796* (T)	99,74
	Bacillota	SB 4.1.B	*Bacillus tequilensis KCTC 13622* (T)	99,69
	Bacillota	SB 4.3	*Bacillus cabrialesii TE3* (T)	100
	Ascomycota	SB 6.1.B	*Ustilaginoidea virens* (T)	100
	Bacillota	SB 6.2	*Staphylococcus epidermis NCTC 11047*	100
	Ascomycota	SB 7	*Geotrichum* sp. *strain DTO 400-C2*	100
	Bacillota	SB 8	*Bacillus subtilis* DSM *23778* (T)	99,86
	Basidiomycota	SB 8A	*Apiotrichum loubieri R50* (T)	100
	Bacillota	SB 9.1	*Leuconostoc mesenteroides* subsp. *mesenteroides ATCC 8293* (T)	99,86
	Bacillota	SB 9.2	*Leuconostoc suionicum DSM 20241* (T)	100
	Ascomycota	SB 11	*Geotrichum candidum BAFC cult 4510* (T)	99,71
BB	Bacillota	BB 3	*Bacillus tequilensis KCTC 13622* (T)	99,93
RB	Ascomycota	RB 1	*Geotrichum* sp. *strain DTO 400-C2*	99,66
	Bacillota	RB 2	*Staphylococcus hominis* subsp. *hominis DSM 20328* (T)	100
	Ascomycota	RB 3	*Geotrichum* sp. *strain DTO 400-C2*	99,39
	Bacillota	RB 8	*Bacillus tequilensis KCTC 13622* (T)	99,86

isolated, of which ten were bacterial strains and nine were eukaryotes (one filamentous fungi and eight yeast). All the microbial isolated were identified through sequencing the 16SrRNA or ITS regions. Each strain exhibited an overall sequence similarity higher than 99 % when compared to the databases. Consequently, all the strains were correctly identified and classified (Table 1). A total of 16 microbial isolates were obtained. All bacterial isolates belong to the phylum Bacillota. Among them, there are isolates from the genera *Bacillus* (5), *Staphylococcus* (2) and *Leuconostoc* (2). In relation to fungi, isolates belonging to Ascomycota was predominant (88.8%), followed by Basidiomycota (11.1%). Among the eight isolates of the phylum Ascomycota, there were representatives from the genera *Geotrichum* (4), *Pichia* (2), *Metschnikowia* (1) and *Ustilaginoidea* (1). Additionally, one isolate from the phylum Basidiomycota belongs to the genus *Apiotrichum.*

3.2. Preliminary screening for antagonist activity by bacterial isolates

This study focuses on the isolation of bacterial strains catalogued as potential probiotic as well as potential as biocontrol agents. Thus, among the isolates, only bacterial strains were selected. As a preliminary result, antagonist screening activity against the three important phytopathogens in berries, *B. cinerea*, *A. alternata*, and *C. acutatum*, by bacterial isolates was studied in PDA solid media. The antagonist screening abilities were expressed in qualitative terms and classified as positive (+) or negative (−), according to the behaviour observed (Table 2). The

Table 2
Screening antagonism by the bacterial isolates against phytopathogenic fungi employing agar plate assay. (+antagonism present, − antagonisms absent).

Bacterial strain isolates	Fungal phytopathogens		
	Botrytis cinerea	*Alternaria alternata*	*Colletotrichum acutatum*
SB 4.1B	+	+	+
SB 4.3	+	+	+
SB 8	+	+	+
SB 9.1	−	−	−
SB 9.2	−	−	−
RB 8	+	+	+
BB 3	−	+	+

bacterial strains exhibited varying levels of antagonist activity. Five strains inhibited the growth of the three phytopathogens. Another two strains did not exhibit any antagonist activity against any phytopathogen. The remaining isolate showed antagonistic activity against at least two of the three phytopathogens.

3.3. In vitro antagonism activity

The *in vitro* antagonism test between the different *Bacillus* species and the pathogens *A. alternata*, *B. cinerea* and *C. acutatum* reported positive IRs in all confrontations (Figs. 1 and 2). Against *A. alternata,* all the three *Bacillus* strains produced significant and important IRs of 40–63 % at three days of interaction, 66–84 % at five days and 75–90 % at seven days. No significant differences were reported between the IRs caused by the three bacterial strains used (Fig. 2a).

In the case of the *B. cinerea* pathogen, the IRs produced by the bacterial strains were significant, but less than 50 %. The reported IRs were 15–32 % at three days of interaction, 19–31 % at five days of interaction and 15–31 % at 7 days of interaction. At three days of interaction, there were no significant differences between the IR caused by each strain against *B. cinerea:* SB4.1.B (*B. tequilensis*) (IR: 19 %), SB4.3 (*B. cabrialesii*) (IR: 15 %) and SB8 (*B. subtilis*) (IR: 22 %). The IRs reported at 5 and 7 days of interaction reported significant differences between the SB 4.1.B strain and the other two strains used. Specifically, SB4.3 (*B. cabrialesii*) and SB8 (*B. subtilis*) inhibited the growth of *B. cinerea* by 29 and 31 %, respectively, while SB4.1.B (*B. tequilensis*) inhibited it by 15–19 % (Fig. 2b).

Finally, the three bacterial strains used also inhibited the growth of the pathogen *C. acutatum* with values higher than 50 % after seven days of bacteria-fungi interaction. At three days of interaction, no bacterial strain significantly reduced growth of *C. acutatum*. Subsequently, at five days of interaction, the three bacterial strains used significantly inhibited the growth of the pathogen (IR:41–46 %). The same trend was reported at seven days of interaction, with IRs between 59–65 % (Fig. 2c).

3.4. SB8 behaviour in edible alginate coating

SB8 strains exhibited the best results among the isolates tested. Consequently, its interaction with an edible alginate coating was evaluated. SB8 was inoculated into the alginate solution at an initial concentration of 26 x 10^6 UFC/ mL. A significant reduction in bacterial count was observed at 24- and 48-hours post-inoculation, with the count decreasing to 44 x 10^5 UFC/mL. By 72 h, the bacterial count showed a non-significant increase to 72 x 10^5 UFC/mL. Remarkably, at 4 days post-inoculation, a significant increase was detected, with the bacterial count returning to levels like those observed on the day of inoculation, indicating good compatibility between the bacteria and the alginate coating, and promoting bacterial survival (Table 3).

3.5. B. cinerea control in strawberries by coating and bacteria

To determine the biocontrol capacity of the bacterium *B. subtilis* (SB8) applied with alginate coating, an *in vivo* infection study of the

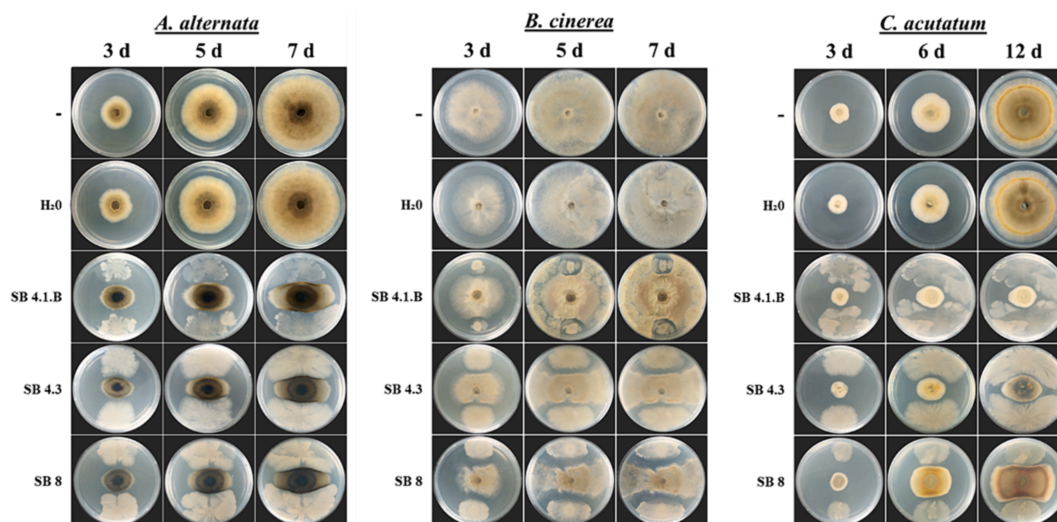

Fig. 1. Photographs of the antagonism *in vitro* study between the pathogens *A. alternata* (3, 5 and 7 days), *B. cinerea* (3, 5 and 7 days) and *C. acutatum* (3, 6 and 12 days) and *Bacillus* endophytic bacteria: SB 4.1.B: *B. tequilensis*; SB 4.3: *B. cabrialesii*; and SB8: *B. tequilensi.* Moreover, H_2O control was measured and recorded.

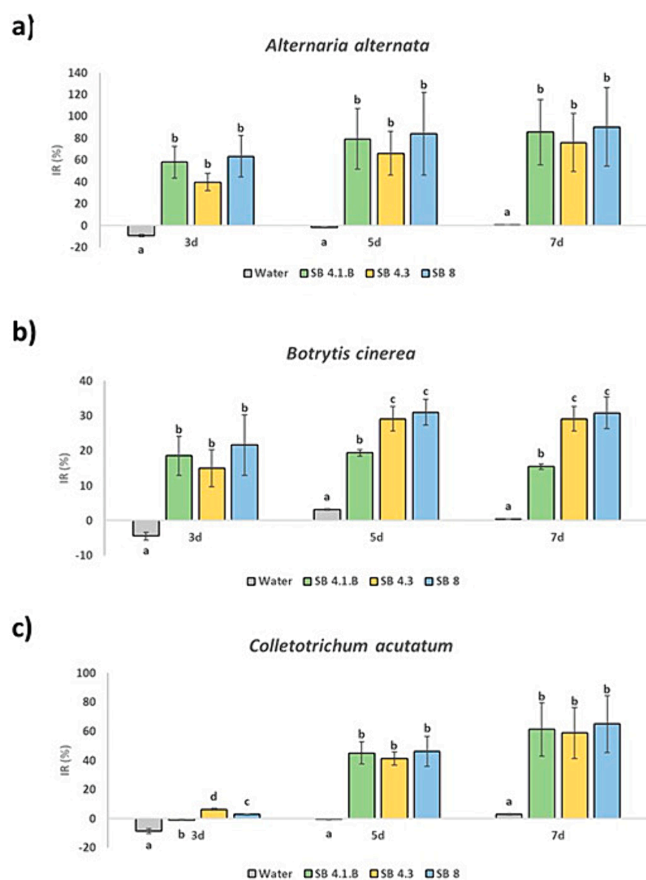

Fig. 2. Inhibition rate (IR, %) in *A. alternata* (a), *B. cinerea* (b) and *C. acutatum* (c) in their antagonistic confrontation *in vitro* against *B. tequilensis* (SB 4.1.B), *B. cabrialesii* (SB 4.3) and *B. tequilensis* (SB 8). Data are the mean of two biological replicates for each condition with eight plates in each one. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

necrotrophic pathogenic fungus *B. cinerea* on strawberry fruits was carried out. Both visually (Fig. 3c) and by analysing the incidence and severity of the disease (Fig. 3a, b), it was reported how treatments with SB8 (*B. subtilis*) reduced the disease caused by *B. cinerea* in strawberries.

The application of the alginate coating on the fruits did not significantly reduce disease incidence and severity compared to the control (Fig. 3a, b). Treatment with *B. subtilis* bacteria (SB8) significantly reduced disease incidence and severity 4 and seven days after infection with *B. cinerea,* compared to the control (Fig. 3, b). The combined application of alginate and SB8 bacteria significantly reduced disease incidence and severity compared to the application of bacteria alone, seven days after pathogen infection (Fig. 3a, b).

3.6. Genome analysis of selected genes of B. subtilis SB8

The general genome features of *B. subtilis* SB8 are presented in Table 4. The genome consists of 15 contigs with a total combined size of 4,041,219 base pairs (bp) and an average CG content of 43.7 %. Additionally, 4,193 coding sequences (CDSs) and 86 tRNAs were predicted. This Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBankunder the accession JBEFAJ000000000. The version described in this work is version JBEFAJ010000000.

According to RAST annotation, the distribution of functional subsystems is illustrated in Fig. 4. Approximately 29 % of the genome information was assigned to various subsystem categories, while the remaining genes did not correspond to any known subsystem. The most abundant genes were associated with amino acids and derivatives (294), followed by those involved in carbohydrates (253), protein metabolism (194), cofactors, vitamins, prosthetic groups, pigments (141), dormancy and sporulation (98), nucleosides and nucleotides (96), cell wall and capsule (77), DNA metabolism (60), RNA metabolism (57), fatty acids, lipids, and isoprenoids (48), stress response, motility, and chemotaxis (45), membrane transport (43), respiration, virulence, disease, and defense (37), iron acquisition and metabolism (31), regulation and cell signaling (30), miscellaneous (25), nitrogen metabolism (19), metabolism of aromatic compounds (12), phosphorus metabolism (11), secondary metabolism (10), sulfur metabolism (8), phages (6), cell division and cell cycle (4), and potassium metabolism (3).

Enzymes involved in the synthesis, degradation, and modification of

Table 3
The behaviour of SB8 over a 4-day period in the alginate edible coating.

Time	*0 day*	*1 day*	*2 day*	*3 day*	*4 days*
UFC/ mL	$26x10^6 \pm 2{,}25^a$	$15x10^6 \pm 1{,}02^b$	$44x10^5 \pm 3{,}18^{cd}$	$72x10^5 \pm 3{,}3^{cd}$	$29x10^6 \pm 1{,}39^a$

Different letters are significantly different ($p < 0.05$).

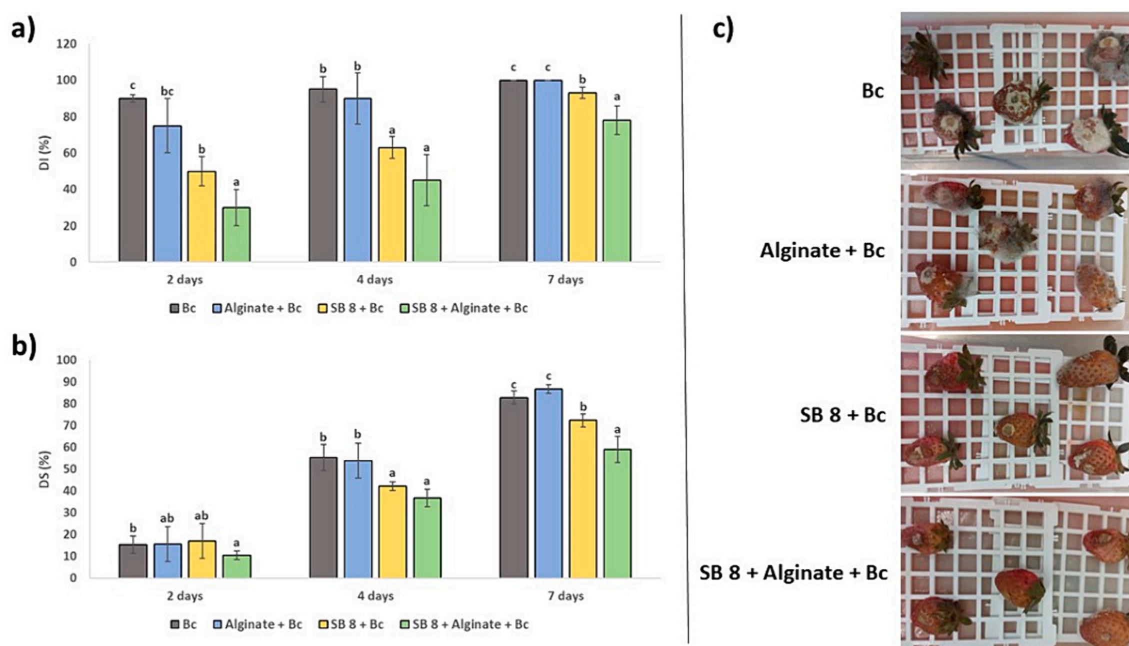

Fig. 3. Disease incidence (DI, %) (a) and disease severity (DS, %) (b) caused by *B. cinerea* on strawberries, 2, 4 and 7 days after infection. Fruits were treated with alginate or *B. tequilensis* bacteria (SB 8), alone and in combination, and infected with *B. cinerea* (Bc). Data are the mean of three biological replicates for each condition with ten fruits in each one. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$). Representative photographs of *B. cinerea* disease in strawberries, 7 days after infection (c).

Table 4
Main features of the draft genome sequences of *B. subtilis* SB8.

Features	*B. subtilis SB8*
Genome size (bp)	4,041,219
L50	2
GC content	43,7
Number of contigs	15
Predicted coding sequences.	4193
Subsystem	329
Number of RNAs	86

carbohydrates, known as CAZymes (Carbohydrate Active enZymes), were analyzed in *B. subtilis* SB8. The genome harbored 130 genes identified as putative CAZymes (Fig. 5), including 7 Polysaccharide lyases (PLs), 44 Glycosyl hydrolases (GHs), 39 Glycosyl transferases (GTs), 15 Carbohydrate esterases (CEs), 2 Auxiliary activities (AAs), and 26 Carbohydrate-binding modules (CBMs), ten of which were classified as both GHs and CBMs (Fig. 5). Among the putative CAZymes, the RAST annotation predicted genes associated with cellulase, xylanase, and amylase enzymes.

The antiSMASH database revealed eight predicted clusters involved in the biosynthesis of secondary metabolites (Table 5). Notably, genes for bacilysin, subtilosin A, pulcherriminic acid, subtilin, and bacillibactin were identified with 100 % similarity. Additionally, one gene cluster showed 82 % similarity to that for surfactin, and another

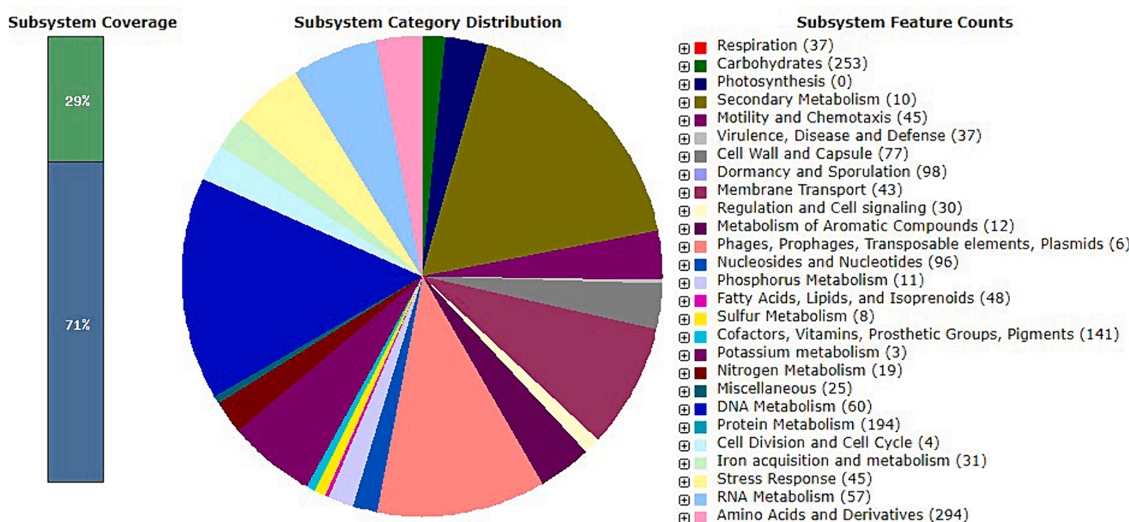

Fig. 4. An overview of RAST subsystem analyses from the *B. subtilis* SB8. The pie chart organizes the presented subsystems by cellular process, and the number of protein-coding genes (in parentheses) that are predicted to be involved in that cellular process are indicated.

displayed 86 % similarity to that for fengycin. Among these clusters, various antimicrobial peptides and antifungal compounds were identified. Additionally, different siderophores, including pulcherrimic acid and bacillibactin, were predicted. Furthermore, five biosynthetic gene clusters were identified with no similarity in the antiSMASH database, indicating potential novel pathways for secondary metabolites.

Furthermore, a variety of predicted genes encoding proteins associated with resistance to diverse environmental stresses and exhibiting probiotic potential were identified (Table 6). Specifically, genes related to cold stress resistance proteins from the CSP family were identified. Additionally, several genes associated with heat resistance were predicted, including the heat shock protein 10 kDa family chaperone GroES/GroEL, one heat shock protein HslU, one ATP-dependent protease subunit HslV, CtsR (a transcriptional regulator of stress and heat shock responses), one gene encoding the heat shock protein ClpP, the chaperone DnaK, and the heat shock protein DnaJ. Moreover, we identified one gene coding for a bile acid sodium symporter and another gene coding for choloylglycine hydrolase. Additionally, genes were annotated for adhesion proteins, including collagen adhesion protein, fibronectin/fibrinogen-binding protein, Sortase A, and the Autoinducer-2 production protein LuxS.

4. Discussion

In this study, we utilized the standard MRS medium, which is widely employed for the cultivation of LAB (Zhang et al., 2014; Hayek et al., 2019). Our objective was to isolate potential bacterial strains that exhibit dual capabilities as human probiotics and biocontrol agents (BCAs) when used in conjunction with an edible coating. The integration of such strains into agricultural practices not only leverages their probiotic benefits but also enhances their biocontrol efficacy against phytopathogens, thereby contributing to sustainable agricultural practices. This study focuses on identifying and characterizing bacterial strains that can simultaneously provide health benefits and act as effective biocontrol agents Traditionally, research has predominantly concentrated on the health benefits of these microorganisms for human use.

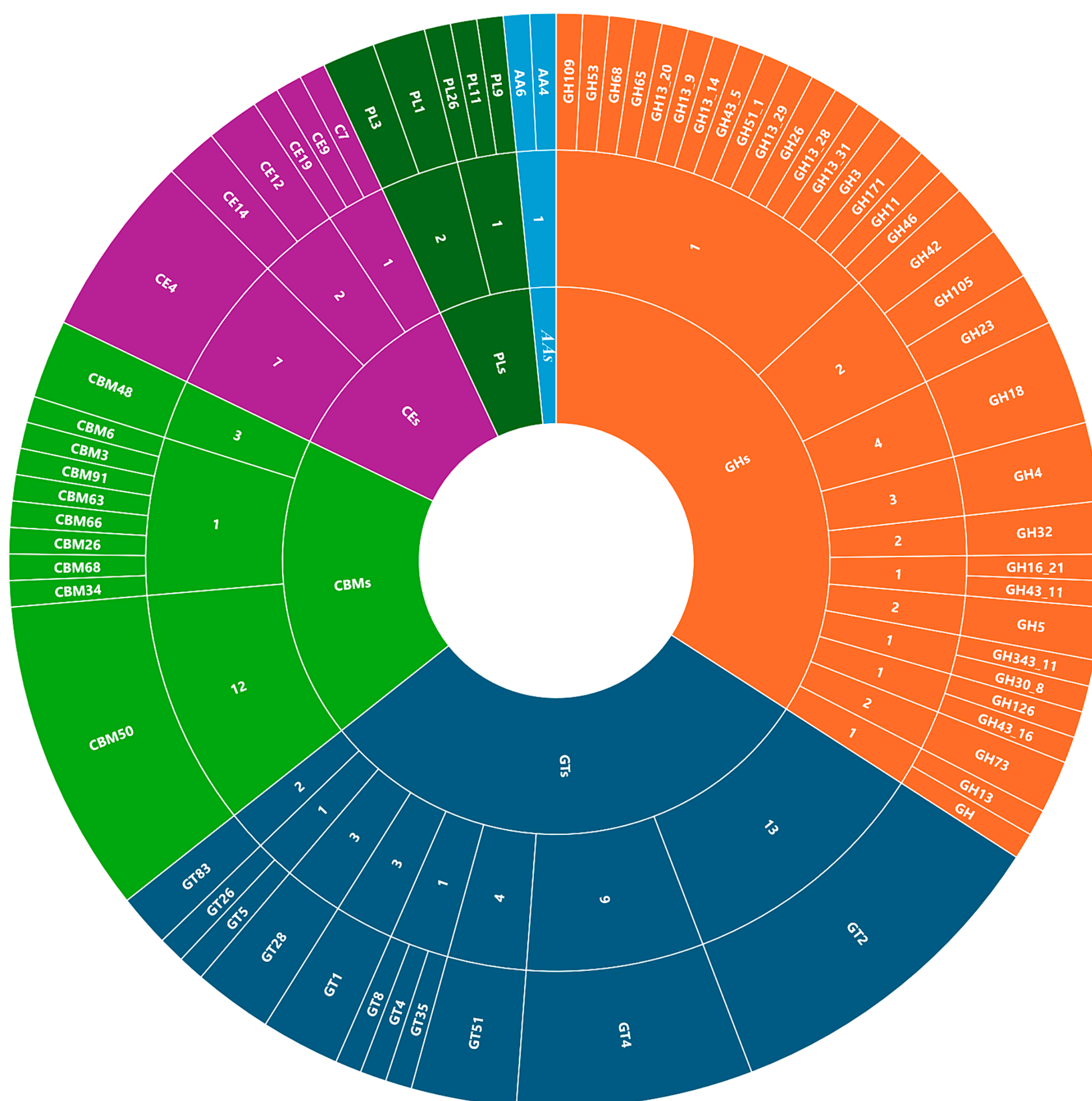

Fig. 5. Classification of predicted carbohydrate-active enzymes (CAZy) from *Bacillus subtilis* SB8.

Table 5
List of the putative genes cluster enconding for secondary metabolites by antiSMAH by *B. subtilis* SB8.

Clusters	Types	Genomic locations	Most similar known clusters	Similarity
Cluster 1	Other	314, 426–355,844	Bacilysin	100 %
Cluster 2	Sactipeptide	363,166–384,777	Subtilosin A	100 %
Cluster 3	CDPS	614,231–634,977	Pulcherriminic acid	100 %
Cluster 4	Lanthipeptide-class-i	752,657–778,882	Subtilin	100 %
Cluster 5	NRP-Metallophore (NRPS)	925,147–976,924	Bacillibactin	100 %
Cluster 6	NRPS	171,612–237,003	Surfactin	82 %
Cluster 7	NRPS, Betalactone	1–28,944	Fengycin	86 %
Cluster 8	TransAT-PKS, NRPS, T3PKS, PKS-like	91,174–205,924	Bacillaene	100 %

However, recent studies have shown an increased interest in their application within agricultural systems (Lahmamsi et al., 2024; Romero et al., 2022). By isolating strains with proven dual capabilities, we aim to enhance their practical applications and efficacy in both fields, reflecting a growing trend towards multifunctional microbial applications.

Despite the specificity of MRS medium for LAB isolation, previous studies such as those by Ragul et al. (2017), have reported the isolation of various *Bacillus* species using this medium, which is consistent with our findings.

Notably, to our knowledge, our study is the first to report the isolation of microbial genera *Pichia*, *Metschnikowia*, *Ustilaginoidea*, *Staphylococcus*, and *Apiotrichum* using MRS medium. This suggests that the nutrient composition of MRS medium may support the growth of a broader variety of microorganisms than previously acknowledged. These findings open a new possibility for the use of MRS medium in isolating diverse microbial taxa.

The identification of microbial from manually harvested berries provides valuable information into the microbiological cultivate composition of fresh produce and its implications for food safety. Notably, the presence of human-associated bacterial as *S. epidermis* SB6.2 and *S. hominis* subsp. *hominis* RB2, species commonly found in the microflora skin (Severn et al., 2022), suggest potential contamination during harvesting, highlighting the critical need for stringent hygiene practices throughout the production chain to mitigate microbial risks.

In terms of fungal isolates, such as of *Geotrichum candidum* and *Ustilago virens,* from strawberry fruit raises significant concerns regarding spoilage and phytopathogenic contamination, underscoring the critical need for enhance post-harvest handling practices to maintain product quality and safety. *G. candidum* has been reported as the agent responsible of sour rot of strawberry (Alonzo et al., 2021), while *U. virens* is recognized as major phytopathogen responsible for false smut disease in rice, which poses a significant threat to rice crops worldwide (Sun et al., 2020).

Additionally, the isolation of novel yeast species such as *Metschnikowia chrysoperlae* and *Pichia terricola* expands our understanding of the diverse microbial communities associated with fruit ecosystems. These yeast species, described as significant non-*Saccharomyces* species in the microbiota of the phyllosphere of *Vitis vinifera,* are employed as alternatives in wine production to enhance aroma and flavour profiles (Vicente et al., 2020; Gao et al., 2022). Importantly, to the best of our knowledge, this study represents the first report of their isolation from strawberry fruit, indicating a novel ecological niche for these yeast species within fruit ecosystems.

Despite the use of MRS medium, only two LAB were isolated from strawberry fruits, with no isolated obtained from the other berries used

Table 6
Stress and probiotic genes in draft genome predicted by RAST annotation in *B. subtilis* SB8.

Gene ID	*Gene name*	*Function*
Cold stress resistance		
6666666.1115896.peg.2099	*cspB*	Cold shock protein, CSP family
6666666.1115896.peg.3342	*cspC*	Cold shock protein, CSP family
Heat stress resistance		
6666666.1115896.peg.3441	*groES*	Heat shock protein 10 kDa family chaperone
6666666.1115896.peg.3442	*groEL*	Heat shock protein 10 kDa family chaperone
6666666.1115896.peg.1344	*hslU*	Heat shock protein HslU
6666666.1115896.peg.1345	*hslV*	ATP-dependent protease subunit HslV (EC 3.4.25.2)
6666666.1115896.peg.4063	*ctsR*	Transcriptional regulator of stress and heat shock response
6666666.1115896.peg.2410	*dnaK*	Chaperone DnaK
6666666.1115896.peg.2412	*dnaJ*	Heat shock protein DnaJ
6666666.1115896.peg.699	*clpP*	Heat shock protein
Bile salt tolerance		
6666666.1115896.peg.157	¬¬¬	Choloyglicine hydrolase (EC.3.5.1.24)
6666666.1115896.peg.2966	¬¬¬	Bile acid sodium symporter
Lytic enzymes *6666666.1115896.peg.1124* *6666666.1115896.peg.1188* *6666666.1115896.peg.2901*	*eglS*	β-1,4-glucanase (cellulase) (EC 3.2.1.4) Xylanase Endo-1,4- β −xylanase (EC 3.2.1.8)
Cell adhesion		
6666666.1115896.peg.1395	¬¬¬	Fibronectin/fibrinogen-binding protein
6666666.1115896.peg.504	*spaA*	Collagen adhesion protein-
6666666.1115896.peg.505	*srtA*	Interaction with cells host
6666666.1115896.peg.3954	*Autoinducer-2 production protein LuxS*	Quorum sensing signal −colonization process

in this study. These strains were identified as SB 9.1 and SB 9.2 corresponding to *L. mesenteroides* subsp. *mesenteroides* and *L. suionicum*, respectively. LABs are recognized for their GRAS status and plays an important role in the dairy food environment.

L. mesenteroides has previously been isolated from apples and grapes, respectively, suggesting potential probiotic applications for humans' health (Romero et al., 2022). Notably, while other LAB species such as *Lactobacillus planatarum* have been isolated from strawberry fruit (Chen et al., 2020), our study represents the first report of *L. suionicum and L. mesenteroides* subsp. *mesenteroides* isolates from strawberry fruit. These findings underscore the diversity of LAB species present in strawberry fruit and highlight the potential for probiotic applications.

Within the genus *Leuconostoc,* the specie *L. mesenteroides* QZ1178 has been reported to exhibit antimicrobial activity against the pathogen *Gallibacterium anatis*, which infects birds and causes significant economic losses (Zhang et al., 2021). However, there is limited literature on their potential as biopesticides against fungal phytopathogens, like *L. suionicum.* These findings align with our results, as these two bacterial

strains did not exhibit antagonistic activity against the fungal phytophatogens tested.

In our study, *Bacillus* emerged as the predominant genus. Within the genus, encompasses both pathogenic species and MBCAs, with the latter holding promise for biological disease management. Several studies have recognized *Bacillus* strains‘ efficacy in combating postharvest phytopathogens, leading to their classification as MBCAs (Romero et al., 2022; Ying et al., 2022). MBCAs offer a safe and sustainable approach to disease control, drawing considerable attention for their potential role in agriculture practices. The appeal of *Bacillus*-based biotechnological applications lies in their prolific synthesis of bioactive compounds, facile cultivation in standard media, and straightforward maintenance and preservation protocols, rendering them promising candidates for commercialization (Prasad et al., 2023).

Among our isolates, we identified the strain SB4.3 as *B. cabrialessi,* the bacterial strain SB8 as *B. subtilis* and the strains SB4.1B, BB3 and RB8 as *B. tequilensis.* These bacterial species are ubiquitous in soils and often establish symbiotic relationship with agronomic plants (Veras et al., 2023). Nevertheless, this study represents the initial documentation of their isolation from post-harvest berries such as strawberry, raspberry and blueberry fruit, expanding our understanding of their ecological niche and potential applications.

The primary screening assay *in vitro* showed that all *Bacillus* isolates tested in this study exhibited antagonistic activity at least two of the three phytopathogen assessed. Particularly noteworthy were five strains − SB4.1B, SB4.3, and SB8 − which displayed antagonistic effects against all three phytopathogens, with the majority isolating from strawberry sources. It is noteworthy that each of the strains isolated from strawberries, exhibiting positive antagonistic activity against the three fungi, belonged to different species, none of which have previously been reported as isolates from strawberry fruit. Consequently, the efficacy of these three bacterial strains in inducing Induced Resistance (IR) was further investigated.

Our study found that *Bacillus* strains demonstrated high inhibition rates (IRs) of 40–63 % just three days post-inoculation. The fungal growth was reduced by 66–84 % at five days, increasing to 75–95 % by day seven. These results align well with previous research. For instance, Gorai et al. (2021) found that *Bacillus velenzensis* SEB1disrupts the pathogenic mycelial biomembranes of *A. alternata*, showing a notable inhibitory effect in potato. Similarly, Xie et al. (2021) reported that *Bacillus siamensis* LZ88 effectively controls *A. alternata* in tobacco. Additionally, the antagonistic activity of *B. cabrialesii* TE3T has been previously investigated by Villa-Rodriguez et al. (2021) in wheat, targeting the phytopathogenic fungus *Bipolaris sorokiniana*. Nevertheless, to date, the literature lacks documentation of the specific interaction between *B. cabrialesii* and *A. alternata*, highlighting the original contribution of our study in addressing this research gap. Our findings not only corroborate the biocontrol efficacy of *Bacillus* species against *A. alternata*, as observed in previous studies, but also unveil a novel aspect of *B. cabrialesii*'s biocontrol potential.

Regarding *C. acutatum*, while initial IRs were insignificant at three days, a notable growth inhibition of 59–65 % was observed by day seven. Although this inhibition was lower compared to strains targeting *A. alternata*, it remained statistically significant. Notably, all three isolates exhibited a similar degree of inhibition *in vitro*. Previous research on MBCAs against *C. acutatum* has primarily focused on various fruits such as apple, loquat, nectarine, peach, tamarillo, and strawberry. The most effective strains identified in these studies include *B. subtilis, P. polymyxa*, and *B. amyloliquefaciens* (Shi et al., 2021).

Furthermore, while Veras et al. (2023) investigated the efficacy of *B. tequilensis* against *C. fructicola*, there are no reports regarding its effectiveness against *C. acutatum*. Therefore, our study presents the first description of the effectiveness of both *B. tequilensis* and *B. cabrialesii* against *C. acutatum*. These findings contribute to the expanding knowledge base on biocontrol agents against *C. acutatum*, offering novel insights into the potential applications of *Bacillus* species in managing this pathogen.

In the case of *B. cinerea*, the bacterial isolates in our study exhibited initial inhibition rates (IRs) below 31 %. While no differences were observed between the strains against *B. cinerea* on the third day, by the seventh day, SB4.3 (*B. cabrialesii*) and SB8 (*B. subtilis*) demonstrated higher levels of inhibition compared to SB4.1.B (*B. tequilensis*). Guo et al. (2023) reported a stronger inhibitory effect of *B. tequilensis* against *B. cinerea* in their study on cherry tomatoes. Additionally, various *Bacillus* strains have been explored as biocontrol agents against *B. cinerea*, including *B. amyloliquefaciens* (Lee et al., 2017), *B. subtilis* (Gao et al., 2018), *B. atrophaeus*, *B. velezensis* (Toral et al., 2021), *B. thuringiensis*, *B. siamensis*, and *B. pumilus* (Poveda et al., 2022). However, to the best of our knowledge, our report is the first to investigate the inhibitory effect of *B. cabrialesii* against this fungal pathogen.

Among the three *Bacillus* species used in this study, *B. subtilis* has gained recognition as an effective biopesticide, with commercial formulations, such as Serenade® approved for application. Additionally, *B. subtilis* has been documented as a probiotic beneficial for human health (Garvey et al., 2022; Cao et al., 2020), leading to the development of numerous commercial dietary supplements featuring this species. However, there have been no reports of any *B. subtilis* strain employed for both biopesticide and potential human probiotic purposes simultaneously.

Therefore, subsequent assays in this study were conducted using the bacterial strain SB8. The dual-use potential of *B. subtilis* highlights promising avenues for further research and development, aiming to optimize its applications across different fields. This dual functionality could lead to innovative approaches in sustainable agriculture and safe production, fostering the development of integrated solutions that leverage the beneficial properties of *B. subtilis*.

Following the assessment of its antagonistic activity *in vitro*, we evaluated the compatibility of SB8 (*B. subtilis*) with an edible coating composed of sodium alginate as the primary polymer material. Sodium alginate is well-known for its use as a carrier for beneficial agronomic bacterial strains, including those of the *Bacillus* genus (Riseh et al., 2021). Additionally, alginate has been utilized for probiotic purposes, specifically with *B. subtilis* (Wai Chun et al., 2021). Consequently, this combination was selected for our study. Given that the shelf life of strawberries is approximately four days, we decided to evaluate the compatibility of the SB8 strain with the alginate coating over this period. The results indicate that the integration of SB8 with the sodium alginate edible coating is feasible and aligns with biotechnological approaches documented in the scientific literature.

Among the fungal pathogen tested in this study, the ascomycete *B. cinerea* is recognized as the predominant pathogen impacting harvested strawberries globally, resulting in considerable economic losses within in the strawberry industry. This phytopathogen is a major cause of fruit rejection among farmers, transportation companies and clients, resulting in substantial economic losses for the strawberry industry (Petrasch et al., 2019). To address this issue, we conducted an *in vivo* assay specifically focused on *B. cinerea* to assess the potential biopesticidal effects of SB8 in synergy with alginate-based edible coating. The aim was to evaluate the effectiveness of this combination in reducing the incidence and severity of *B. cinerea* infection on strawberries, thus enhancing fruit quality and shelf life.

The results from the *in vivo* assay demonstrated that the bacterial strain SB8 effectively reduced postharvest decay in strawberries. Notably, the combination of SB8 with alginate as an edible coating exhibited the most effective antagonistic effect against *B. cinerea*. Interestingly, the coating alone did not exhibit a significant difference compared to the control in reducing *B. cinerea* infection in strawberries. These findings highlight the importance of the synergistic interaction between SB8 and the alginate coating in enhancing the biocontrol efficacy against this major phytopathogen.

Given the excellent results obtained, a comprehensive genomic analysis was conducted to further investigate the features of *B. subtilis*

SB8. This analysis aimed to confirm the presence of biocontrol mechanisms and assess its potential as a human probiotic. The genomic mining confirmed that SB8 possesses genes essential for its biocontrol activity. Additionally, genomic features indicative of probiotic potential was identified. These findings reinforce SB8′s efficacy as a biocontrol agent against *B. cinerea* and highlight its potential application as a human probiotic.

In this study, genes encoding hydrolytic enzymes such as cellulase, amylase, and protease were identified. These enzymes can break down polymers present in fungal cells, thereby inhibiting the growth of the phytopathogen (Chandrasekaran et al., 2016; Riseh et al., 2024). Furthermore, the significant number of CAZy genes found in the genome of SB8 suggests a robust capability to antagonize fungal phytopathogens by potentially degrading and utilizing fungal polymers as nutrient sources (Thiruvengadam et al., 2022). These genomic insights provide valuable information regarding the mechanisms underlying SB8′s biocontrol activity and its potential for application in agricultural.

Bacillales species are extensively studied due to their ability to synthesize a diverse array of antimicrobial compounds, including bacteriocins, non-ribosomally synthesized peptides (NRPs), and polyketides (PKs) (Zhao and Kuipers, 2016). It has been established that the genome of *B. subtilis* SB8 contains homologous regions associated with these synthesis pathways.

Bacillus species have been shown to produce a variety of ribosomally synthesized antimicrobial peptides, including subtilin, a class I Lanthipeptide, which has been extensively studied in *B. subtilis* (Fu et al., 2023). Subtilosin A, known for its antibacterial properties, was also identified in our mining results. In terms of non-ribosomally synthesized peptides (NRPs), our study revealed the presence of lipopeptides such as surfactin and fengycins, both with significance antimicrobial activity which are commonly found in various *Bacillus* species including *B. pumulis* (Wang et al., 2020); *B. velenzensis* (Armenova et al., 2024) and *B. subtilis* (Dong et al., 2022). However, iturins, along with other major NRPs typical of *Bacillus*, were absent in the SB8 strain. Nonetheless, we did identify other NRPS compounds such as bacilysin and bacillibactin, commonly found among the *Bacillus* species (Ribeiro et al., 2021) such as *B. amyloliquefaciens* MBI600 (Dimopoulou et al., 2021) and *B. subtilis* (Tian et al., 2023). Furthermore, our investigation into polyketides (PKs) of Bacillales revealed the presence of bacillaene an antibiotic, indicating the common occurrence of these compounds in *B. subtilis* (Miao et al., 2023). This suggests their potential as biopesticides against both fungi and bacteria. Additionally, we identified the dipeptide Pulcherriminic acid, known for its role in iron acquisition, which is commonly synthesized by other *B. subtilis* strains (Yuan et al., 2020), indicating its widespread presence among related bacterial species. The genomic findings are likely correlated with the observed antagonistic activity in both *in vitro* and *in vivo* assays.

As human probiotics, bacteria possess a variety of mechanisms, including resistance to different environmental stresses such as cold, heat, and bile salts, as well as the ability to adhere to intestinal epithelial cell (Baig et al., 2022). This potential was evaluated by mining the genome, and the results indicate that strain SB8 possesses genes involved in these features. This correlates with the widespread use of dietary commercial supplements based on this specie, as it is also classified as GRAS (Mishra et al., 2023; Su et al., 2020; Zhang et al., 2020).

Indeed, further studies are essential to gather information on the proposed combination of *B. subtilis* and edible coatings as an alternative to pesticides applied on strawberries. Additionally, research focusing on the physicochemical and sensory aspects of strawberries is necessary to elucidate the efficacy of this application in field or greenhouse experiments in strawberry agronomic crops. Such studies are crucial for overcoming real-world conditions and effectively inhibiting fungal diseases Bacillales species are extensively studied due to their ability to synthesize a diverse array of antimicrobial compounds, including bacteriocins, NRPs and PKs (Zhao and Kuipers, 2016). It has been established that the genome of *B. subtilis* SB8 contains homologous regions associated with these synthesis pathways.

5. Conclusion

In conclusion, the search for BCAs with probiotic potential in agriculture offers a promising strategy for cultivating strawberries safely for human consumption. Specifically, bacterial strains SB4.3 (*B. cabrialesii*), SB8 (*B. subtilis*), and SB4.1.B (*B. tequilensis*) have emerged as promising BCAs for this purpose. Their potential to combat fungal diseases and enhance food safety underscores their significance in strawberry cultivation practices.

Among them, *B. subtilis* stands out as a versatile species, recognized for its dual roles as a biopesticide and a human probiotic. The SB8 strain exemplifies this versatility, displaying antagonistic activity against fungal pathogens both *in vitro* and *in vivo* conditions, while harbouring genes encoding antifungal compounds such as lipopeptides, hydrolytic enzymes, and secondary metabolites. Moreover, SB8 carries genes conferring resistance to various environmental stresses and adhesion to mucous cell surfaces, further bolstering its potential as a probiotic agent.

Products formulated with *B. subtilis* SB8 hold considerable promise for mitigating fungal diseases in strawberry crops. The survival and proliferation of SB8 within edible coatings and strawberry fruit wounds underscore its efficacy as a biocontrol agent for managing grey mold disease, a prevalent concern in agricultural settings. In summary, these findings underscore the significant role of probiotic endophytic bacteria and edible coatings in advancing biological control strategies aimed at enhancing both the productivity and safety of strawberry cultivation. Additionally, the detection of potential pathogens emphasizes the importance of employing hygienic techniques during berry collection to enhance food safety and promote good agricultural practices.

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CRediT authorship contribution statement

Sandra Menéndez-Cañamares: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Alberto Blázquez:** Investigation, Methodology. **Irene Albertos:** Investigation, Methodology. **Jorge Poveda:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Alexandra Díez-Méndez:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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