

Harnessing *Bacillus* and *Paenibacillus*: metabolic and genetic characterization for the development of biopreparations for plant pathogen control

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Abstract. The development of effective and stable microbial biopreparations is essential for sustainable plant protection and for assessing the status of environmental microorganisms and phytopathogens. This study investigated the metabolic and ecological capacities of *Bacillus* and *Paenibacillus* species with potential roles in pathogen biocontrol. Therefore, organic-compliant liquid biopreparations were formulated using bacterial strains in combination with plant extracts, humic acids, and/or vinasse – components influence on microbial metabolism and antagonistic activity. Individual formulation ingredients were evaluated for their antagonistic properties, compatibility with beneficial bacteria, and their effects on microbial viability and functional activity during storage. Antagonistic assays demonstrated that nettle leaf and horsetail herb extracts effectively inhibited the growth of phytopathogens while preserving the proliferation of beneficial bacteria, indicating selective disruption of pathogen cellular integrity and metabolism. Scanning electron microscopy (SEM) and NanoLive holotomography imaging confirmed the structural stabilization of the final biopreparation. The liquid formulations maintained bacterial viability and functional stability for at least three months at both room temperature and 4°C, meeting industrial standards for microbial product shelf-life. These findings highlight that biopreparations enriched with ecological, biologically safe ingredients have strong potential for application in crop protection against pathogen infections, supporting the development of sustainable biocontrol strategies.

Keywords: microbial biopreparations, beneficial microorganisms, plant pathogen control, antifungal activity, microbe-pathogen interactions

1. INTRODUCTION

One of the major challenges facing modern agriculture is meeting the growing demand for highquality agricultural raw materials while simultaneously ensuring environmental sustainability. In recent decades, research on the negative impacts of fertilizer use and chemical plant agents on soil and the quality of raw materials has intensified. Agricultural intensification typically involves the widespread application of chemical nitrogen, phosphorus, and potassium fertilizers, along with pesticides, herbicides, insecticides, and fungicides. However, long-term use of these substances can adversely affect the environment – contributing to soil, water, and air pollution – as well as biodiversity, including plants, animals and microorganisms, and ultimately human health (Al Zaidi *et al.*, 2011; Ahmad *et al.*, 2024; Rani *et al.*, 2021). In this context, it is very important to develop solutions that, at the same time, ensure proper development and protection of plants, as well as care for the environment. An alternative or complement to chemical fertilizers is the application of various organic wastes, which provide organic matter and nutrients. Substitutes for chemical plant protection products might be preparations based on beneficial microorganisms. This direction is an innovative approach in plant protection and could also be applied in organic farming.

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The European Union aspires to promote and expand the adoption of organic farming and to increase the availability of organic food. The development of biopreparation production aligns well with the European Union's current policy. Organic producers under the Regulation of the European Community Council No. 834/2007 of 28 June 2007 on organic production are obliged to limit the use of mineral fertilizers and chemicals in favour of the use of organic preparations of natural origin, such as microbiological agents.

Softfruit crops are highly susceptible to fungal and oomycete phytopathogens, including *Botrytis*, *Verticillium*, *Colletotrichum*, or *Phytophthora*, species, which cause substantial pre and postharvest losses. These losses arise not only from a reduced yield and fruit quality but also from infections that spread rapidly across plantations, ultimately leading to plant decline and withering. Among the most damaging diseases affecting soft fruits are gray mold, verticillium wilt, anthracnose, and phytophthorosis, all driven by the aggressive pathogens (Malarczyk *et al.*, 2019; Ma *et al.*, 2023). Effective management is particularly challenging in organic farming systems, where producers are required to limit the use of mineral fertilizers and synthetic pesticides, combined with rising demand for environmentally sustainable plant protection, have intensified interest in biological control strategies and the development of effective biocontrol agents.

Biological control agents, especially beneficial bacterial strains, are valuable tools for environmentally friendly disease management. Bacteria such as Plant Growth Promoting Rhizobacteria (PGPR) improve the availability of soil nutrients to plants, positively affect root development, and activate forms of inaccessible phosphorus (Gutierrez *et al.*, 2001; Sun *et al.*, 2026). Moreover, some species or strains exhibit strong antagonistic properties against fungi (Gajbhiye *et al.*, 2010; Benaissa, 2024) and are often used in microbiological preparations used in plant cultivation. Strains of the *Bacillus* and *Paenibacillus* are widely reported to exhibit strong antagonistic activity against a variety of phytopathogens. These genera represent the most intensively studied groups of beneficial bacteria due to their high potential for use in plant protection strategies. *Bacillus* and *Paenibacillus* genera are naturally abundant in diverse soil environments, display high tolerance to elevated temperatures, multiply rapidly in liquid media, and form durable spores-traits that make them particularly suitable for biopreparation development. Reflecting their recognized value, the United States Environmental Protection Agency had registered more than ten *Bacillus* strains as biopesticides and biofungicides by 2006. Several different strains of *Bacillus* with antagonistic activity against fungi have been described in the literature. Soil isolate of *Bacillus velezensis* (AH2 CECT-7221) was characterized by antifungal activity against various plant disease pathogens,

such as fungi of the genera *Botrytis*, *Pythium*, *Rhizoctonia*, *Alternaria*, *Fusarium*, *Phytophthora*, *Thielaviopsis*, and *Botryosphaeria* (Fernandez Martinez *et al.*, 2013). A biopreparation based on the *Bacillus amyloliquefaciens* strain NRRL B50349, containing spraydried spores, demonstrated significant efficacy in suppressing the growth of fungal phytopathogens, including *Aspergillus niger*, *Bremia lactucae*, *Erisphe necator*, *Rhizoctonia Solani*, *Sclerotinia sclerotiorum*, *Septoria apicola*, *Spatherotheca fulignea*, *Spatherotheca macularis* (Snyder *et al.*, 2016). Regarding the strawberry pathogens mentioned above (*Verticillium dahliae*, *Botrytis cinerea*, *Phytophthora cactorum*), *Bacillus methylophilus* (XT1 and XT2) exhibited antagonistic activity (Bejar Luque *et al.*, 2016). These advances and the expanding scientific understanding of *Bacillus* biology underscore the need to identify and characterize new strains within this genus, particularly in light of the increasing demands and sustainability requirements of modern agriculture.

The development of biopreparations based on biological components represents a central challenge and a promising avenue for advancing sustainable agriculture. Such microbial technologies contribute to climate-resilient production systems and offer economically attractive alternatives to conventional chemical inputs. To achieve consistent field performance, however, microbial inoculants must be formulated to remain effective across diverse soil and environmental conditions. Modern bioinoculant formulation encompasses several critical stages, including inoculum production, incorporation of functional additives, selection and sterilization of suitable carrier materials, scale-up procedures, quality assurance, and optimized packaging and delivery strategies. Formulations may be solid or liquid, with solid preparations further classified as dry or wet depending on application requirements. Because the agronomic efficacy of any microbial product depends on precise formulation and correct application, establishing a robust and reproducible formulation framework formed a key objective of this research. The objective of this study was to develop and optimize biological components for use in environmentally safe biopreparations intended for ecological crop protection. The research focused on:

- Experimental evaluation of three soil-derived bacterial strains exhibiting antagonistic activity against key phytopathogens (*Botrytis* sp., *Verticillium* sp., *Colletotrichum* sp., and *Phytophthora* sp.);
- Assessing additional natural components with antifungal properties, such as plant extracts and humic acids, to complement the bacterial strains in the final formulation;
- Designing and optimizing the production process for the two biopreparations, including selection of culture conditions, formulation steps, and stabilization strategies;

- Evaluating storage conditions and product stability to ensure the biopreparations meets the requirements of ecological (organic) farming systems;

- Defining and evaluating a structured methodological framework for developing new bioproducts, with a focus on integrating microbial screening, functional evaluation, and formulation steps into a coherent stepwise model that supports the development of sustainable agricultural bioproducts.

This multifaceted approach supports both the practical design of effective microbial agents and the broader advancement of methodologies for next-generation bioproduct development. The experimental data and a structured development pathway, addressing a gap in the literature, which is that comprehensive, end to end optimization of microbial biopreparations remains limited.

The study hypothesized that soil-derived bacterial strains exhibiting antagonistic activity against major phytopathogens including *Botrytis* sp., *Verticillium* sp., *Colletotrichum* sp., and *Phytophthora* sp., can be developed into a stable and effective biopreparation for ecological crop protection, provided that their biological activity, formulation parameters, and storage conditions are properly optimized. This hypothesis reflects the premise that targeted microbial selection, combined with rational formulation design, can yield robust next-generation biocontrol products suitable for sustainable agriculture.

2. MATERIALS AND METHODS

2.1. Culture conditions and genetic identification of bacterial strains

The strains used in the experiments – AF75AB2, Sp115AD, and Sp116AC* – were obtained from the SYMBIOBANK Collection The National Institute of Horticultural Research in Skierniewice, Poland. Strain AF75AB2 was isolated from the rhizosphere of a wild *Fragaria* sp. (cultivar unknown) collected in the Pepper Mountains Nature Reserve (Poland). Strains Sp115AD and Sp116AC* were isolated from bulk soil sampled beneath an apple tree (cultivar unknown) in Nowe Berezowo, Poland. The strains was cultured on plate count agar (PCA) at 27°C. For long term preservation, each isolate was stored at –20°C as a pellet suspended in sterile glycerol, which effectively maintained viability over extended periods and allowed reliable revival within 24 h.

The bacterial strains were identified by wholegenome sequencing (WGS) on the Illumina platform. The quality and quantity of the isolated DNA were assessed using Nano Drop 2000 and a Quantus fluorometer. DNA samples were diluted to 100-500 ng in 30 µl. Libraries were prepared according to the Illumina DNA Prep Reference Guide. DNA was subjected to tagmentation, clean-up, and PCR amplification (8 cycles). Library quality was evaluated using D1000 ScreenTape, TapeStation 4150, and the

Quantus fluorometer. Sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycles). Raw reads were filtered and trimmed with Cutadapt v4.4 (Martin, 2011), and *de novo* genome assembly was performed using SPAdes. Assembly quality was evaluated using QUAST v5.2 (Gurevich *et al.*, 2013), and annotation was carried out using Prokka (Cantalapiedra *et al.*, 2021). Sequence comparisons were conducted using BLAST (Altschul *et al.*, 1990) and NCBI resources (2016). Raw sequencing data were deposited in the NCBI SRA database under accession numbers: PRJNA1470977 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1470977>).

2.2. Biochemical characterization of bacterial strains based on BIOLOG and API ZYM assays

The bacterial strains were characterized based on physiological and biochemical activities by determining the ability to use 71 carbon substrates belonging to the groups of compounds: amino acids, sugar alcohols, carbohydrates, sugar acids, and carboxylic acids, and the degree of growth in the presence of these substrates. Furthermore, the chemical sensitivity of selected bacterial strains to 23 selected chemical compounds (antibiotics and dyes) was determined. Phenotypic characterization and chemical sensitivity of isolates were tested in triplicate using the Biolog GEN III Microplate™ (Biolog Inc., Hayward, CA). GEN III microplates were inoculated with 100 µl of cell suspension prepared in sterile inoculating fluid, IF-A (Biolog Inc., Hayward, CA). Turbidity was adjusted to 90-93% transmittance using a turbidimeter (Biolog Inc., Hayward, CA). Each bacterial strain was tested on a separate plate, in three replicates. The microplates were incubated at 30°C, and absorbance readings were taken using the Biolog MicroStation™ after 36 h at 590 nm.

Enzymatic activities of isolated strains were assessed using the API ZYM kit (BioMérieux). The assays were performed in triplicate, and each API-ZYM strip included the manufacturer-provided control well. The procedure followed the standard protocol. Briefly, bacterial suspensions were prepared in 0.85% sterile sodium chloride and adjusted to a density corresponding to McFarland Standard 5-6. The API-ZYM strips were then inoculated with the prepared suspensions and incubated at 30°C for 4 h, after which ZYM A and ZYM B reagents were added. After short exposure to light, the colour formation was checked and described as follows. This approach ensured consistent assessment of enzymatic activity profiles across all tested strains. The noted enzyme activity was described as a positive or negative reaction ('+', '–'). Additionally, the color grades corresponding to the intensity of the reaction were noted (from light to dark, where “light yellow circle” is the minimal reaction, “orange circle” is the intermediate, and “red circle” is the maximum intensity of the reaction).

2.3. Phytopathogen strains and growth conditions

The antagonistic effects of selected bacteria were tested against the most important plant pathogens: *Botrytis* sp. (G277/18), *Colletotrichum* sp. (G171/18), *Phytophthora* sp. (G408/18), and *Verticillium* sp. (G296/18). The plant pathogens were obtained and deposited in the Laboratory of Molecular and Environmental Microbiology, Institute of Agrophysics, Polish Academy of Sciences. The strains of fungal and oomycete pathogens were isolated from infected strawberry plants, fruits, or soil and were identified at the genus level based on DNA extraction, amplification of the D2 large subunit region of the rDNA (Rozynek *et al.*, 2004), and sequencing as described by Frąc *et al.* (2014) with minor modifications. More detailed information concerning the phytopathogen strains was described previously by Malarczyk *et al.* (2020). The sequences were deposited in GenBank of NCBI (<http://www.ncbi.nlm.nih.gov>) under the following accession numbers MT126670, MT126803, MT 154304, MT 133320. The pathogens were cultured on Petri plates containing PDA medium at 27°C, 7-days-old fungal cultures were used for the next stages.

2.4. Activity of bacteria against phytopathogens

Antagonistic activities of the bacterial strains against selected phytopathogens (*Botrytis* sp. (G277/18), *Colletotrichum* sp. (G171/18), *Phytophthora* sp. (G408/18), and *Verticillium* sp. (G296/18) were evaluated using a standardized co-culturing assay. Preliminary growth assessments (data not shown) confirmed that both the bacterial strains and the phytopathogens exhibited consistent and comparable growth on PDA; therefore, PDA was selected as the assay medium. A 7-day-old culture of the pathogen was harvested from the agar medium, adjusted to 70% turbidity in 10 ml of sterile water, and vortexed. Subsequently, 300 µl of the pathogen suspension was evenly spread onto Petri dish plate with PDA medium. For each antagonistic test plate, a sterile filter paper disk (5 mm diameter; Macherey-Nagel MN 615) was impregnated with 25 µl of the bacterial suspension (adjusted to 70% turbidity) and placed at the center of agar surface plate inoculated with the pathogen in the previous step. Plates inoculated with sterile water served as negative controls, enabling assessment of pathogen growth in the absence of bacterial antagonists. The plates were incubated at 27°C for 5-7 days, after which the inhibition zone was measured. Each antagonistic test was performed in three independent biological replicates, with each replicate consisting of a separate plate. Measurements were analysed using standard statistical procedures appropriate for replicated inhibition assays, ensuring reliable comparison of antagonistic performance across strains. This design provides clear control conditions, replication structure, and quantifiable outcomes for evaluating antagonistic activity.

2.5. Optimization of biopreparations formulas incorporating tested *Bacillus* spp. and *Paenibacillus* sp. strains and natural additives

2.5.1. Composition of the liquid growth medium and parameters for bacterial cultivation (AF75AB2, Sp115AD, Sp116AC*)

To support the development of an industrially applicable biopreparation, this study evaluated the composition of liquid growth medium to ensure robust cultivation of all tested bacterial strains. Although, individual strains may differ in their nutritional requirements, the objective was to formulate a single universal medium capable of supporting consistent growth across all isolates. To maximize bacterial biomass production, we designed a nutritionally enriched culture medium. The formulation process began with M9 Minimal Salts (Sigma-Aldrich), consisting of 33.9 g Na₂HPO₄, 15 g KH₂PO₄, 5 g NH₄Cl, and 2.5 g NaCl per liter as the basal mineral medium, which was subsequently supplemented with additional carbon and nutrient sources to enhance growth performance. Thereafter, we added to this mineral medium: CaCl₂, MgSO₄, FeCl₂, MnCl₂, ZnCl₂, CuCl₂, CoCl₂, Na₂MoO₄. This mineral liquid medium was supplemented with 20 g l⁻¹ neutralized acid whey, pH 5.5 (OSM Krasnystaw, Poland), as a carbon source to obtain a nutritionally enriched growth medium. The addition of whey to the mineral formulation was intended to enhance bacterial growth efficiency and support robust culture development. The detailed recipe for this liquid medium was described in the Supplementary (S2). For the bacterial cultivation, on a laboratory scale, the 40 ml of final culture medium was sterilized at 121°C for 20 min in a 100 ml flask. Sterile mineral medium with whey (MMW) was inoculated with 4 ml of bacteria suspension (90% of turbidity or 0.5-1 McFarland standard, inoculum of 10⁵ CFU ml⁻¹) and was incubated at 30°C with shaking (120 rpm). The number of viable cells in culture was counted at 24, 48, 72, and 96 h to determine growth dynamics and identify the maximum growth point of the bacteria. The cell concentration in each treatment point was assessed by serial dilution in sterile saline (0.85% NaCl) and spread plating on plate count agar. Subsequently, the inoculated plates were incubated for 96 h at 27°C, after which the colonies were counted. The culture assays were performed in three independent replicates (three flasks). Viable cell counts were also determined in three technical replicates, resulting in a total of nine measurements per condition.

2.5.2. Optimizing sporulation conditions for bacterial cultures

The selected bacterial strains were classified as spore-forming bacteria. During incubation, actively growing cells were induced to differentiate into spores by exposure to thermal stress, altered salinity, or modified pH. Sporulation was triggered using a single factor optimization experiment.

Specifically, after 48 h of incubation in MMW (corresponding to the point of maximal bacterial growth) the following conditions were tested: increase temperature to 35, 40, and 45°C, change the pH by added lactic acid (decrease to 5.5, 4.5 and 3.5) and modify the salinity by added potassium chloride (increase to 1, 2 and 4% of potassium chloride), sporulation stage was carried out for 48 h. The impact of different conditions on sporulation was assessed by quantifying spores growing on agar medium. Cultures grown in glass flasks were placed in a thermostatically controlled water bath and heated at 80°C for 20 min. This pasteurisation step eliminated vegetative cells, leaving only spores. From each flask, 1 ml of spore suspension was collected, and serial dilutions from 10^{-1} to 10^{-8} were prepared. Subsequently, 0.1 ml of each dilution was spread onto 90 mm Petri plates containing PCA. The plates were incubated at 27°C for 72 h, and the number of heat-resistant spores was expressed as $\log_{10}$ CFU ml $^{-1}$. The sporulation conditions assays were performed in three independent replicates (three flasks). Viable cell counts were also determined in three technical replicates, resulting in a total of nine measurements per condition.

2.5.3. Functional interactions of natural additives with phytopathogens and bacterial strains

The formulation of biopreparation varies depending on the intended application. However, the most common components, besides microorganisms, are natural extracts, organic compounds, and specific ingredients. The interactions between components and effective bacteria were studied. For that reason, the antagonistic activity of selected compounds was determined against phytopathogens and beneficial bacteria (AF75AB2, Sp115AD, Sp116AC*). Antagonistic activity tests were carried out in Petri plates (90 mm diameter) with PDA medium, in three replicates. During the experiment, various natural additives were tested: nettle leaf extract, horsetail herb extract, calendula extract, humic acids (The Total Humus, THE sp. z o.o. company), and vinasse. Leaf extracts were prepared as follows: 100 g of dried plant material was poured over 1 liter of boiling water and left to stand at room temperature for 24 h. The infusion was then decanted, separated from the sediment, and sterilized at 121°C for 20 min. Vinasse was obtained as a raw byproduct of yeast production. Humic acids were prepared as a 1% solutions in sterile water. For the investigation assays, agar plates were inoculated with either 300 μ l of pathogen suspension (70% of turbidity) or 100 μ l of bacterial suspension (prepared by transferring 48-h colonies into 10 ml of sterile water, vortexing, and adjusting to 70% of turbidity). For liquid components, the sterile filterpaper disk (5 mm diameter), soaked with 25 μ l of the test solution, was placed in the center of each agar plate. Control plates containing a single culture of each strain were prepared to verify strain purity and to document the characteristic colony morphology of each organism.

The plates were incubated at 27°C for 72 h for bacteria and 7 days for the pathogen. After incubation, inhibition zones were measured as diameters in millimetres. The microbial interaction assays were performed in three replicates.

2.5.4. An outline of the preparation of bioproducts based on optimized parameters

Based on the analyses and experimental tests conducted in this study, we establish an optimized procedure for producing two biopreparations composed of bacterial spores combined with selected natural components. The initial cultivation step involves growing each bacterial strain for 48 h at 27°C on PCA. From these cultures, a bacterial suspension is prepared and adjusted to 90% transmittance (T), which is then used to inoculate the liquid medium at 10% (v/v). The propagation stage is carried out in MMW liquid medium under shaking conditions 120 rpm, 30°C for 48 h, ensuring efficient biomass accumulation and preparation for the subsequent sporulation and formulation steps. Subsequently, a spore-induction step is performed by imposing stressful conditions (elevating the culture temperature to 40°C for Sp115AD or stabilizing the pH at 5.5 for AF75AB2 and Sp116AC*). After 48 h of cultivation under stress conditions that promote spore formation by the bacterial cells, the cultures are pasteurized by incubation at 80°C for 20 min. The spore suspension obtained through this procedure serves as the primary active component of each biopreparation. To enhance their functional properties, the bioproducts were further enriched with natural bioactive additives, including extracts of dried nettle, horsetail, calendula, as well as humic acid solutions (prepared as described above). Using these components, the two liquid antifungal biopreparations were formulated. Each biopreparation ingredients, were included in precisely calculated proportions, ensuring consistency, biological activity, and suitability for scalable biopreparation development. Biopreparation 1 (BP1) consists of spore suspension of AF75AB2 and Sp115AD (in equal amounts) – 80%, and 5% of each ingredient: extract of dried nettle, extract of dried horsetail, and extract of dried calendula, and also liquid humic acids, exactly 400 ml of each strain suspension, 50 ml of each plant extract and 50 ml of 1% liquid humic acid per one liter of the final bioproduct. Biopreparation 2 (BP2) consisting of spore suspension of Sp115AD and Sp116AC* (in equal amounts) – 80 and 15% of vinasse and 5% of liquid humic acid, exactly 400 ml of each strain suspension, 150 ml of vinasse and 50 ml of 1% liquid humic acid per 1 l of final bioproduct. The prepared liquid bioproducts (BP1 and BP2) were stabilized (protected against the spores formation) by lowering the pH with lactic acid (80%) to 4–4.5. The manufacturing procedures and formulations of these biopreparations are described and claimed in patents P.445051 and P.445053 (Gryta *et al.*, 2025a, b).

2.5.5. Microscope visualization analysis with scanning electron microscope (SEM) and Nanolive holotomography microscope

The morphological characteristics of bacteria and their microstructures in biopreparations were examined using a scanning electron microscope (SEM). Bacterial cells in the final liquid biopreparations BP1 and BP2 underwent ultrastructural analysis to assess cellular integrity following formulation and stabilization. Samples were collected directly from freshly prepared formulations containing spore suspensions and plant-derived additives. The aliquots of each biopreparation were deposited onto sterile aluminium stubs coated with conductive double-sided carbon adhesive tape. The samples were air-dried under controlled conditions in the presence of silica gel to remove residual moisture and prevent structural collapse. After complete drying, specimens were coated with an approximately 5 nm layer of gold using a sputter coater (CCU-010 LV, Safematic GmbH, Zizers, Switzerland) to ensure electrical conductivity. Microscopic observations were performed using a Phenom ProX scanning electron microscope (Thermo Fisher Scientific, Waltham, MA, USA). Images were acquired with a backscattered electron (BSE) detector at an accelerating voltage of 10 kV. Micrographs were recorded at magnifications of 200× and 5000× to evaluate the overall distribution of bacterial cells within the formulation matrix and the detailed surface morphology of vegetative cells and spores. This approach enabled visualization of bacterial structural integrity, cell surface characteristics, and interactions with residual formulation components (Maj *et al.*, 2024).

Nanolive holotomography microscope with 3D Cell Explorer (NanoLive) was used to determine the bacteria. Suspensions of biopreparations BP1 and BP2 were prepared in sterile Potato Dextrose Broth. Holotomographic visualization required the use of a liquid medium. Potato dextrose broth (PDB) was selected for its suitable viscosity and ability to support stable sample positioning during imaging, and because its use did not modify the composition of the biopreparation. Subsequently, 1.5 ml of each suspension was transferred onto a microscope dish, and the bacterial cells were imaged using Nanolive's holotomography microscope (3D Cell Explorer). To compare the effects of biopreparations BP1 and BP2 on the number of bacterial cells and the surface area they occupied, the acquired images were analyzed using ImageJ software (v. 1.54g).

2.5.6. Antagonistic activity of biopreparation against phytopathogens

Antagonistic activity of the biopreparations was evaluated against phytopathogens (*Botrytis* sp., *Colletotrichum* sp., *Phytophthora* sp., and *Verticillium* sp.) on 90 mm Petri plates containing PDA medium, using three replicates per treatment. Plates were first inoculated with 300 µl of 70%

turbidity of pathogen suspension (spread over the entire plate) and incubated for 24 h at 27°C. After this pre-incubation period, a 5 mm diameter filtration paper disk soaked with 25 µl of biopreparation was placed at the center of each plate. The plates were then incubated under the same conditions for an additional 72 h. Following incubation, inhibition zones were measured as diameters in millimetres. The antagonistic assays were conducted in three independent replicates, and a control plate containing the pure strain was included to verify strain purity and baseline growth characteristics.

2.5.7. Storage of biopreparations

The antagonistic properties of the prepared biopreparations were evaluated under different storage conditions. The storage test was conducted as follows: each biopreparation, sealed in a dark plastic bottle, was stored for six months at three temperatures: 4°C (C), 35°C (H), and room temperature (RT). Antagonistic activity was assessed after 3 and 6 months of storage. For both formulations (BP1 and BP2), antagonistic activity was tested immediately after preparation and again during storage, following the method described above in 2.5.6.

2.6. Statistical analysis and calculations

Data analysis and subsequent visualizations were conducted using RStudio v.4.3.2.1. Illustrations and graphical elements were generated using BioRender.com. The values of all calculated indices presented on plots or heat maps were expressed as the mean of all experimental replicates, and statistical significance was assessed using Tukey's HSD test at the 0.05 significance level, and significant differences were indicated by lowercase letters on the plot. This approach allowed for reliable comparison among treatments and ensured robust interpretation of differences in experimental outcomes. Antibiotic resistance gene (AMR) profiles were predicted from genomic data with Resistance Gene Identifier (RGI) using reference data from the Comprehensive Antibiotic Resistance Database (CARD). The resulting resistome profile was then visualized with the heatmap function of RGI tool, highlighting perfect and strict hits to CARD.

3. RESULTS

3.1. Culture conditions and genetic identification of bacterial strains

The bacterial isolates cultured on PCA supported robust growth within 24–72 h of incubation at 23°C. The strains grew consistently and reproducibly, forming light to dark-beige, shiny, smooth colonies. Representative growth characteristics of the tested *Bacillus subtilis* (AF75AB2 and Sp115AD) and *Paenibacillus polymyxa* (Sp116AC*) strains are shown in Supplementary Material (Fig. S1).

The identification of the bacterial strains was performed to confirm their taxonomic affiliation and to ensure that they belonged to a group commonly recognized as PGPR, with potential antagonistic activity and suitability for use in agricultural biopreparations. For the aims of this study-centered on functional screening, antagonistic potential, and formulation development-genus level identification based on whole-genome sequencing was performed as an appropriate and adequate level of taxonomic resolution. The whole-genome sequencing (WGS) analyses identified strains AF75AB2 and Sp115AD as showing 100% sequence similarity to members of *Bacillus subtilis*, whereas strain Sp116AC* exhibited 100% similarity to *Paenibacillus polymyxa*. All sequence comparisons were performed against the NCBI database.

3.2. Metabolic potential and antagonistic activity of bacterial strains

The metabolic potential of the bacterial strains was assessed using BIOLOG™ microplates and the API ZYM test kit, enabling rapid and comprehensive characterization of their biochemical traits and sensitivity profiles.

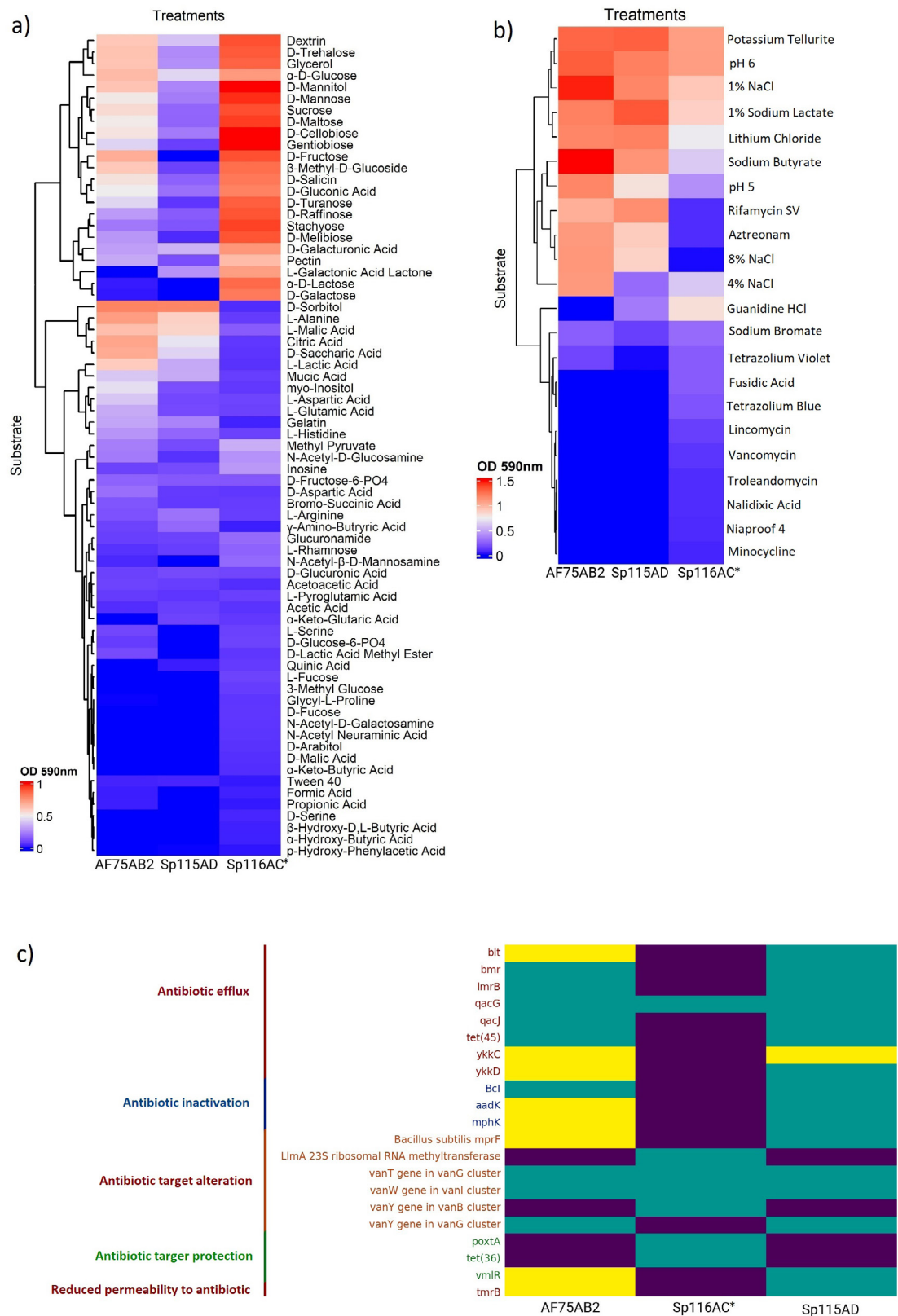
The BIOLOG GEN III plates technique is based on the rate of utilization of carbon substrates, and analyzes well color development and optical density (OD), and creates an individual characteristic “metabolic fingerprint”. Furthermore, the GEN III plate includes reagents that enable the determination of bacterial chemical sensitivity. The results of biochemical and physiological activity of the tested bacterial strains are presented in Fig. 1. The metabolic activity results for the three tested strains revealed differences in carbon-source utilization rates. Generally, the substrates included in the carbohydrate group are highly metabolized. However, the strain Sp115AD (*B. subtilis*) is distinguishable from AF75AB2 and Sp116AC* (*P. polymyxa*) and can metabolize only a few substrates, such as α -D-Glucose, D-Sorbitol, L-Alanine, L-Malic Acid, and Citric Acid. The substrates included amino acids, and carboxylic acids were definitely less metabolized by all strains. What is more characteristic of strain Sp116AC* (*P. polymyxa*), is that there is no very high activity under incubation with carbohydrates such as D-Mannitol, D-Cellobiose, Gentiobiose, D-Mannose, Sucrose, D-Maltose, D-Trehalose (the OD ranged 0.5-1), and a very low level of utilization of other substrates. The AF75AB2 (*B. subtilis*) strain is characterized by a distinct functional profile compared with Sp116AC* (*P. polymyxa*). This strain can utilize a broader range of substrates at lower levels. The second part of phenotypic profiling analyses based on GENIII plate included tests of bacteria sensitivity on growth under different conditions: pH (6 or 5), salinity (1, 4, 8 % NaCl), assimilation of various substrates (dyes: tetrazolium blue, tetrazolium violet; surfactants: Niaproof 4, Tween 40) and some toxic ions (potassium tellurite, sodium bromate, lithium chloride) and organics drugs such

as salicin, inosine, guanidine HCl as well as sensitivity to common antibiotics (aztreonam, fusidic acid, nalidixic acid, rifampicin, linomycin, vancomycin, minocycline, troleandomycin) (Fig. 1b). The obtained results showed that the studied *Bacillus* isolates varied in their sensitivity to antimicrobial agents. All tested strains are highly sensitive to sodium bromate, tetrazolium violet and blue, fusidic acid, linomycin, vancomycin, troleandomycin, nalidixic acid, niaproof 4, and minocycline. What is more, *P. polymyxa* (Sp116AC*) was also susceptible to rifampicin, aztreonam, and 8% NaCl. The tested isolates exhibited resistance to potassium tellurite, pH 6, sodium lactate, 1% NaCl, and lithium chloride. Among them, strain AF75AB2 (*B. subtilis*) demonstrated the strongest ability to grow in the presence of antimicrobial chemical agents. Salinity tolerance assessed using the BIOLOG GENIII microplate system (1-8% NaCl) confirmed tolerance to 1% sodium chloride, while markedly reduced growth was observed at 4 and 8% NaCl. Collectively, the phenotypic and metabolic profiling results provide valuable insights that may support the application of these isolates in biotechnology processes.

The bacterial genome was analysed to assess its antibiotic resistance profile, and the resulting resistome was visualized as a heat map (Fig. 1c). This analysis was incorporated to address an existing knowledge gap regarding the antibiotic-resistance potential of the studied strain and to provide a more comprehensive characterization of its resistome. The analysis revealed that the strains do not possess genes essential for antibiotic resistance, and the few resistance-associated genes that were detected, particularly in strain AF75AB2 (*B. subtilis*), were present only as isolated elements. When such genes occur individually rather than as components of a larger gene cluster or operon, they are unlikely to confer measurable resistance activity (Munita and Arias, 2016).

Enzymatic activity was assessed using the API ZYM system, and the results are presented in Fig. 2. The obtained enzyme activity profiles varied among the tested bacterial isolates. Strains of *B. subtilis*: AF75AB2 and Sp115AD exhibited similar enzymatic patterns. Among the 19 enzymes evaluated, both strains showed positive reaction for nine, including alkaline phosphatase, esterase, esterase lipase, leucine and valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α - and β -glucosidase. Additionally, no reaction for either lipase, cystine arylamidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, or trypsin was detected. What is more, for strain Sp116AC* (*P. polymyxa*), the unique activity of α -chymotrypsin, β -galactosidase, and α -galactosidase was detected.

In the next stage of strain characterization, the antagonistic activity against phytopathogens was evaluated. This assessment represented a key functional criterion for selecting isolates suitable for use as biological components in the biopreparation, ensuring that only strains with



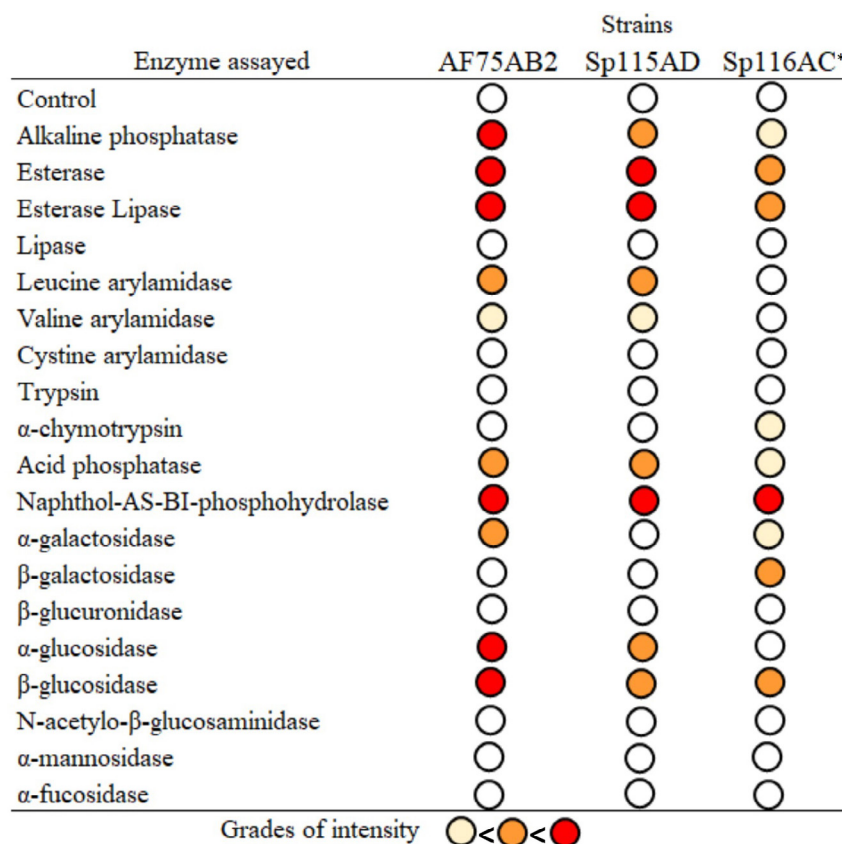


Fig. 2. Enzyme activity of the bacteria AF75AB2, Sp115AD, and Sp116AC* measured with the API-ZYM assay.

demonstrable inhibitory effects were advanced for further biocontrol development. The inhibitory effectiveness of the bacterial isolates on pathogen growth was evaluated using an agar plate method. Because the use of chemical fungicides is limited and increasingly restricted in ecological crop production, this study focused on four phytopathogens that pose particular challenges under such conditions. The antagonistic properties of the tested *B. subtilis* and *P. polymyxa* strains are presented in Fig. 3. The growth of the pathogen on agar plates was significantly reduced in the presence of the bacterial strains. Pathogen cultures exposed to bacterial isolates exhibited both diminished mycelial expansion and decreased sporulation. All studied bacterial strains demonstrated antagonistic activity against the selected phytopathogens. Among them, *B. subtilis* strain (AF75AB2) showed the strongest inhibitory effect, while *P. polymyxa* strain (Sp116AC*) displayed slightly lower but still notable antagonistic activity. These *in vitro* results indicate that the tested bacterial isolates are capable of suppressing the growth of phytopathogenic fungi and oomycetes under controlled laboratory conditions. While these findings do not allow conclusions about performance in field environments, they provide a promising basis for further investigation and highlight the potential of these strains for future biological control applications pending additional *in vivo* and field-level validation.

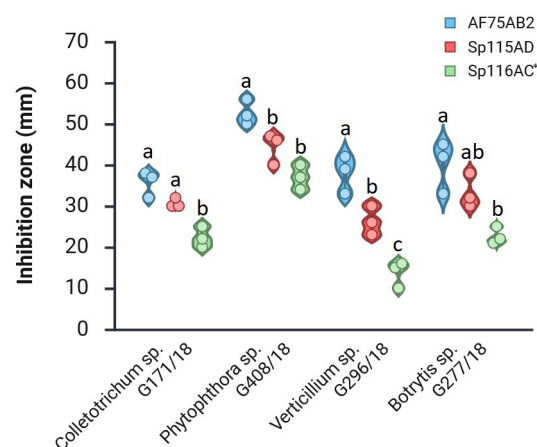


Fig. 3. Inhibition effect of tested *B. subtilis* strains (AF75AB2, Sp115AD), and *P. polymyxa* (Sp116AC*) on pathogen growth (inhibition zone – mm). Small letters mean significant differences (results of the Tukey's HSD test at the 0.05 level).

During the biotechnological process of optimizing bacterial culture production, a crucial stage is achieving a high yield of bacterial biomass. In this study, we evaluated acid whey as a carbon source in basal mineral medium (MMW) and compared it with the mineral medium (MM) without whey supplementation as the control. Figure 4 presents the bacterial growth response to acid whey – supplemented

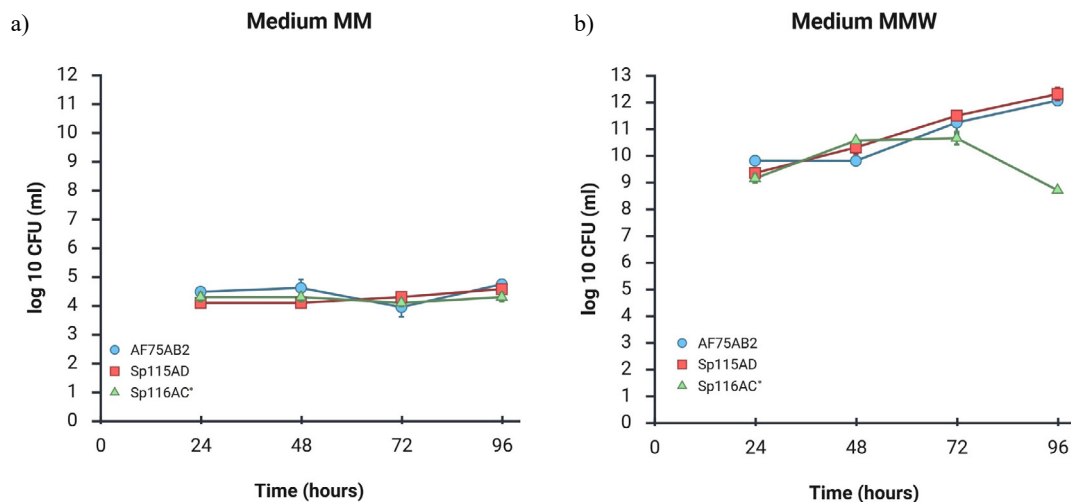


Fig. 4. Line plot presenting kinetic dynamics of bacteria growth in two variants of liquid medium: a) mineral medium (MM) and b) mineral medium enriched with whey (MMW).

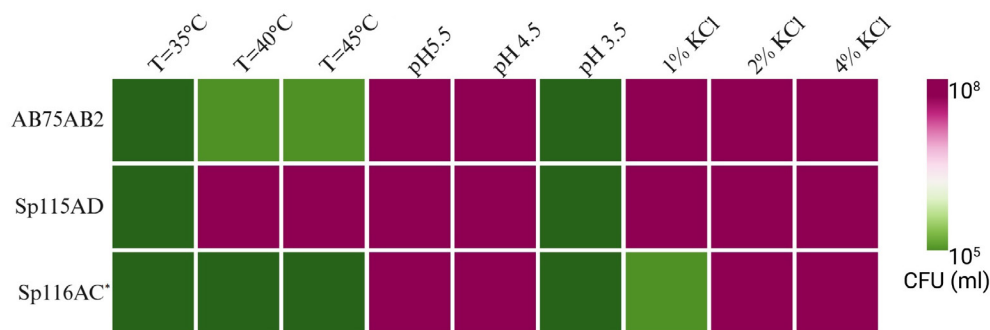


Fig. 5. Heatmap visualization of the conditions for the sporulation test of *B. subtilis* and *P. polymyxa* strains. The heatmap visualizes colony forming unit (CFU ml⁻¹) level.

medium compared with the unsupplemented control. We selected liquid culture conditions to achieve high bacterial growth yields. The results showed a significant difference for the tested treatment. A lower level of growth and no differentiation in growth dynamics were observed for bacterial cultures in mineral medium (MM) as a control medium without a carbon source. The maximum cell concentration was estimated at 4–4.5 log₁₀ CFU during the 96 h of incubation. In contrast, the wheysupplemented medium (MMW) supported significantly higher and more stable bacterial growth relative to the unsupplemented mineral medium. The addition of this ingredient to the medium corresponds with the highest cell concentrations, even doubling the improvement (9–12.5 log₁₀ CFU). What is more, the dynamic growth of bacteria was noticed. We observed an increase in cell concentrations during the incubation. For *P. polymyxa* (Sp116AC*), the maximum growth level was obtained after 72 h of incubation; longer incubation resulted in a decrease in live cell concentration. For *B. subtilis*: AB75AB2 and Sp115AD during the 96 h of incubation, a constant increase in growth was noted.

3.3. Stress-induced sporulation in bacterial cultures: temperature, salinity, and pH effects, interaction between components

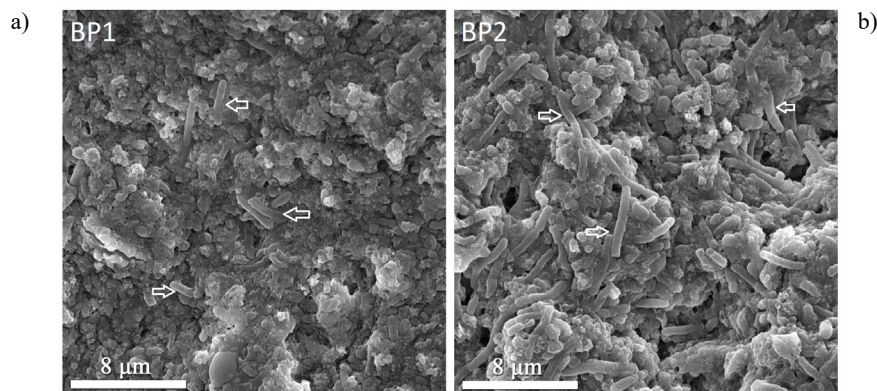
The most commonly used method for evaluating sporulation in bacterial cultures is colony counting on agar plates following pasteurization of the samples. The effectiveness of the sporulation stage under the tested conditions is visualized in Fig. 5. Three parameters were assessed: temperature, salinity, and pH. The optimal values of these parameters varied among the examined strains. The highest spore concentration exceeding 10⁸ CFU ml⁻¹ was observed along all bacterial isolates under pH stress (5.5 and 4.5) and salinity stress (2 and 4% KCl). Moreover, for *B. subtilis* (Sp115AD), the temperature stress was also effective, the increase in incubation temperature from 30°C to 40–45°C resulted in high spore concentrations. Lower sporulation level was observed under temperature stress (35°C).

The interactions between the formulation components and their antifungal properties were evaluated experimentally, and the results are summarized in Table 1, providing a basis for further refinement of biopreparation design.

Table 1. Microbial interactions among the biopreparation components and their antagonistic activity against phytopathogens. Inhibition zone diameter (mm) with 5 mm diameter of paper disk

Strain	Component of biopreparation				
	Nettle leaf extract	Horsetail herb extract	Calendula extract	Humic acids preparation	Vinasse
AF75AB2	n.i.	n.i.	n.i.	n.i.	n.i.
Sp115AD	n.i.	n.i.	n.i.	n.i.	n.i.
Sp116AC*	n.i.	n.i.	n.i.	n.i.	n.i.
<i>Phytophthora</i> sp.	40.33 ± 2.52	20.67 ± 2.08	n.i.	46.33 ± 17.04	n.i.
<i>Colletotrichum</i> sp.	59.17 ± 14.65	n.i.	n.i.	n.i.	n.i.
<i>Verticillium</i> sp.	n.i.	n.i.	n.i.	n.i.	n.i.
<i>Botrytis</i> sp.	n.i.	35.67 ± 12	n.i.	44.67 ± 9.29	n.i.

n.i. – no zone of inhibition.

**Fig. 6.** Scanning electron microscopy (SEM) images of the microstructure of biopreparations: a) BP1 and b) BP2. Scale bars for images are 8 µm in length.

It is assumed that the extract from nettle leaf, horsetail herb and calendula, as well as humic acids and vinasse, would not inhibit the growth of beneficial bacteria. In contrast, these components showed differential effects on the phytopathogens. Clear inhibition zones were observed for several combinations. The strongest inhibitory activity was recorded for nettle leaf extract, particularly against *Phytophthora* sp. and *Colletotrichum* sp. Additionally, horsetail herb extract and humic acid reduced both the growth and sporulation of *Phytophthora* sp. and *Botrytis* sp. These observations highlight the potential of selected natural components to contribute to the antifungal properties of the final biopreparation formulation.

3.4. Morphology and microstructure of bacteria in biopreparations

Microscopic methods (SEM and NANOLIVE) were used to examine the microstructure of the prepared biopreparations (BP1 and BP2). As demonstrated by the scanning electron microscopy (Fig. 6) and holotomography (Fig. 7) images, both biopreparations exhibit whole undamaged cells (marked with a white arrow in the images). The bacterial cells in BP1 and BP2 exhibited a regular morphology and a smooth, intact surface, indicative of an

undisturbed cell wall structure. Holotomography images clearly show single bacterial cells without leakage of intracellular contents. Additionally, SEM imaging demonstrated that the bacterial cells in biopreparations were surrounded by granules originating from residual substrate components, including whey, plant extract, and humic acid.

The experiments conducted in this study enabled the development of two biopreparations against phytopathogens, designated BP1 and BP2. Following formulation, the next step was to evaluate the efficacy of the final bioproducts in inhibiting pathogen growth and to determine appropriate storage conditions for maintaining their stability and activity. The effects of each biopreparation on pathogen growth under *in vitro* conditions are illustrated in Fig. 8, providing an initial indication of their potential performance and informing further biopreparation optimization.

The inhibition zones and reduction in sporulation (visible as mycelial clearings) observed on plates treated with the constructed biopreparations indicate that both formulations exhibited measurable antagonistic effects under *in vitro* conditions. Among them, BP1 exhibited the largest inhibition zone against *Colletotrichum* sp. (approximately 35 mm), with additional activity noted against the *Botrytis* sp. strain. More moderate effects were observed

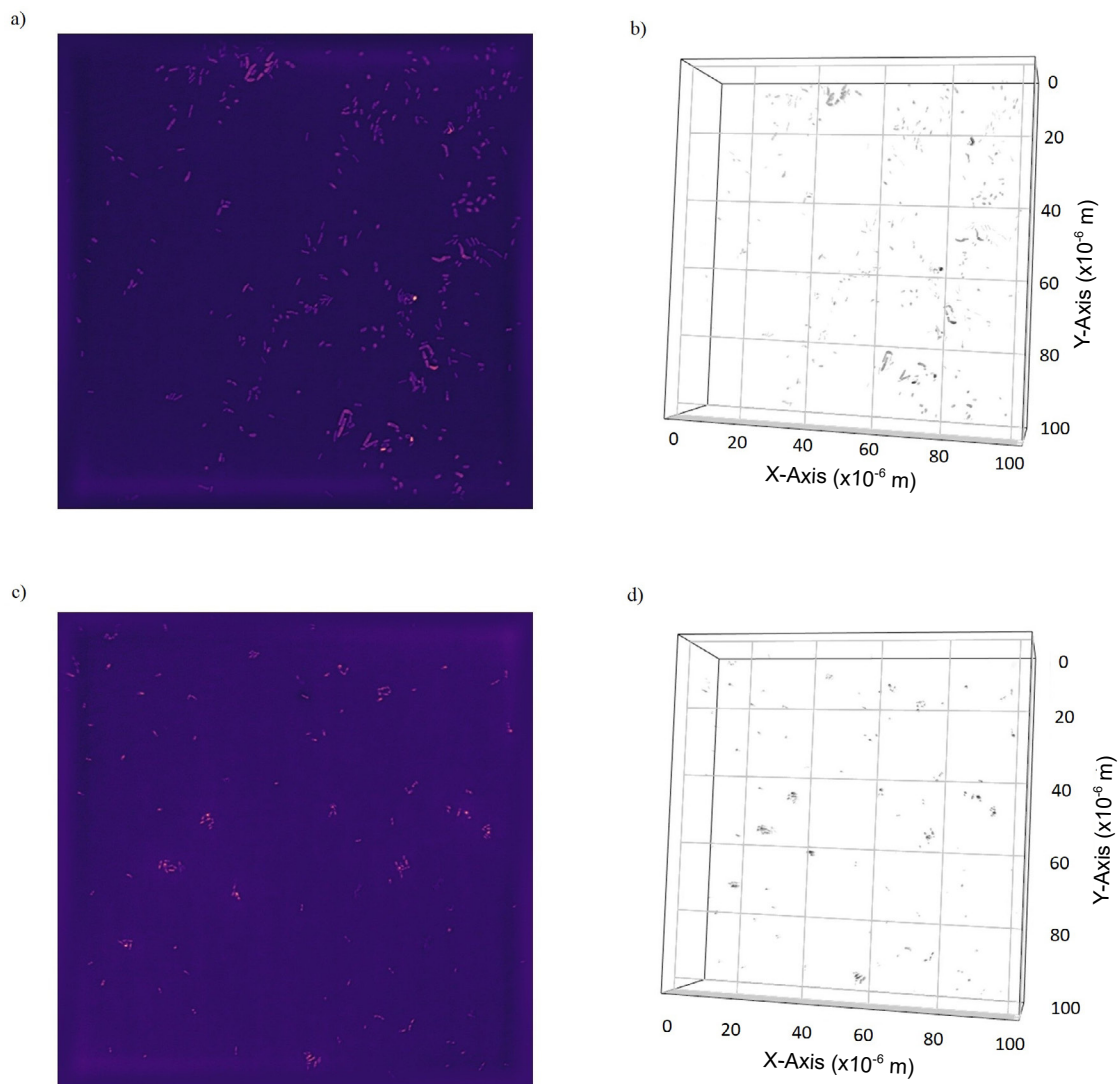


Fig. 7. Images of bacterial cells in biopreparations: a) and b) BP1, c) and d) BP2 acquired with the NanoLive 3D microscope. The 2D images a) and c) show an X-Y slice. The 3D images b) and d) represent the same sample under X-Y-Z angles.

against *Phytophthora* sp. and *Verticillium* sp., with inhibition zones of approximately 16 and 13 mm, respectively. In comparison, BP2 showed more pronounced activity against *Verticillium* sp. and *Botrytis* sp. strains. These results provide an initial indication of the differential antagonistic potential of the two formulations and form a basis for further biopreparation evaluation under more complex conditions.

The final formulated biopreparations (BP1 and BP2) were evaluated for antagonistic activity during a 6-month storage period at the following temperatures: 4, 35°C, and room temperature. The inhibition zones of plant pathogens' growth on agar plates were compiled on a heat map (Fig. 9). The results clearly indicate that both formulations maintain measurable antagonistic activity for up to 3 months,

with the most stable activity observed under refrigerated storage. After 3 months, preparations retain their antagonistic properties against all pathogens. After 3 months, preparation stored at room temperature or 35°C showed only a slight reduction in activity, particularly against representatives of *Verticillium*, *Botrytis*, and *Phytophthora* genera, while a more noticeable decline was observed for *Colletotrichum* sp. Beyond this period, 6-months storage led to a marked decrease in antagonistic activity for both BP1 and BP2 across all pathogens. These findings provide an initial indication of storage-related stability under controlled laboratory conditions and inform future optimization of biopreparation shelf-life.

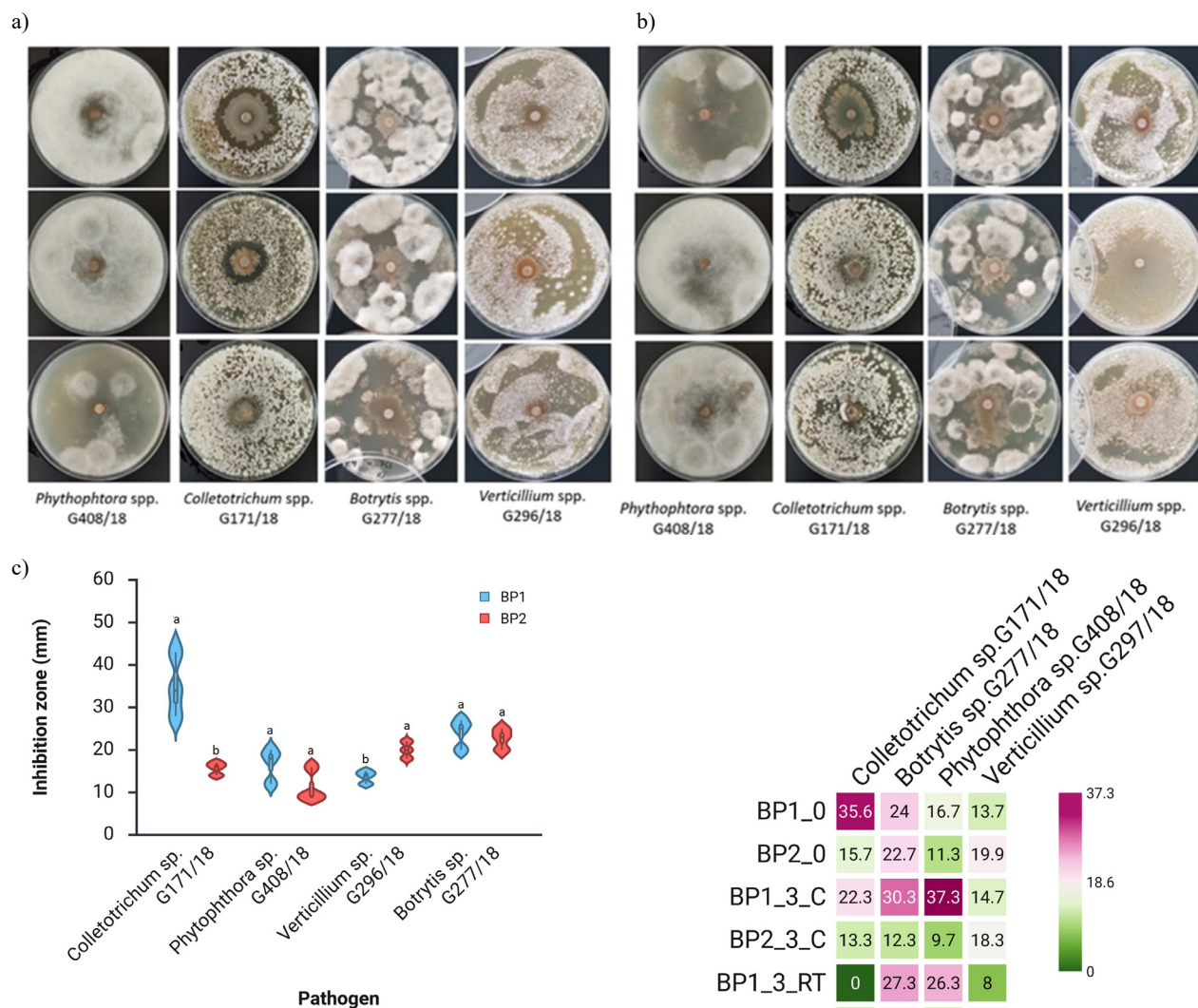


Fig. 8. Antagonistic activity of biopreparations against phytopathogens. Inhibition zone of pathogen growth on agar plate: a) biopreparation 1 (BP1), b) biopreparation 2 (BP2), c) violin graph with inhibition zone diameter (mm) with 5 mm diameter of paper disk. Small letters mean significant differences (results of the Tukey's HSD test at the 0.05 level).

4. DISCUSSION

The increasing demand for sustainable agricultural management has spurred interest in biopreparations as eco-friendly alternatives to chemical fungicides, particularly for ecological farming. The presented research is the effect of searching for effective solutions to problems related to pathogens of soft-fruit crops. In recent years, considerable global interest has focused on alternatives to agrochemicals, such as plant growth-promoting microorganisms and/or biostimulants. The potential for this type of bioproduct to expand rapidly and gain increasing relevance in future agriculture is evident. Moreover, advances in our understanding of the soil microbiome and plant holobiome have transformed current views on ecosystem functioning

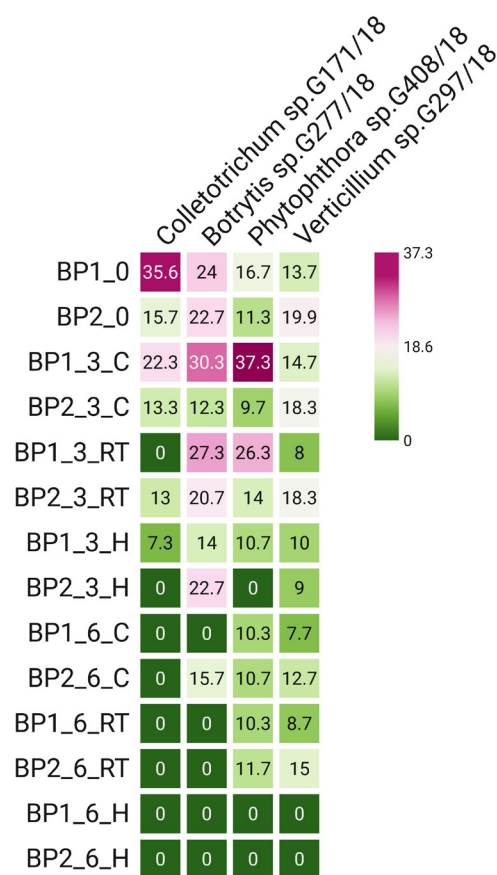


Fig. 9. Heat map visualization of antagonistic activity of biopreparations against phytopathogens. Inhibition zones of pathogen growth on agar plate for biopreparation 1 (BP1) and biopreparation 2 (BP2) during 6 – month storage under different temperature conditions. Inhibition zone diameter (mm) with a 5 mm diameter of paper disk. Explanations: 0 – biopreparation directly after production, 3 or 6 – 3 or 6 months storage, C – storage conditions 4°C, RT – storage conditions room temperature, H – storage conditions 35°C.

and enhanced the effectiveness of biological solutions. This progress has stimulated significant interest within both the biotechnology industry and the scientific community, particularly in the use of live microbial agents to promote plant growth and enhance resistance to biotic stresses, including pathogen infection. Beneficial bacteria can be classified into several functional groups. The Hamid *et al.* (2021) list as the most important microorganisms supporting plant growth the following bacteria: *Rhizobium* spp., *Pseudomonas* spp., *Azotobacter* spp., *Azospirillum* spp., and *Bacillus* spp. The recent research papers describe knowledge about the role of it in improving crops (Coelho *et al.*, 2020; Rouphael and Colla, 2020; Dellagi *et al.*, 2020). These findings formed the basis for selecting beneficial, antagonistic bacterial strains within our approach. In the presented study, we developed biopreparations using bacterial isolates obtained from soil. Because the research was conducted exclusively under *in vitro* laboratory conditions, the characterization focused on defining the biochemical properties of the isolates and their antagonistic activity, to identify their potential suitability as biological agents. These results provide an initial indication of their promise but require further validation under greenhouse and field conditions before any practical biocontrol application can be confirmed. Based on the genus-level identification obtained from WGS analysis, we confirmed that our isolates belong to the *Bacillus* and *Paenibacillus* genera. This verification establishes their affiliation with a group of soil bacteria widely recognized for their biocontrol potential and longstanding relevance in agricultural applications, supporting their suitability for further development as components of biopreparations. The *Bacillus* and *Paenibacillus* genera represent a highly interesting group of bacteria that have been extensively studied under both *in vitro* and *in vivo* conditions (Maake and Sibisi, 2025; Oszust *et al.*, 2023; Tariq *et al.*, 2025). These genera are known as beneficial strains, however, each isolate could have specific properties. For this reason, we evaluated our isolates based on biochemical, enzymatic, and antifungal activity. The combination of methods such as phenotypic profiling (BIOLOGTM System microplate) and API-ZYM, as well as antagonistic tests on agar plate were used and allowed for the characterization of the potential activity and sensitivity of the isolated strain. The Biolog GEN III system and API ZYM assays provided a comprehensive functional characterization of the strains. They allowed to evaluate traits that are essential for effective colonization of plant-associated niches, competition with phytopathogens, production of enzymes and metabolites involved in antagonism, survival and activity under field-relevant fluctuating conditions. Moreover, phenotypic profiling with the BIOLOG GEN III plate shows not only biochemical activity but also chemical sensitivity of isolates. In our research, we detected that carbon sources, especially carbohydrates, were favorably metabolized by the tested *B. subtilis* (AF75AB2, SP115AD) and *P. poly-*

myxa (Sp116AC*) strains. It could be assumed that the studied strains metabolized carbohydrates intensively and exhibited high adaptation to utilize these carbon sources. However, other carbon substrates were also utilized (*e.g.*, organic acids, amino acids), efficient utilization of diverse plant-derived substrates (*e.g.*, sugars, organic acids, amino acids) indicates a potential strain's ability to colonize the rhizosphere or phyllosphere, where nutrient availability fluctuates. Knowledge of the strains' capacity to utilize diverse carbon sources is valuable for the development and optimization of culture media used in the biotechnological production of the biopreparation. Such information guides the selection of substrates that promote efficient and stable bacterial growth (Bonnet *et al.*, 2019; Di Caprio, 2021). Furthermore, knowledge about the preference for metabolizing substrates could be useful in developing supplements for bacteria in biopreparations. This type of supporting and supplementing is working like probiotics. The addition of substrates that are favorably used by beneficial microbes will increase their adoption in the soil environment through enhancing the proliferation, physiological activities, and improvement of microecological and enzyme balance (Du *et al.*, 2021). This mechanism is very effective and positive, and enhances the activity of biopreparation. In addition to the phenotypic characterization obtained from the GEN III plates, the genomes of the tested strains were analyzed for the presence of antibiotic-resistance determinants. These preliminary genomic assessments did not reveal any resistance genes. Moreover, all strains were sensitive to a broad spectrum of antimicrobial compounds, which represents an additional advantage for their use in technological processes and agricultural applications. In the next aspect under consideration, bacterial strains intended for agricultural applications should exhibit a certain degree of tolerance to salinity or pH fluctuations, as these traits are essential for adapting to variable environmental conditions. The three strains selected for this study demonstrated high tolerance to 1% salinity, with slightly reduced, but still measurable tolerance to 4 and 8% salinity. Similar results were obtained by Woźniak *et al.* (2019) in studies focused on endophytic bacteria that potentially promote plant growth. These parameters are very important because the salinity stress has a negative effect on crops and agriculture (Numan *et al.*, 2018). What is more, the use of biopreparations based on salt-tolerant bacteria would be another advantage and could be helpful in the mitigation of salt stress in agriculture.

Selected strains were also evaluated based on enzymatic activity (API-ZYM approach). This test revealed positive enzymatic activity of each isolate with alkaline and acid phosphatase, esterase, esterase lipase, naphthol-AS-BI-phosphohydrolase, and β -glucosidase. This enzymatic activity was also detected by Park *et al.* (2021) in studies on antifungal activities of *Bacillus* isolates. The hydrolase activities observed in the tested strains, especially esterase, play an important role in the degradation

of various soil pollutants (Gangola *et al.*, 2018). Moreover, this enzymatic potential may positively influence key soil processes, including the solubilization of organic and inorganic compounds as well as the decomposition of cellulose and lignin. Considering these advantages, together with the biochemical profiles obtained for the isolates, the selected strains appear to be strong candidates for use as beneficial microorganisms in agricultural applications. However, our primary objective was to develop bioproducts capable of protecting plants against fungal and oomycete pathogens. Pathogen diseases, including anthracnose, grey mold diseases, or verticillioses, pose major challenges for crop production. Soft-fruit crops are highly susceptible to fungal and oomycete infections due to their close contact with the soil (Malarczyk *et al.*, 2019). Although chemical fungicides are commonly used to manage these diseases, their excessive application has raised concerns regarding environmental impact and human health. Consequently, natural products and microbial agents are increasingly explored as alternatives to synthetics. In this context, we evaluated the *in vitro* antagonistic activity of two *B. subtilis* (AF75AB2, Sp115AD) and one *P. polymyxa* (Sp116AC*) isolates against four important phytopathogens. Previous studies have shown that microbial agents such as *Bacillus subtilis* exhibit strong inhibitory effects against *Colletotrichum acutatum* (Hong *et al.*, 2015; Barra Bucarei and Ortiz, 2020). Our results are consistent with these findings, as all tested strains demonstrated substantial antagonistic activity, with inhibition zones ranging from 13 to 52 mm depending on the bacterial isolate and phytopathogen. Among these interactions, the particularly large inhibition zone observed against *C. acutatum* (up to 52 mm) indicates a strong strain-specific antagonistic response under the conditions used in our assay. This value exceeded that reported by Deng *et al.* (2023) for *Bacillus velezensis* FJAT-52631 against *C. acutatum* FJAT-30256. However, inhibition-zone diameters reported in different studies should not be interpreted as directly comparable measures of antagonistic efficacy, because they may be strongly influenced by bacterial and pathogen strains, inoculum density, culture medium, incubation conditions, and assay design. Broad-spectrum antagonistic activity of *Bacillus* strains against *Colletotrichum*, *Verticillium*, *Botrytis*, and *Phytophthora* has also been reported by Drobek *et al.* (2021). The pronounced inhibition observed in our study may reflect the combined contribution of several antagonistic mechanisms commonly associated with *Bacillus*, including antimicrobial metabolite production, extracellular hydrolytic enzymes, and competition for nutrients and space (Kim *et al.*, 2015; Zhang *et al.*, 2023). Nevertheless, these mechanisms were not directly quantified in the present study, and their contribution to the observed inhibition therefore remains to be experimentally verified.

In contrast to bioproducts based on biological components such as bacteria or fungi, synthetic fungicides remain widely used in crop protection. Commercial products including cyprodinil, fludioxonil, and phthalimide-based fungicides are commonly applied to control a broad spectrum of plant pathogens, particularly *Botrytis* spp. (grey mold), *Colletotrichum* spp. (anthracnose), and *Phytophthora* spp. (phytophthora). Beger *et al.* (2024) reported a comparative evaluation of chemical fungicides and biological products in controlling fungal pathogens. Their results demonstrated that synthetic fungicides were the most effective in suppressing disease development. However, they also noted that *Bacillus* based biopreparations reduced *Botrytis cinerea* infection by approximately 35%, highlighting their potential as complementary tools in integrated diseasemanagement strategies. Future studies should therefore explore the combined or synergistic effects of biopreparations with reduced or minimal doses of chemical fungicides.

In summary, although the isolates demonstrated promising antagonistic traits under *in vitro* conditions, it is important to recognize that the final activity and behavior of microbial agents in complex environments such as soil depend strongly on the overall physical, chemical, and biological properties of the habitat. These environmental factors can influence microbial survival, competitiveness, and interaction with both pathogens and native microbiota. Nevertheless, the detailed characterization of the isolates' abilities provides a well-supported foundation for advancing these strains into further stages of investigation.

4.1. Development of biopreparations

The second part of that work was focused on the development of a biopreparation and its production procedure. In this phase, the composition of liquid growth medium, sporulation conditions, and formulation of the biopreparation were evaluated. According to results on strain cultivation, the highest biomass yield was achieved using a mineral medium enriched with whey. The dynamic culture experiment showed that maximum growth was reached after 72 to 96 h of incubation. Using whey as a medium supplement is beneficial for this assay. Whey, generated as a waste product from dairy processing, was incorporated into the mineral medium to enhance its nutritional value and support bacterial growth. As a natural source of lactose, peptides, amino acids, vitamins, and minerals provides both available carbon and organic nitrogen, thereby improving the nutritional profile of the otherwise minimal medium. Its composition supports robust microbial proliferation without the need for costly commercial supplements such as peptone or yeast extract. From an economic perspective, whey represents a lowcost and sustainable substrate, as it is abundantly available and typically treated as industrial waste. Incorporating whey into the culture medium therefore reduces overall production costs while simultaneously

contributing to waste valorization within the dairy industry (Kumar *et al.*, 2012). In biotechnology processes, employing inexpensive substances, particularly industrial waste products, is a valuable strategy.

The bacteria are characterized by optimal growth and metabolic parameters, including medium composition, pH, temperature range, and salinity. However, under stress conditions, some bacteria can form spores, which allow them to survive in unfavorable environments. Spores are more resistant than vegetative cells and provide a more stable form for storage. *Bacillus* and *Paenibacillus* isolates, as mesophilic spore-forming bacteria, are suitable for industrial biotechnological applications (Setlow and Christie, 2023). To determine spore formation in the liquid growth medium, the effects of temperature, pH, and salinity were studied. The pH of the medium is closely linked to the charge state of the bacterial cell membrane, thereby affecting its permeability, which ultimately influences nutrient absorption and metabolite production by bacteria (Branco *et al.*, 2015; Potočník *et al.*, 2019; Jiang *et al.*, 2020). Under the following conditions, 4.5–5.5 pH value, 2 and 4% salinity, the number of spores produced by bacteria was the largest (CFU ml⁻¹ > 10⁸) for each bacterial strain. The *Bacillus* strain Sp115AD forms a high number of spores (CFU ml⁻¹ > 10⁸) even at increased temperatures of 40–45°C and 1% salinity. These methods for obtaining high spore concentrations are also beneficial for the economic aspects of production.

4.2. Final formulation of agricultural biopreparations

The development of a final formulation for biological products intended for agricultural application presents multifaceted challenges within biotechnology processing. The culturing and formulation stages are relevant in synthesizing components into a final biopreparation suitable for agricultural applications. An effective formulation must achieve several goals: ensuring cost-effectiveness, preserving the biological activity of ingredients, and maintaining product stability for a minimum shelf life of 6 months. Another challenge is that the final product must be user-friendly and enable accessible handling and use in agriculture. Among the available formulation types, liquid-based formulations are considered promising due to their ease of use (Trujillo Roldan *et al.*, 2013). Additionally, biopreparations, which are dedicated to protecting plants from pathogens, should be applied to the whole plant (roots and leaves especially). Due to this, the liquid formulation of biopreparation could be suitable for seed or root coating, foliar spray, and soil amendment. The main advantages of this type of formulation are: i) extended shelf life (especially when a suspension of spores is used); ii) high cell/spores density; and iii) ease of application (foliar spray or soil drenching). However, there are also several notable drawbacks, such as: i) microbial overgrowth risk; ii) difficulties in transport and storage; and iii) the sedimentation of ingredients may cause errors

in measuring volumes and doses. The formulation of agricultural biopreparations is a critical factor influencing their effectiveness, stability, and environmental compatibility. In this study the biopreparations were designed to combine functionally active bacterial strains with natural components known for their beneficial properties in suppressing phytopathogens. The selected formulation ingredients nettle leaf extract, horsetail herb extract, calendula extract, humic acids, and vinasse, were chosen for their compatibility with microbial systems. An additional key requirement was that the of organic nutrients, herbal extracts, and bioactive compounds must maintain bacterial viability throughout the formulation process. Our results demonstrated that the proposed technological process for producing biopreparations is safe for bacterial cells and ensures their viability and activity. Microscopic visualization confirmed the presence of intact, undamaged cells within the final formulations. These observations indicate that the bacterial cells successfully survived the biotechnology production and remain in an expected form in the final product. This confirms that the selected process parameters and formulation components are appropriate for obtaining a stable and biologically active biopreparation. Importantly, the application of microscopic analysis in this context represents a novel and valuable approach for evaluating biopreparation quality. While viability tests are commonly performed using culture-based or biochemical methods, direct microscopic assessment provides unique insight into cell morphology, structural integrity, and the physical interactions between bacterial cells and formulation components. The combined use of SEM and holotomographic imaging represent a useful methodological approach for quality assessment during microbial formulation development. While SEM provides high-resolution information on cell and spore morphology and their association with formulation components, holotomography enables visualization of intact bacterial cells in a liquid environment with minimal sample processing. The complementary application of these techniques may therefore help detect formulation-induced structural alterations that are not captured by conventional viable-count measurements alone. Microscopic assessment could complement culture-based viability and functional assays during formulation optimization and stability testing, thereby enhancing the reliability of quality control and supporting the development of reproducible, highperformance biopreparations.

A key novelty of our approach is based on the integration of two complementary components: the microbiological element, represented by the selected bacterial isolates, and the bioactive plant-derived compounds, such as herbal extracts and humic substances or vinassa. The incorporation of plant extracts into biopreparation formulations represents an innovative direction in the development of biological plantprotection products. The use of plantderived extracts introduces an additional potential functional dimension, as these extracts contain diverse

bioactive metabolites with antimicrobial, antioxidant, or stimulatory properties. Importantly, the biological activity of plant extracts is highly dependent on the extraction methodology, including parameters such as solvent composition, extraction temperature, duration, and the physicochemical characteristics of the plant material (Melikoglu, 2026). These factors determine the qualitative and quantitative profile of active compounds and, consequently, the performance and stability of the final formulation. In the present study, we provide a detailed description of the extraction procedure and demonstrate how the resulting extract can be effectively incorporated into microbial biopreparation. Establishing a standardized and reproducible extraction protocol is essential for obtaining a stable, high-quality product with consistent biological value. Such methodological transparency is crucial for ensuring repeatability, enabling future optimization, and supporting the development of next-generation biopreparations that integrate both microbial and plant-derived components. For example, plant extracts have previously been incorporated into biopreparation aimed at controlling *Botrytis cinerea* (Pylak *et al.*, 2019; Narwade *et al.*, 2023). In studies focused on antifungal activity, neem extracts, citronella oils, and garlic extracts were evaluated by Marwal *et al.* (2022), Steglińska *et al.* (2022), and Chakraborty *et al.* (2023). In our work, we demonstrated that extracts derived from nettle leaves and horsetail herbs also exhibit antimicrobial properties. Importantly, we confirmed that these extracts do not inhibit the growth of beneficial bacteria used in the biopreparation. This compatibility indicates that the microbial component and the plant-derived active compounds can be safely combined within a single formulation, supporting their joint use in developing integrated biological *Bacillus*-*Paenibacillus* bioproducts targeting phytopathogens. The plant-derived ingredients used in the formulation should be understood as complementary components rather than agents that necessarily enhance antimicrobial activity. Their primary role is to support the formulation matrix and improve its technological properties without suppressing the beneficial bacterial strains that constitute its biologically active core.

The final formulated biopreparations (BP1 and BP2) were evaluated for their antagonistic activity. However, in the present study the compatibility of the bacterial strains within BP1 and BP2 was not evaluated using dedicated pairwise cross-inhibition or co-inoculation assays. Therefore, although the final formulations retained antagonistic activity against the tested phytopathogens, these results cannot distinguish whether interactions between the constituent strains are synergistic, additive, or competitive. Future studies should include pairwise compatibility assays and strain-specific monitoring during co-culture and storage to determine population dynamics and functional stability within the microbial consortia. Additionally, a reduction in the shelf-life of liquid microbial products is a well-recognized limitation in biopreparation processing

as an industrial standards typically require a minimum of six months of stability and biological activity. Our results indicate a stability profile, demonstrating that the biopreparations can be stored at either room temperature or 4°C for up to three months without significant loss of viability or antagonistic potential. Although some studies report shelf-life of one to two years for certain commercial bioproducts stored at ambient temperature (Selladurai *et al.*, 2025; Deaker *et al.*, 2004; Catroux *et al.*, 2001; Teixidó *et al.*, 2020), achieving such extended stability generally requires additional formulation strategies. Further optimization will therefore be necessary to prolong the storage period of our preparations.

5. CONCLUSIONS

This study provides a grounded framework for the development of environmentally compatible biopreparations targeting phytopathogenic fungi and oomycetes. The increasing demand for sustainable plant protection strategies requires deeper and detailed insight into how environmental microorganisms regulate pathogen infection and interact within complex agroecosystems.

We demonstrated that tested *Bacillus* spp. and *Paenibacillus* sp. strains exhibit strong antagonistic activity against major plant pathogens. Their antagonistic efficacy is associated with metabolic versatility, defined enzymatic profiles (phosphatases, esterases, glucosidases), and tolerance to physicochemical stress. Controlled induction of sporulation under optimized pH, salinity, and temperature conditions enabled the production of stable, high-density spore suspensions ($>10^8$ CFU ml⁻¹), ensuring functional durability of liquid formulations. We tested that plant-derived extracts, humic acids, and vinasse might be ingredients of the final biopreparation formulation due to their potential properties and ability to inhibit pathogen growth while preserving bacterial viability. Microscopic analyses confirmed the structural integrity of bacterial cells within biopreparation formulations, while macroscopic observations indicated alterations at the pathogen-bacterium interface, suggesting interference with hyphal organization and infection potential. The formulated biopreparations retained significant antagonistic activity, particularly against *Colletotrichum* sp. and *Botrytis* sp., and remained effective for up to three months under practical storage conditions. All formulations were based on non-GMO microbial strains and natural components, making them suitable for potential use in ecological and organic crop management systems tests. However, their practical application still requires additional testing under greenhouse and field conditions, as environmental factors may influence their activities. These results therefore provide a promising foundation for further evaluation of the biopreparations as candidates for sustainable biological plant protection. The integrative approach presented here bridges

microbial ecology, functional characterization, and formulation engineering, contributing to the field of environmental microbiology and pathogen interaction research.

Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions. Research concept and design: A.G., M.F.; Collection and assembly of data: A.G., M.F., K.O., K.S., M.M., J.P.; Data analysis and interpretation: A.G., M.F., K.O., K.S., J.P.; Funding Acquisition: M.F.; Project Administration: M.F., L.S.P.; Writing the article: A.G.; Critical revision of the article: M.F., K.O., M.M., L.S.P., K.S., J.P., A.G. Final approval of the article: M.F., K.O., M.M., L.S.P., K.S., J.P., A.G. All authors have read and agreed to the published version of the manuscript.

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