The biocontrol agent Lactiplantibacillus plantarum AMBP214 is dispersible to plants via bumblebees

The biocontrol agent *Lactiplantibacillus plantarum* AMBP214 is dispersible to plants via bumblebees

Jari Temmermans[1*], Marie Legein[1*], Yijie Zhao[2,3], Filip Kiekens[4], Guy Smagghe[5], Barbara De Coninck[2,3], Sarah Lebeer[1,#]

[1] Laboratory of Microbiology & Biotechnology, Department of Bioscience Engineering, Antwerp University, Groenenborgerlaan 171, 2020 Antwerp, Belgium (jari.temmermans@uantwerpen.be; marie.legein@uantwerpen.be; sarah.lebeer@uantwerpen.be)

[2] Laboratory of Plant Health and Protection, Department of Biosystems, KU Leuven, Willem de Croylaan 42, 3001 Leuven, Belgium.

[3] KU Leuven Plant Institute, 3001 Leuven, Belgium.

[4] Laboratory of Pharmaceutical Technology and Biopharmacy, Department of Pharmaceutical Sciences, Antwerp University, Universiteitsplein 1, 2610 Wilrijk, Belgium (filip.kiekens@uantwerpen.be)

[5] Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium (guy.smagghe@ugent.be)

[#] Corresponding author

* Shared first authors Jari Temmermans and Marie Legein contributed equally to this work. Author order was determined via a random sample function in R.

Abstract

Microbial biocontrol agents have emerged as a promising alternative for the management of plant diseases and the reduction of chemical pesticide dependence. However, a significant challenge in using these agents is their inconsistent performance under field conditions, often caused by the poor

establishment and limited spread of the microorganisms. Entomovectoring, a system where microbial biocontrol agents are dispersed to crops via pollinators, provides a potential solution to these challenges. Still, there are limited examples of successful systems, and no studies have tested this technology with versatile and generally beneficial lactobacilli. Here we demonstrate that *Lactiplantibacillus plantarum* AMBP214 shows potential as a biocontrol agent displaying antimicrobial activity in an *in vitro* and a gnotobiotic seedling experiment, and the ability to establish itself in strawberry flowers. However, AMBP214 was ineffective in protecting strawberry flowers against *Botrytis cinerea* in a greenhouse trial. An innovative formulation and dispersal strategy was proven successful with this strain as it could be formulated into a spray-dried powder that could be loaded onto bumblebees via a dispenser. When loaded bumblebees were released into a greenhouse with strawberry plants, *L. plantarum* AMBP214 was effectively dispersed to flowers, resulting in high bacterial abundances (on average 1 x 10^5 CFUs per flower) and consistent coverage across all sampled flowers. This is the first report of using spray-dried lactobacilli with entomovectoring, providing a novel and promising approach to biocontrol. These results pave the way for further research and the development of spray-dried non-spore-forming bacteria in entomovectoring strategies, which hold great promise for enhancing plant health and mitigating the negative impacts of plant diseases.

Importance

Plant protection products are essential for ensuring food production, but their use poses a threat to human and environmental health, and their efficacy is decreasing due to the acquisition of resistance by pathogens. Stricter regulations and consumer demand for cleaner produce are driving the search for safer and more sustainable alternatives. Microbial biocontrol agents, such as microorganisms with antifungal activity, have emerged as a promising alternative management strategy but their commercial use has been limited by poor establishment and spread on crops. This study presents a novel system to overcome these challenges. The biocontrol agent *Lactiplantibacillus plantarum* AMBP214 was spray-dried and successfully dispersed to strawberry flowers via bumblebees. This is the

first report of combining spray-dried, non-spore-forming bacteria with pollinator-dispersal which scored better than the state-of-the-art in terms of dispersal to the plant (CFU/flower), and resuscitation of the BCA. Therefore, this new entomovectoring system holds great promise for the use of biocontrol agents for disease management in agriculture.

1 Introduction

Microbial biocontrol agents, microbes that protect plants against diseases, are promising sustainable alternatives to chemical pesticides in the management of pests and diseases in agriculture. The use of biocontrol agents can help reduce the negative impacts of chemical pesticides on the environment and human health, while also improving crop yields. However, current biocontrol products often yield variable and unpredictable results in field conditions, which is often attributed to the poor establishment and limited dispersal of the biocontrol agent (1, 2, 3). Second, working with live microorganisms as biocontrol agents imposes specific constraints that may not always align with the routine operations and management practices of a farm. An elegant solution for both these problems could be provided by entomovectoring, a system where microbial biocontrol agents are dispersed to the crops via pollinators.

Entomovectoring has the potential to improve the efficiency and effectiveness of biocontrol by targeting the pathogen at the site of infection, reducing the need for repeated applications, and increasing the persistence of the biocontrol agent on the plant. Such a system is especially interesting for the biocontrol of *Botrytis cinerea*, the causal agent for grey mould, as this pathogen primarily infects the flowers, leading to disease symptoms on the fruit. In strawberry plants, flowers only stay open for approximately three days and new flowers are budding throughout the season, making timely biocontrol delivery a challenge.

Already in the 1990s, researchers experimented with the delivery of *Gliocladium roseum* to raspberry flowers for the control of *Botrytis cinerea* (4, 5). More recently, the commercial product Flying Doctors

(Biobest, Westerlo, Belgium) combines bumblebees with the fungus *Gliocladium catenulatum* strain J1446 (Prestop®) to control *Botrytis cinerea*. Other entomovectored biocontrol agents are fungi such as *Trichoderma harzianum* (6, 7, 8, 9) and *Aureobasidium pullulans* (10), or bacteria from the *Bacillus* genus (11). However, to the best of our knowledge, no lactic acid bacteria have been tested as biocontrol agents in combination with entomovectoring. These bacteria often have an inhibitory impact on pathogens due to their capacity to acidify the environment and the production of antimicrobial metabolites and reactive oxygen species (14). Moreover, these bacteria are typical members of the pollinator microbiome such as bumblebees (12) and were found to be dispersed by bumblebees to tomato plants in greenhouses (13). Finally, a significant advantage of lactic acid bacteria is that they often have, or easily acquire a QPS label, and are Generally Regarded As Safe (GRAS).

In this study, *Lactiplantibacillus plantarum* AMBP214, isolated from the leaves of a cistus rose (*Cistus ladanifer*), was assessed as a biocontrol agent. We focused on AMBP214's efficacy against several plant pathogens and its potential to be entomovectored by bumblebees (*Bombus terrestris*). First, antimicrobial properties against *Botrytis cinerea* and *Alternaria alternata* were assessed *in vitro* and its activity against *Pseudomonas syringae* DC3000 was assessed in a gnotobiotic seedling experiment. Next, *L. plantarum* AMBP214 was spray-dried and formulated. The number of spray-dried bacteria that could be loaded onto bumblebees as well as their dispersal to strawberry flowers pollinated by these bumblebees, was quantified, and the performance of this application was compared to commercial benchmarks and previous entomovectoring trials. Finally, the antimicrobial properties of AMBP214 were tested *in vivo* against *B. cinerea* in a greenhouse setup.

2 Materials and methods

2.1 Microbial strains and culture conditions

An overview of all microbial strains used in this study is given in **Table 1.** *Lactiplantibacilli* were grown at 28 °C or 30 °C, shaking at 135 rpm, in de Man, Rogosa and Sharpe (MRS) broth (Difco, Le Pont de

Claix, France). Commercial biocontrol agent *Pantoea agglomerans* p10c, used in the *in vitro* assays, was resuspended from the commercial product Blossom Bless™ (AgriNova NZ Ltd, New Zeeland) in sterile water. Commercial biocontrol strain *P. agglomerans* E325, used in the seedling assay, was grown at 28 °C, shaking at 135 rpm in Luria-Bertani (LB) broth (Fischer BioReagents, Pittsburgh, USA). The bacterial pathogen *Pseudomonas syringae* DC3000 was grown at 28 °C, shaking at 135 rpm in King's B (KB) medium (10 g/L glycerol, 20 g/L Bacto proteose peptone (no. 3; Gibco, New York, USA), 1.5 g/L $K_2HPO_4 \cdot 3H_20$, 1.5 g/L $MgSO_4 \cdot 7H_2O$) or Reasoner's 2A (R2A) Medium (Roth, Karlsruhe, Germany). The fungal pathogens *Botrytis cinerea* B05.10 and *Alternaria alternata* MUCL 1852 were grown on potato dextrose agar (PDA) in an adjusted formulation (7.5 g agar + 19.5 g potato dextrose broth (PDB; Difco, Le Pont de Claix, France)). The fungi *B. cinerea* and *A. alternata* were incubated at 25 °C in the dark. For the greenhouse trial, *B. cinerea* strain B05.10 was cultivated on PDA for 5 d in the dark at 25 °C, then exposed to UV-A (315 nm-400 nm) for 12 h and subsequently allowed to sporulate for 5-9 d in the dark.

Table 1: Bacteria and fungi used in this study, their taxonomy and NCBI assembly accession if their genome was used in this study.

Strain	Taxon	Comments	Reference
AMBP214	*Lactiplantibacillus plantarum*	Leaf of a cistus rose, Portugal	This study
WCFS1	*Lactiplantibacillus plantarum*	Human saliva	Model lab strain
B05.10	*Botrytis cinerea*	Plant pathogen	Model lab strain, obtained from Prof. De Coninck
MUCL1852	*Alternaria alternata*	Plant pathogen	Model lab strain
DC3000	*Pseudomonas syringae*	Plant pathogen	Model lab strain, obtained from Prof. Koskella
P10c	*Pantoea agglomerans*	Commercial biocontrol agent	Blossom Bless™ (AgriNova NZ Ltd, New-Zeeland)
J1446	*Gliocladium catenulatum*	Commercial biocontrol agent	Prestop® 4B (Biobest, Westerlo, Belgium)
E325A	*Pantoea agglomerans*	Biocontrol agent	(15)
QST713	*Bacillus amyloliquefaciens*	Biocontrol agent	Serenade (Bayer Cropscience AG, Germany)

2.2 In vitro antimicrobial activity against *Botrytis cinerea* and *Alternaria alternata*

To examine the inhibitory activity of *L. plantarum* AMBP214 against *B. cinerea* or *A. alternata*, the bacteria were incubated on a PDA plate together with the fungi after which the fungal mycelium radius was measured. An overnight culture of *L. plantarum* AMBP214 was diluted with phosphate-buffered saline (PBS) to a concentration of 1×10^8 CFUs/ml and 20 µL of this dilution was spotted in a square

115 configuration on PDA. This assay was also performed with spray-dried *L. plantarum* AMBP214, for
116 which the powder was resuspended in sterile PBS in a concentration of 1 mg/ml, corresponding to 2 x
117 10^8 CFUs/ml, and also spotted in 20 µl on PDA. As a positive control, the commercial biocontrol agent
118 *Pantoea agglomerans* P10c was used. This commercial biocontrol powder was resuspended in sterile
119 water (0.1 mg/µl) to obtain a 1×10^8 CFUs/ml suspension. Next, the fungi were added to the plates.
120 Different incubation times were used for the assay with *B. cinerea* B05.10 and *A. alternata* MUCL 1852
121 due to the different growth rates of the fungi. For *B. cinerea*, the plates with bacterial spots were
122 incubated at 25 °C or 28 °C (depending on the experiment) for three days before *B. cinerea* inoculation.
123 For *A. alternata*, plates were inoculated with bacteria and fungus on the same day. Fungal inoculation
124 was done via a mycelium plug, excised with a sterilized cork borer from the edge of the mycelium. For
125 *B. cinerea*, the plug was taken from a three-day-old culture on PDA, and for *Alternaria* from a 10-day-
126 old culture on PDA. The plugs were placed in the middle of four bacterial spots of 20 µL on the PDA
127 plates. In addition, plates without bacteria were also inoculated with mycelial plugs and acted as
128 controls. Plates were further incubated at 25 °C in the dark. The fungal radius was measured after four
129 days for *B. cinerea* and six days for *A. alternata*. The percentage decrease in the radius of the fungal
130 mycelium in the plates with bacteria, compared to control plates without bacteria was calculated. To
131 determine which bacteria significantly reduced fungal growth, a Dunnett's test was used, comparing
132 the fungal radius in the presence of an isolate to the radius in the absence of bacteria.

133 The above method was adapted slightly to assess the antimicrobial activity of cell-free supernatant
134 and pH-adjusted cell-free supernatant of *L. plantarum* AMBP214 against *B. cinerea* and *A. alternata*.
135 Cell-free supernatant was obtained by centrifuging an overnight culture (2500 × g for 10 min at 4 °C),
136 followed by filter sterilization (pore size 0.2 µm, VWR, Radnor, USA). Subsequently, instead of pipetting
137 four spots of 20 µl on the PDA plates, wells were made in the plates using a sterile cork borer and these
138 were filled with 30 µl of the cell-free supernatant. Finally, the fungal plug was placed in the middle. As
139 in the previous experiment, the fungal radii were measured after three days for *B. cinerea* or six days

for *A. alternata*. This assay was also performed with cell-free supernatant that was neutralized to pH 7.0 by adding NaOH (original pH was between 3.7 and 4.1).

2.3 Antimicrobial activity against *Pseudomonas syringae* D3000 on tomato seedlings

Tomato seedling preparation

Biocontrol activity against model pathogen *P. syringae* DC3000 on tomato seedlings was assessed based on a protocol described by Morella and colleagues (15). Tomato seeds (*S. lycopersicum* cultivar Moneymaker) were surface-sterilized in 70 % ethanol for 1 min, followed by a 20 min soak on an orbital shaker, in sterilization solution (one part 8.25 % bleach, two parts 0.2 % Tween 20 (Sigma-Aldrich, Missouri, USA) in water). Seeds were then washed four times in 45 ml autoclaved MilliQ water and placed in loosely capped sterile 15 ml tubes with 7 ml water agar (one seed per tube). Tube racks were placed in a dark 21 °C chamber and checked daily for signs of germination. After shoot emergence, tubes were moved to a 28 °C chamber with a 15 h light/ 9 h dark cycle.

Seedling inoculation and disease severity scoring

Bacteria were harvested from an overnight culture by centrifugation (2500 x g for 10 min at 4 °C), washed once in sterile 10 mM $MgCL_2$ and resuspended in $MgCL_2$. Suspensions were diluted to approximately 1×10^4 CFUs/ml. Finally, 0.015% (v/v) sterile Silwet (De Sangosse Ltd, Newmarket UK), a wetting agent, was added to the bacterial inoculant. Subsequently, the seedlings (7 to 12 days old) were inoculated with the resuspended bacteria. Four conditions were tested, positive control (*Pantoea agglomerans* E325A), negative control (NC, sterile $MgCl_2$ buffer + 0.015 % silwet), *L. plantarum* AMBP214 (biocontrol strain of interest), and *L. plantarum* WCFS1 (model lab strain closely related to AMBP214). The seedlings were flooded with 7 ml of inoculant and placed on an orbital shaker at room temperature for 4 min. Next, the inoculant was poured out and the tubes with seedlings were left open to dry in a biosafety cabinet for approx. 1 h. These seedlings were then incubated in a growth chamber at 28 °C with a 15 h light/9 h dark cycle. After three days, seedlings were inoculated with the pathogen

P. syringae DC3000 by the same flooding procedure (approx. 1×10^4 CFUs/ml with 0.015 % (v/v) silwet). Sterile buffer was used as a negative control. Seedlings were placed back in the growth chamber and disease symptoms were scored blindly, every day for 10 days using the same indices as described by (15) (1 = mildly diseased, showing only a few necrotic areas, 2 = moderately diseased showing multiple necrotic areas or loss of one leaf, 3 = severely diseased or loss of both leaves, and 4 = death). Each treatment consisted of 7 replicates, seedlings showing disease symptoms before inoculation with biocontrol agents were discarded. The area under the disease progression curve as a cumulative measure of disease symptoms over time was calculated using the 'sintegral' function from the Bolstad2 package in R. Statistical significance between strains at one time point was determined using one-way ANOVA followed by a Tukey test.

2.4 Spray drying

Bacterial cells of *L. plantarum* AMBP214 and *L. plantarum* WCFS1 were harvested from overnight cultures in MRS liquid medium by centrifugation at 3893 × g for 12 min at 20 °C and the pellet was resuspended in its original volume sterile demineralized water. To protect the cells during spray drying, 2.5 % (w/w) trehalose (Cargil, Krefeld, Germany) was added to this suspension. The resuspended cells with trehalose were then spray-dried using a laboratory scale spray dryer (B-290; Büchi, Flawil, Switzerland) using the following settings: inlet temperature of 135 °C, flow rate of 7.5 ml/min, air flow of 32.5 m^3/h and outlet temperature of 55 °C.

2.5 Resuscitation of biocontrol powders

Resuscitation of two spray-dried *Lactiplantibacillus plantarum* strains, AMBP214 and WCFS1, was compared to three commercial benchmarks, Prestop-mix containing *Gliocladium catenulatum* J1446, Blossom bless containing *Pantoea agglomerans* p10c and Serenade containing *Bacillus amyloliquefaciens* QST713. The powders were resuspended in sterile PBS to a concentration of 10 mg/ml and 10 µl of this suspension was added to 190 µl sterile PDB (Difco, Le Pont de Claix, France), a medium on which all tested powders and typical plant pathogens grow well, in a 96-well plate.

Growth in each well was followed up over time by measuring the optical density (OD) at 600 nm every 15 min using a Biotek plate reader. Each powder was tested using five replicates at 28 °C and the plate was shaken before each measurement. The lag time of the growth curves was determined by identifying the time at which the OD exceeded a certain threshold. This threshold was calculated for each replicate as the OD value at which there was a ten percent increase compared to the average OD value in the first hour of the assay. For the commercial product containing *B. amyloliquefaciens* spores, two lag times were calculated. A first increase in OD at a threshold of ten percent was identified as the spore burst and a second increase at a threshold of 30 % followed by a much steeper increase in OD was identified as the start of exponential vegetative growth. Statistical significance between the lag times of different products was determined using one-way ANOVA followed by a Tukey test.

2.6 Bumblebee loading and CFU enumeration

The spray-dried powder was formulated in a 1:10 ratio with corn-starch (Maizena, Unilever, Rueil-Malmaison Cedex, France) and mixed vigorously with a spatula. This 1:10 formulation was added to a dispenser with a length of 20 cm in a layer of approx. 2 mm thick. Important to note is that this layer should not be too thick to not impede the walk of the bumblebees. A bumblebee was placed in a darkened 50 ml tube on one side of the dispenser. As bumblebees are attracted to the light, they walk through the dispenser where they were collected in a second non-darkened 50 ml tube. This was repeated with 15 individual bumblebees. The bumblebees (*Bombus terrestris*) were ordered from Biobest NV (Ilse Velden 18, 2260 Westerlo, Belgium).

The number of bacteria on the outside surfaces of these bumblebees was quantified by first washing the bumblebees in three ml wash buffer (1:50 diluted wash buffer (1M tris-Hcl, 500 mM EDTA, 1.2 % Triton, adjusted to pH8 (16), during 5 min in a 50 mL tube at maximum speed on the Vortex Genie® 2 (MoBio)). This washing buffer was then serially diluted and plated out on selective MRS medium supplemented with cycloheximide (0.1 g/l). MRS medium is selective for lactic acid bacteria and

cycloheximide was added to inhibit fungal growth. CFUs were established after incubation for 2 days at 28 °C in the dark. Bumblebees from multiple Biobest hives were plated out using the same protocol, yielding no detectable lactobacilli.

2.7 Entomovectoring of AMBP214 by bumblebees to strawberry flowers

The dispersal of AMBP214 by bumblebees to strawberry flowers was assessed in a small greenhouse assay with strawberry plants (*Fragaria x ananassa*, cultivar Sonsation) and a specific entomovectoring bumblebee hive (Flying Doctors system, Biobest). The greenhouse contained one Flying Doctors hive and three trays with four to five strawberry plants (**Figure 1**). Extra flowering strawberry plants from various cultivars were added to avoid damage to the flowers as the hive contained too many bumblebees for the number of flowers. Only the strawberry plants in the trays were sampled, as these came from the same distributor, and were planted similarly.

First, the bumblebees were allowed to explore the greenhouse for five days to be accustomed to the environment. Three flowers were sampled on day five and acted as blanks. Next, the 1:10 formulation (described above) was added to a dispenser to achieve a depth of approximately 2 mm, through which the bumblebees passed to exit the hive. The bacterial formulation in this dispenser was renewed daily. Flowers were sampled each morning, for four consecutive days, and the number of lactic acid bacteria on the flowers was determined by washing them in 3 ml wash buffer (described in method to enumerate bumblebee bacterial load), plating on selective MRS medium, supplemented with cycloheximide and counted after two days of incubation at 28 °C. Of the three blank samples taken (i.e. flowers collected after bumblebee release but before the dispersal of *L. plantarum* AMBP214), two blanks did not show growth and the third sample contained 710 CFUs/flower. The detection limit of this technique is 30 CFUs/flower. This experiment was performed a second time in a similar set-up, but with cultivar Elsanta, and 71 flowers were sampled only at day 4. Here, sunflowers were added to avoid damage to the flowers caused by too many bumblebees.

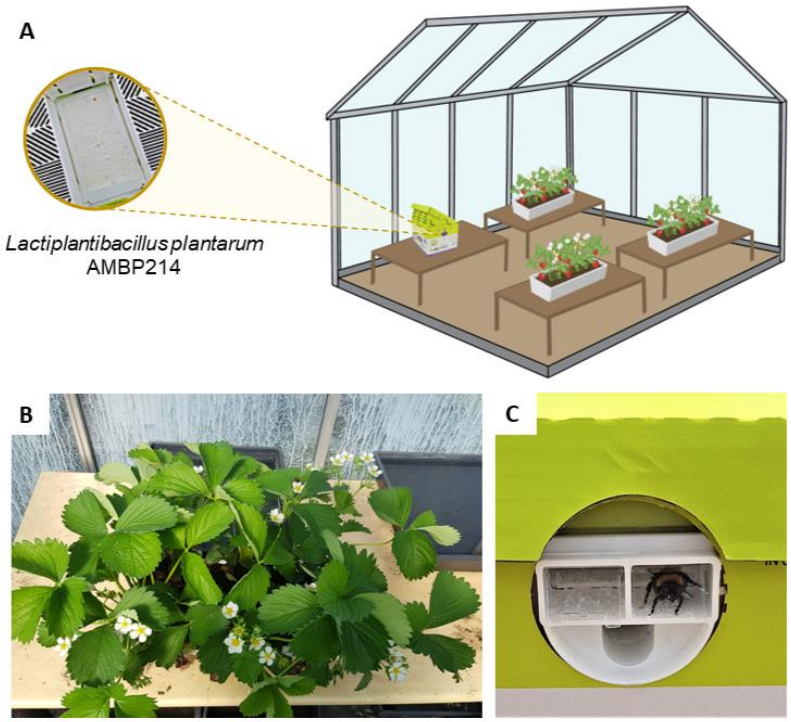

Figure 1: Experimental set-up for the entomovectoring trials. A) overview of the greenhouse and a detail of the powder product (spray-dried bacteria + Maizena, 1:10, w:w) in the Flying Doctors® system (Biobest). B) close-up of a tray containing four to five strawberry plants (*Fragaria x ananassa* cv. Sonsation or Elsanta) C) A worker bee (*B. terrestris*) leaving the exit of the Flying Doctors hive (Biobest).

2.8 *L. plantarum* AMBP214 persistence on the strawberry flower

The survival of *L*. *plantarum* AMBP214 was assessed on the flowers of the strawberry plant (*Fragaria x ananassa*, cultivar Elsanta). First, bacterial cells were harvested via centrifugation (15 min, 2000 x *g*) from cultures grown overnight in MRS broth at 30 °C, shaking at 135 rpm. These cells were then washed twice in PBS and diluted in PBS to three concentrations (≈ 5 x 10^4, 5 x 10^6, and 5 x 10^8 CFUs/ml), based on the optical density (OD) and previously determined OD per CFUs/ml ratios. These three suspensions were then enumerated based on serial dilution and inoculated on the flower, by pipetting four times 5 µl suspension on a flower, resulting in the inoculation of the flowers with approximately 10^3, 10^5, and 10^7 CFUs per flower. Each concentration was inoculated on 10 flowers. Strawberry plants were kept indoors in a pop-up greenhouse (1 m^2) at room temperature and natural light. Twice a day, the greenhouse was sprayed till drip-off to maintain a high relative humidity. The number of lactic acid bacteria was determined on two blank flowers and ten inoculated flowers per treatment after 72 h.

Enumeration of lactic acid bacteria on flowers was done by plating out on selective MRS medium (as described above).

2.9 Adherence to bumblebees compared to the state of the art

The entomovectoring of spray-dried AMBP214 was compared to the state-of-the-art based on the number of live biocontrol agents at three crucial steps of the application. For this purpose, data was extracted from the most relevant scientific publications which tested the dispersal of live microbes via pollinators to plants (4, 6, 7, 9, 10, 11, 18, 19, 20, 21, 22, 23). The three stages that were compared were (i) the number of CFUs in the powder, (ii) the number of CFUs on the pollinator and (iii) the number of CFUs on flowers.

2.10 Botrytis cinerea inhibition in a greenhouse trial on strawberry

The biocontrol efficacy of AMBP214 against *B. cinerea* B05.10 was assessed in a greenhouse assay with strawberry plants (*Fragaria x ananassa*, cultivar Favori) and entomovectoring using two bumblebee hives (Flying Doctors system with *Bombus terrestris*, Biobest). Plants were grown in a greenhouse at 21–23 °C under light (on average 14 h light/10 h dark, 250 W/m^2) and a relative humidity of 65 %, except for 1 h before sunset, the humidity was increased to 90% (17). Plants were substrate grown and automatically watered twice a day with 65 mL of nutrient solution (**Supplementary Table S1**), adjusted to keep the electrical conductivity of the nutrient solution between 1.1 and 1.3 and the pH between 5.5 and 6. The greenhouse trial included two biocontrol treatments: i) entomovectored AMBP214 and ii) entomovectored commercial Prestop® 4B with active component *Gliocladium catenulatum* J1446 (Biobest, Westerlo, Belgium) (**Figure 2**). The negative control involved untreated plants. The two entomovectored biocontrol treatments were covered with a transparent cloth of fine mesh size to contain the bumblebees. Bumblebee hives were placed underneath these covers 24 h prior to treatment to acclimatise. It is important to note that one bumblebee hive did not forage abundantly during this period for unclear reasons. On the day of the treatment, open flowers were marked in all treatments using coloured ribbons. Pollination was ensured by using a soft brush for marked flowers

in the negative control and the AMBP214 treatment, as bumblebees in the latter were not foraging abundantly. Next, the beehive dispensers were loaded with biocontrol powders. The fully functioning hive was loaded with Prestop® 4B and the hive with low to no foraging activity was loaded with AMBP214. To make up for the limited bumblebee activity, AMBP214 was manually inoculated onto the flowers by dispensing four times 5 µl of an AMBP214 in PBS suspension (prepared as described in section 2.8) with an average viability of 1.47 x 10^{10} CFUs/ml. The number of lactic acid bacteria was determined on three blank flowers before dispensing biocontrol agents on flowers or in bumblebee hives. This was done by plating out on selective MRS agar as described above. To assess inoculation of AMBP214, three flowers were sampled right after inoculation and similarly, AMBP214 persistence on the flowers was assessed on two flowers right before *B. cinerea* inoculation. Subsequently, all marked flowers were inoculated five times with 2 µl *B. cinerea* spore suspension (10^5 spores/ml). To promote optimal *B. cinerea* infection, all plants were covered with plastic and humid air was misted inside maintaining 100 % relative humidity for 24 h. This setup (pollination + biocontrol treatment + *B. cinerea* inoculation) was done twice. The first cycle started on 22/02/2023, the second on 28/02/2023. Upon ripening, around one month after infection, strawberries were collected, weighed individually, and incubated for 11 days at 25 °C in individual sterile petri dishes placed on humid paper in tip boxes. Three fruits were stored per box. During incubation, disease symptoms were scored visually over time, based on three categories: 1) *B. cinerea* symptoms, 2) no symptoms, and 3) infected with another pathogen. Very small fruits and flowers were discarded resulting in a total of 155 fruits for incubation, 137 fruits resulting from the first pollination + treatment + inoculation week and 18 fruits resulting from the second.

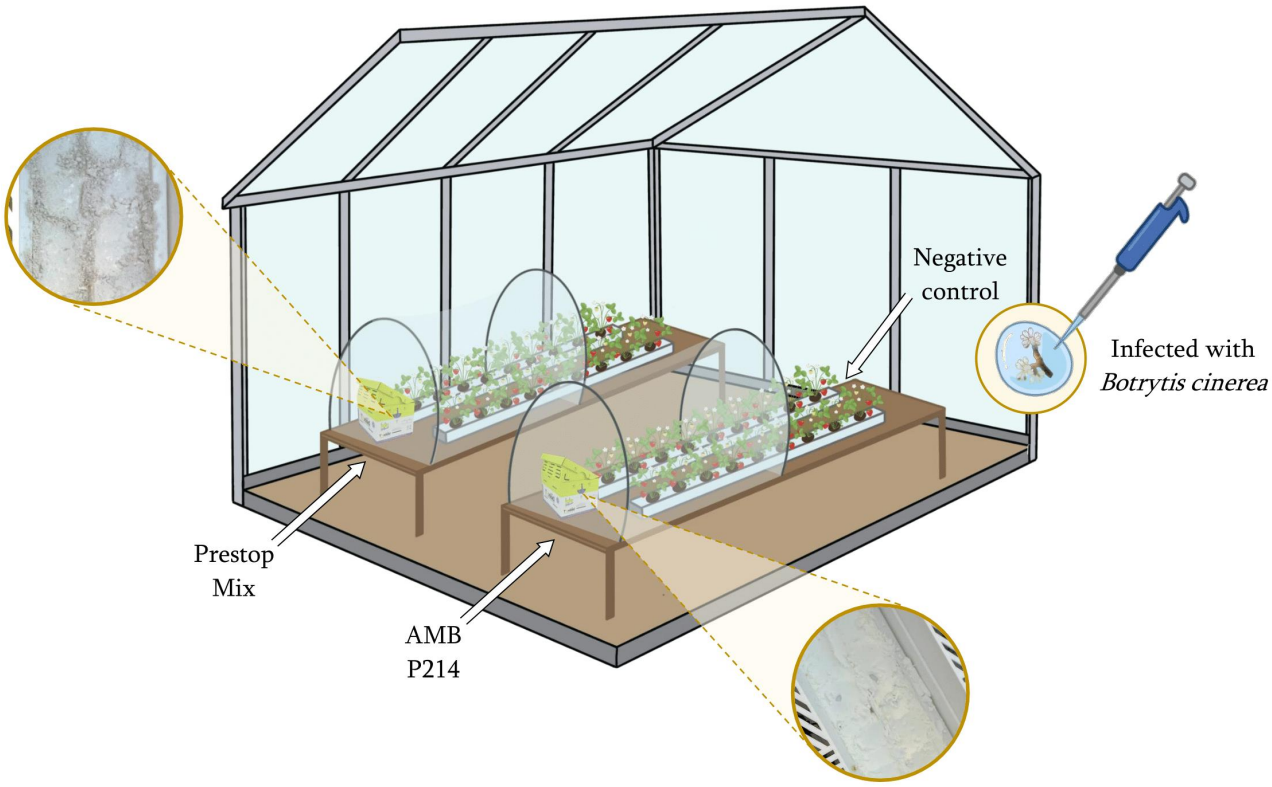

Figure 2: Experimental set-up for greenhouse trial. Overview of the greenhouse trial including 3 treatments: 1) commercial Prestop® 4B with active component *Gliocladium catenulatum* J1446 (Biobest), entomovectored with a Flying Doctors® system (Biobest) 2) spray-dried *Lactiplantibacillus plantarum* AMBP214 + Maizena, 1:10, w:w), entomovectored with the Flying Doctors® system (Biobest) with additional manual inoculation. 3) negative control: plants without a biocontrol treatment. Two days after inoculation, all treatments, including the negative control, were manually infected with *Botrytis cinerea*.

3 Results

3.1 *L. plantarum* AMBP214 inhibits key fungal and bacterial pathogens

We set out to evaluate the inhibitory effect of *Lactiplantibacillus plantarum* AMBP214 against several key fungal and bacterial pathogens. We found that the presence of *L. plantarum* AMBP214 led to a 29 % reduction in the mycelial growth of *Botrytis cinerea* and an 18 % radius reduction in *Alternaria alternata* in a plate assay on potato dextrose agar (PDA) (**Figure 3)**. Additionally, we observed that *B. cinerea* inhibition by AMBP214 increased to 40 % when the bacteria were incubated at 28 °C instead of 25 °C prior to fungal inoculation and growth at 25 °C (**Supplementary Figure S1**).

The cell-free supernatant of AMBP214 also reduced the mycelium growth of *B. cinerea*, but this effect disappeared after neutralization of the supernatant (original pH between 3.7 and 4.1; **Figure 3**). While the supernatant did not reduce the mycelium radius of *A. alternata*, it did affect its morphology. The

fungal mycelium appeared lighter in all treatments compared to the blank, including treatments with unadjusted and pH-adjusted cell-free supernatant, possibly indicating reduced sporulation.

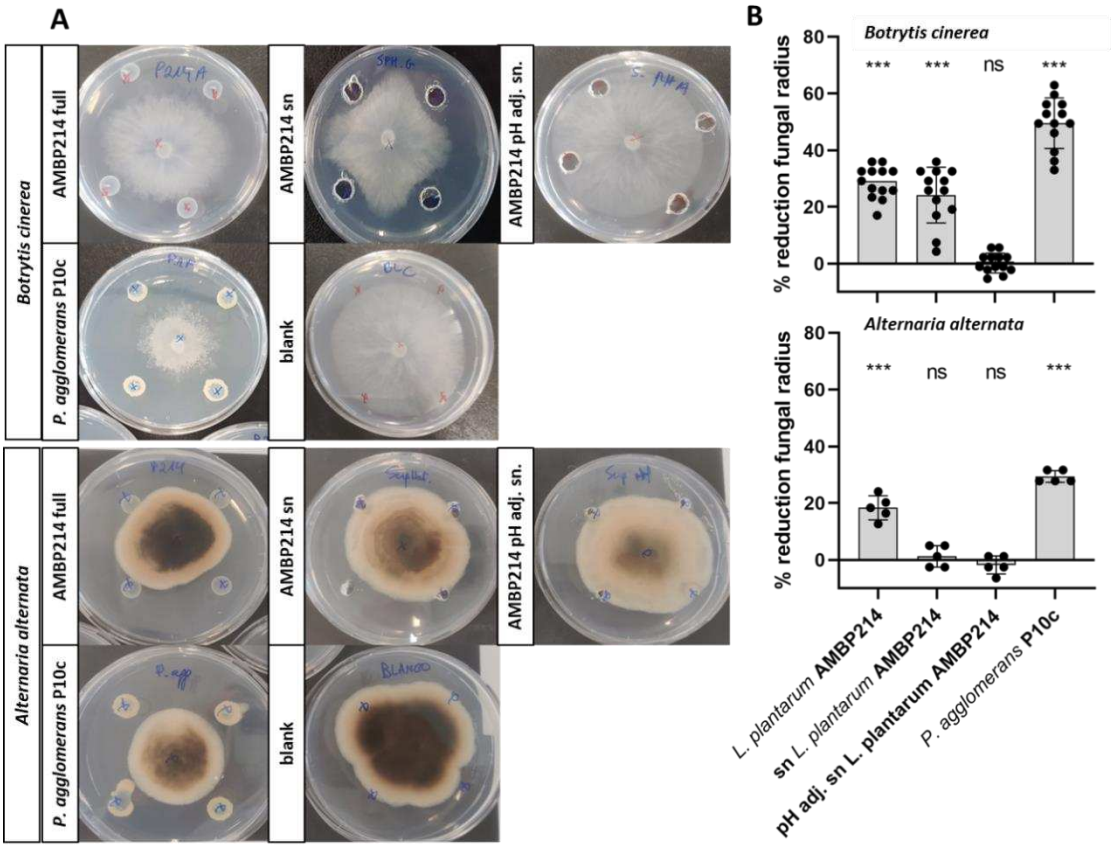

Figure 3: Antimicrobial assays against *B. cinerea* and *A. alternata.* A) Exemplary plates showing the growth of the fungus in the presence of different treatments; *L. plantarum* AMBP214 (overnight culture, "full"), supernatant ("sn") of AMBP214, the pH adjusted supernatant ("pH adj. sn.") of AMBP214, the commercial biocontrol agent *P. agglomerans* p10c, and blank plates (no bacteria nor supernatant). **B) The percentage decrease in fungal radius** compared to the blank for the four conditions. For *B. cinerea* two experiments with ten and three repetitions were combined. *A. Alternaria* was tested once in five repetitions. Dunnett's test was used to compare each treatment with the blank treatment, * p<0.05, ** p<0.01, *** p<0.001.

The inhibitory effect of *L. plantarum* AMBP214 was also observed *in planta*, on tomato seedlings, as the presence of AMBP214 on the seedlings significantly reduced disease symptoms caused by *P. syringae* DC3000 (**Figure 4**). This biocontrol effect of AMBP214 on seedlings was comparable with the effect of the commercial biocontrol agent *P. agglomerans* E325A. As a reference, also the closely related model strain *L. plantarum* WCFS1 was included in this assay and showed a similar inhibitory effect, suggesting a more generic antimicrobial mechanism.

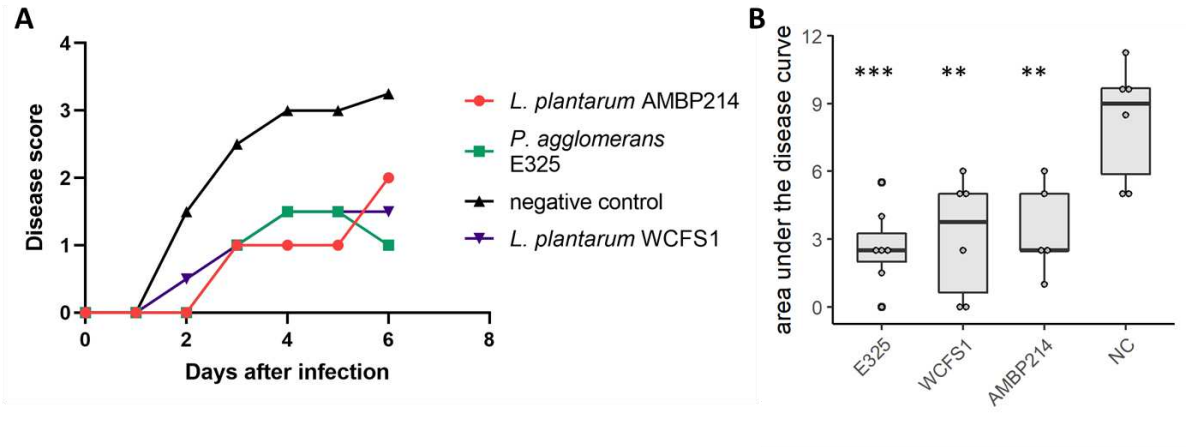

Figure 4: Disease progression curves (A) and area under the disease progression curve (B) for seedlings treated with different bacteria followed by inoculation with pathogen *Pseudomonas syringae* DC3000. Four conditions were tested; a positive control (*P. agglomerans* E325, a known biocontrol agent (15)), a negative control (NC, no bacteria), *L. plantarum* AMBP214 (biocontrol strain of interest), and *L. plantarum* WCFS1 (model lab strain closely related to AMBP214). A) shows the mean disease score for each treatment and B) shows the area under the disease progression curve between days 0 and 6 for these treatments (arbitrary unit). Each treatment consisted of 7 replicates, seedlings showing disease symptoms prior to inoculation with biocontrol agents were discarded. Statistics in B) were performed via Dunnett's test, comparing each treatment to the negative control, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2 Spray-dried AMBP214 can be dispersed to strawberry flowers via bumblebees

Next, *L. plantarum* AMBP214 was spray-dried to obtain a powder that could be loaded onto bumblebees. Spray-drying resulted in a powder with a viability of 2 x 10^8 CFUs/mg and these spray-dried bacteria retained their antimicrobial activity against *B. cinerea in vitro* (**Figure 5**). Secondly, both strains of spray-dried *Lactiplantibacillus plantarum*, AMBP214 and WCFS1 regained their metabolic activity at least three times faster compared to the three commercial powders (**Table 2**). This is an important characteristic as priority effects play a role in the effectiveness of biocontrol applications particularly for biocontrol in flowers since these are temporary structures. Next, this spray-dried powder was formulated in a 1:10 ratio with corn-starch (Maizena). The corn-starch functioned as a diluent as well as a carrying agent, as it is said to improve attachment to bumblebees (18). The resulting 10-fold dilution in corn-starch had an average concentration of 4.7 x 10^6 CFUs/mg. The viability of the dilution was lower than expected, possibly due to imperfect mixing. With the help of a dispenser, the 1:10 was loaded onto bumblebees (*Bombus terrestris*). This resulted in a detectable amount of AMBP214 on 13 out of 15 bumblebees (87%), and a median load of 5 x 10^7 CFUs per bumblebee sampled directly after exiting the dispenser (**Supplementary Figure S2**). Similar results were obtained

in a second dispenser experiment with a different batch of spray-dried AMBP214. The viability in this batch was lower, 6.2 x 10^5 CFUs/mg in the 1:10 formulation, resulting in a lower median load on the bumblebees (1.6 x 10^6 CFUs per bumblebee). Similarly to the previous experiment, the success rate was high (9 out of 10 bumblebees carried detectable amounts of bacteria), and a similar amount of powder stuck to the bumblebees in both experiments.

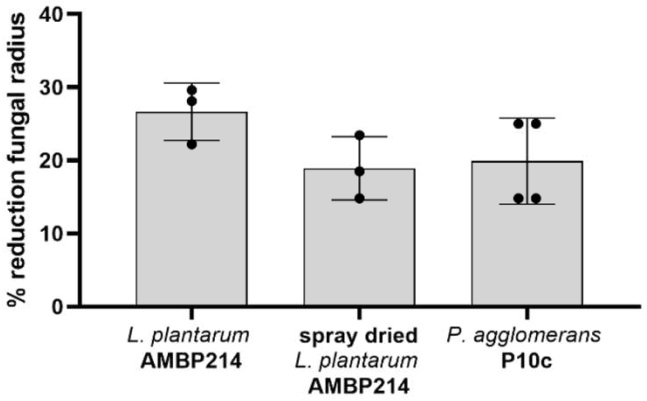

Figure 5: Antimicrobial characteristics of the spray-dried powder: The percentage decrease in fungal mycelium radius of *Botrytis cinerea,* compared to the radius in the absence of any bacteria, in the presence of *L. plantarum* AMBP214 (overnight culture from freezer stock), spray-dried and resuspended *L. plantarum* AMBP214, or commercial biocontrol strain *Pantoea agglomerans* P10c (Blossom Bless™). All treatments were adjusted to an average CFUs count of 10^8 CFUs/ml. The data was collected from two separate *in vitro* assays. No significant differences were detected between the three treatments, using one-way ANOVA.

Table 2: Powder resuscitation speed. The growth of spray-dried *Lactiplantibacillus plantarum* AMBP214 and WCFS1 were compared to commercial benchmarks. Growth was measured by the increase in optical density at 600 nm over time at 28 °C and lag times were calculated when the OD exceeded certain thresholds. Significant differences compared to the lag time of spray-dried AMBP214 were determined using one-way ANOVA followed by a Tukey test.

Product	lag time (h) at 28 °C	Compared to spray-dried AMBP214
spray-dried *Lactiplantibacillus plantarum* AMBP214	3.5 (+/- 0.16)	
spray-dried *Lactiplantibacillus plantarum* WCFS1	3.6 (+/- 0.33)	ns
commercial Prestop-mix: *Gliocladium catenulatum* J1446	10.9 (+/- 0.71)	***
commercial Blossom bless: *Pantoea agglomerans* P10c	10.5 (+/- 0.52)	***
commercial Serenade: *Bacillus amyloliquefaciens* QST713	Spore burst: 5.1 (+/- 0.33) Total lag time until exp. growth: 16.8 (+/- 4.6)	*** ***

Next, the abundance of lactic acid bacteria on flowers after these were pollinated by loaded bumblebees was quantified. In a small greenhouse assay, flowers of strawberry plants were collected every day for four days after adding a dispenser to the bumblebee hive containing the AMBP214 1:10 formulation. A total of 37 flowers were collected during four days after releasing the inoculated bumblebees. Plating out the wash solutions from these flowers on selective MRS medium showed that

these flowers were loaded consistently with lactic acid bacteria, with an average of 1 x 10^5 CFUs per flower (**Figure 6A**) and lactic acid bacteria were detected on all sampled flowers in a minimal abundance of 7 x 10^3 CFUs/flower. These values exceed previously reported values for other biocontrol agents on both the bumblebee and the flower level (**Figure 6B).** Additionally, previous entomovectoring efforts often report poor replicability, e.g. Yu and Sutton only detect biocontrol agents on 7.5% of flowers (4) and Kapongo and colleagues on 40% (22). For *L. pantarum* AMBP214, 100% of flowers contained minimally 7 x 10^3 CFUs per flower.

In a second experiment, 71 flowers were collected on the fourth day only, and similar abundances of lactic acid bacteria were counted (Day 4:rep). *L. plantarum* AMBP214 contributed to the majority of the counted lactic acid bacteria on the flowers, as flowers that were sampled after the release of bumblebees for pollination, but before the addition of *L. plantarum* AMBP214 to the dispenser, carried much lower or undetectable amounts of lactic acid bacteria (Day 0). Finally, *L. plantarum* AMBP214 was persistent and even metabolically active on strawberry flowers. These bacteria reached a population of approximately 10^6 CFUs per flower after 72 hours, regardless of the initial inoculation amount. This represents a 1000-fold increase for the lowest inoculated concentration and a reduction in population size for the highest inoculated concentration (**Figure 6C**). The lactic acid bacteria counted on the flowers are largely the inoculated *L. plantarum* AMBP214 since no lactic acid bacteria were detected on two blank flowers and colonies were morphologically similar.

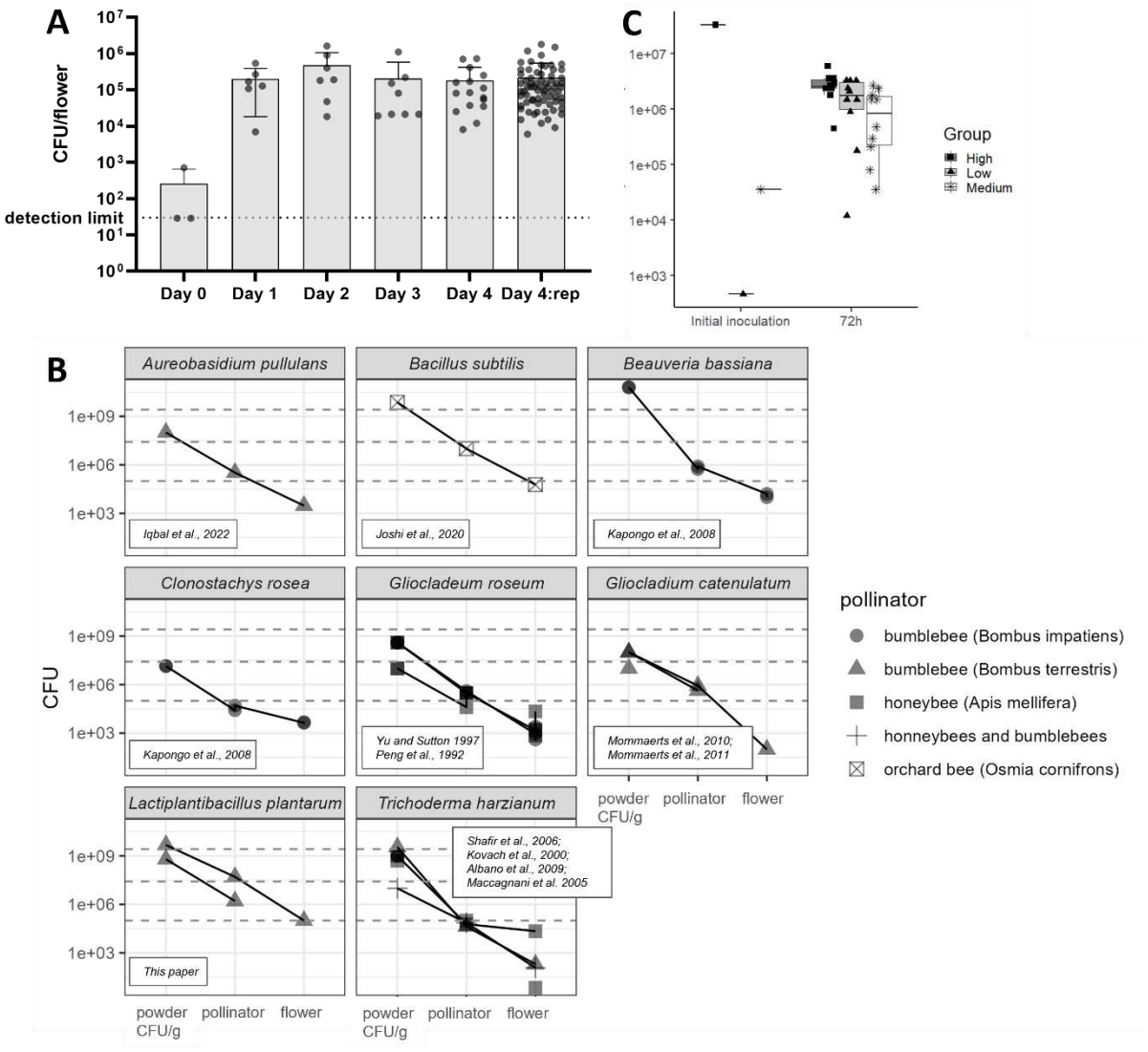

Figure 6: (A) Bacterial load of lactic acid bacteria on strawberry flowers. Flowers were pollinated by bumblebees which were loaded with spray-dried *L. plantarum* AMBP214 in a 1:10 ratio with corn-starch, using a flying Doctors hive with an in-built dispenser (Biobest). Lactic acid bacterial load on strawberry flowers was determined by plating out on selective MRS medium. Before releasing the bumblebees (day 0), three flowers were sampled. Samples in which no CFUs were detected are indicated with an 'X'. The experiment was repeated and only the flower load on day four (Day 4: rep) was determined. **(B) Comparison of the number of live biocontrol agents at three crucial steps of the application**; (i) in the administered powder (CFUs/g), (ii) on the pollinator after passing through a dispenser containing the powder (CFUs/vector) and (iii) on the flower after visitation of pollinators (CFUs/flower). Horizontal dashed lines indicate the three levels for *Lactiplantibacillus plantarum* AMBP214. The average was calculated for the repeated dispenser experiments. The shape of the data points indicates the type of pollinator used. *Gliocladium catenulatum* is also known as *Clonostachys rosea*. The taxonomy of the publication was used in this visualization. Data was collected from the most relevant scientific publications on this topic (4, 6, 7, 9, 10, 11, 18, 19, 20, 21, 22, 23). **(C) Survival of *Lactiplantibacillus plantarum* AMBP214 on the flower of the strawberry plant**. Flowers were manually inoculated with low (~10^3 CFUs/flower), medium (~10^5 CFUs/flower) or high (~10^7 CFUs/flower) amounts of cells. For each concentration, the number of lactic acid bacteria per flower was determined by serial dilution of the inoculum and ten flowers after 72h. No lactic acid bacteria were counted in two blank flowers before the experiment.

3.3 *L. plantarum* AMBP214 could not protect strawberries from *Botrytis cinerea* in a greenhouse trial

Next, we set out to investigate the inhibitory effect of *L. plantarum* AMBP214 against *B. cinerea* on strawberries in a greenhouse setting. We confirmed the presence and persistence of AMBP214 on flowers as its abundance was 9.07 x 10^7 CFUs/flower just after manual inoculation, and 2.10 x 10^7 CFUs/flower after 48 h, just before *B. cinerea* infection. No lactobacilli were detected on three not-inoculated blank flowers. Unlike entomovectored *Gliocladium catelaneum* (Prestop-Mix (Biobest, Westerlo)), AMBP214 did not significantly reduce the disease incidence immediately after harvest compared to the negative control, i.e. plants without a biocontrol treatment (p=1), while the Prestop-Mix treated strawberries did (p=0.024) using a pairwise Fisher test with the Benjamini-Hochberg correction for multiple comparisons. Also, on all other observation days (3, 4, 6, 7, 8, and 11 days post-harvest), *B. cinerea* disease incidence was found to be similar between the negative control and the AMBP214 treatment (p-values ranging from 0.224 on day six to 1 on day three) using a pairwise Fisher test with Benjamini-Hochberg correction (**Figure 7**). The positive control did significantly lower disease incidence on all of these days (p-values ranging from 0.00058 on day eight to 0.044 on day 11). Similarly, AMBP214 did not significantly delay the development of *B. cinerea* symptoms (p=0.77, Dunn test with Benjamini-Hochberg correction) as disease symptoms were visible on average after 4.17 days for the AMBP214 treatment and 3.93 days for the negative control (**Supplementary Figure S3**). Alternatively, strawberries treated with Prestop-Mix took significantly longer (on average 6.45 days) to exhibit disease symptoms compared to the negative control (p=0.0078). Similar trends were seen when analysing the two treatment + inoculation cycles separately. Average days until symptoms for the positive control, AMBP214 and the negative control were 6.30 days, 4.21 days and 3.73 days, resp., for symptomatic strawberries in cycle one (n=109) and 8 days, 4 days, and 5.33 days, resp., for symptomatic strawberries in cycle 2 (n=16). Regarding the fruit weight, treatment with AMBP214 did not have an effect compared to the negative control (p=0.60) whereas bee-vectored Prestop-Mix positively affected fruit weight (p=0.022, using a Dunn test with Benjamini-Hochberg correction) with an average fruit weight of 6.83 g, 6.52 g, and 9.18 g for the negative control, AMBP214, and the positive control, respectively (**Supplementary Figure S4**). Similarly to the disease symptoms, similar trends

were observed when comparing the fruit weight of the two treatment + inoculation cycles. Due to the low number of strawberries in the second cycle (n = 19), these differences in fruit weight were not significant.

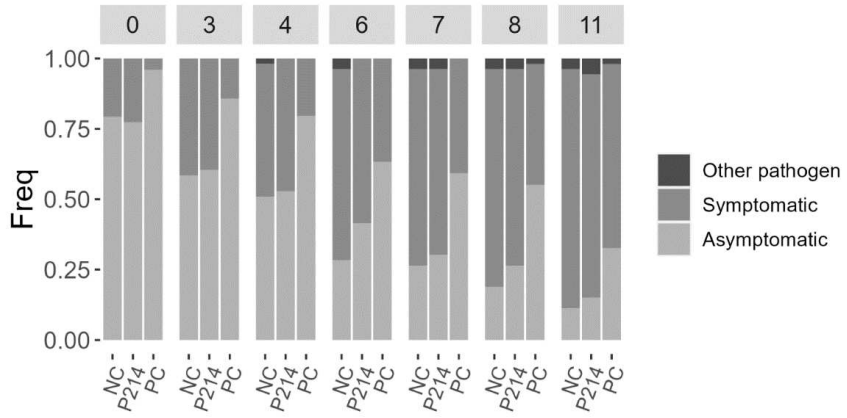

Figure 7: Proportion of strawberries after 0, 3, 4, 6, 7, 8, and 11 days of incubation showing visible symptoms of *Botrytis cinerea* for the treatments *Lactiplantibacillus plantarum* AMBP214, the negative control (NC, plants untreated with biocontrol agent), and the positive control (PC, Prestop-Mix 4B, Biobest, Belgium).

4 Discussion

This study showed that *Lactiplantibacillus plantarum* AMBP214 possesses promising biocontrol properties as it is active against several key plant pathogens, is metabolically active on flowers, and can be abundantly and uniformly dispersed to flowering crops via bumblebees after spray-drying and formulation. However, despite these properties, this did not result in biocontrol activity in a greenhouse trial under the tested conditions.

Regarding antipathogenic properties, the strain inhibited *B. cinerea* and *A. alternata* in plate assays and *P. syringae* in a gnotobiotic seedling assay suggesting broad applicability as a realistic option. The *in vitro* assays showed that different mechanisms played a role, as the cell-free supernatant reduced the mycelial growth of *B. cinerea*, but not of *A. alternata*. However, both the acidic and the neutralized cell-free supernatant affected the morphology of *A. alternata*, indicating a reduction in sporulation. For *B. cinerea*, neutralization of the supernatant resulted in a loss of the inhibitory effect. De Simone and colleagues (24) also showed that the supernatant of multiple strains of *L. plantarum* inhibited *B.*

cinerea, while pH-adjusted supernatant did not. This indicates that organic acids, primarily lactic acid, are important in the antifungal activity, as has been proposed previously (25, 26, 24, 27). Still, this does not rule out other possible mechanisms, such as phenyl lactic acids and cyclic dipeptides (27, 26), or hydrolytic enzymes (28), as a neutralized pH could reduce the activity of metabolites and enzymes.

Second, *L. plantarum* AMBP214 was formulated into a powder with high viability and good compatibility with a bumblebee-entomovectoring system. Proper formulation of biocontrol agents is crucial for future applications, mainly to ensure long shelf life and convenient use in everyday farm operations. Specifically for entomovectoring, biocontrol agents need to be formulated into a powder that can be loaded efficiently on the vector. Spray-drying is currently not often used to formulate biocontrol agents as it is thought to be only applicable to robust spore-forming bacteria such as *Bacilli* (29, 30). However, both this study as Broeckx and colleagues (30) showed that lactobacilli can be successfully spray-dried resulting in a powder with high viability. While this study did not study the shelf-life of spray-dried *L. plantarum* AMBP214, Broeckx and colleagues (30) showed that spray-dried *Lacticaseibacillus rhamnosus* GG had a shelf life of at least 28 weeks at 4°C. Additionally, spray-drying offers several advantages, as it is a rapid, continuous, cost-effective and scalable process that allows for easy control of the powder characteristics such as moisture content, flow properties, and size distribution (30). These possibilities are highly valuable in entomovectoring as they provide opportunities to increase the loading capacities on the vector. An additional advantage of using non-spore formers is that these vegetative cells quickly regain their metabolic activity when conditions are favourable. This was shown here as both strains of spray-dried *L. plantarum* had a similar lag time, at least three times faster compared to commercial benchmarks, containing spores. This is a significant advantage compared to current products, as priority effects play a role in microbiome assembly and biocontrol efficacy, especially in ephemeral floral environments (31, 32).

Next, a 1:10 formulation with corn-starch of these spray-dried bacteria could be loaded in high quantities onto bumblebees resulting in approx. 1 x 10^9 CFUs per bumblebee using a free-standing

dispenser. Releasing bumblebees into a greenhouse using a flying Doctor hive (Biobest), with an in-built dispenser containing the 1:10 formulation resulted in high bacterial abundances on the flowers. The flowers carried on average 1×10^5 CFUs per flower and the coverage was consistent as lactic acid bacteria were detected on all sampled flowers with minimally 1×10^4 CFUs. Moreover, we showed that *L. plantarum* AMBP214 was metabolically active on strawberry flowers, as they were able to increase their population size 1000-fold in 72 h. Regardless of the inoculation concentration, AMBP214 reached 10^6 CFUs/flower, the flower's carrying capacity for AMBP214 under tested conditions.

The high numbers and consistency of biocontrol agents observed on both the vector and the flower represent a significant improvement over previous entomovectoring efforts (4, 6, 7, 9, 10, 11, 18, 19 20, 21, 22, 23). To fully realize this potential, further studies are needed to evaluate the efficacy of dispersal on a larger scale. In addition, other performance parameters of the system must be evaluated, such as the impact of entomovectoring on the performance, health, and foraging behaviour of the bumblebees and the optimization of the powder formulation for loading capacity and shelf life.

Despite its persistence on the flower, AMBP214 could not significantly reduce the incidence of *B. cinerea* in the greenhouse under the tested conditions while commercial Prestop® 4B (Biobest, Westerlo, Belgium) could. Perhaps the flower environment or experimental conditions were unsuitable for AMBP214's antimicrobial mechanism, or maybe the abundance of AMBP214 during the greenhouse trial (10^7 CFUs/flower) exceeded the flower's carrying capacity (10^6 CFUs/flower), which resulted in a dying and inactive population. This illustrates the common, but underreported, difficulty in ensuring biocontrol efficacy outside the lab environment. Although *L. plantarum* AMBP214 has promising properties regarding antipathogenic activity *in vitro* and *in planta*, formulation, persistence on flowers and dispersal, it is essential to recognize that these factors alone do not guarantee success in the greenhouse. We believe that a better understanding of the metabolism of *L. plantarum* AMBP214 on the flower and its antipathogenic mechanism could clarify this inadequate efficacy and offer options for improvement.

In conclusion, this study did show that *L. plantarum* AMBP214 could be spray-dried and dispersed to flowering crops via bumblebees. Compared to the state-of-the-art, this system was a significant improvement on multiple levels. These findings underscore the potential for spray-dried, non-spore-forming bacteria to be effectively used in entomovectoring systems, with important implications for sustainable agriculture and pest management practices.

Acknowledgements

The authors would like to thank Prof. Britt Koskella and Dr. Reena Debray for their help in conceptualizing and optimizing the seedling assay. The authors would also like to thank Dr. Shari Kiekens for her help with spray-drying, Sofie Van der Sluys for helping with the greenhouse experiment, and Daan Rooms for his help with the fungal spot assays.

References

1. Wei F, Xiaoping H, Xiangming X. 2016. Dispersal of Bacillus Subtilis and Its Effect on Strawberry Phyllosphere Microbiota under Open Field and Protection Conditions. Scientific Reports 6 (1): 22611. https://doi.org/10.1038/srep22611.
2. Trivedi P, Chakradhar M, Kellye E, Leach J E. 2021. Enabling Sustainable Agriculture through Understanding and Enhancement of Microbiomes. New Phytologist. https://doi.org/10.1111/nph.17319.
3. Kaminsky L M, Trexler R V, Malik R J, Hockett K L, Bell T H. 2019. The Inherent Conflicts in Developing Soil Microbial Inoculants. Trends in Biotechnology 37 (2): 140–51. https://doi.org/10.1016/J.TIBTECH.2018.11.011.
4. Yu H, Sutton J C. 1997. Effectiveness of Bumblebees and Honeybees for Delivering Inoculum of Gliocladium Roseum to Raspberry Flowers to Control Botrytis Cinerea. Canadian Journal of Plant Pathology. Biological Control 10 (2): 113–22. https://doi.org/10.1006/BCON.1997.0562.
5. Peng G, Sutton J C, and Kevan P G. 1992. Effectiveness of Honey Bees for Applying the Biocontrol Agent Gliocladium Roseum to Strawberry Flowers to Suppress Botrytis Cinerea. 14 (2): 117–29. https://doi.org/10.1080/07060669209500888.
6. Shafir S, Dag A, Bilu A, Abu-Toamy M, Elad Y. 2006. Honey Bee Dispersal of the Biocontrol Agent Trichoderma Harzianum T39: Effectiveness in Suppressing Botrytis Cinerea on Strawberry under Field Conditions. European Journal of Plant Pathology 116 (2): 119–28. https://doi.org/10.1007/s10658-006-9047-y.
7. Kovach J, Petzoldt R, Harman G E.. 2000. Use of Honey Bees and Bumble Bees to Disseminate Trichoderma Harzianum 1295-22 to Strawberries for Botrytis Control. Biological Control 18 (3): 235–42. https://doi.org/10.1006/BCON.2000.0839.

8. Maccagnani B, Mocioni M, Ladurner E, Lodovica G M, Maini S. 2005. Investigation of Hive-Mounted Devices for the Dissemination of Microbiological Preparations by Bombus Terrestris. Bulletin of Insectology 58 (1): 3–8.
9. Albano S, Chagnon M, Oliveira D D, Houle E, Thibodeau P, Mexia A. 2009. Effectiveness of Apis Mellifera and Bombus Impatiens as Dispersers of the Rootshield® Biofungicide (Trichoderma Harzianum, Strain T-22) in a Strawberry Crop. Hellenic Plant Protection Journal.
10. Iqbal M, Jutzeler M, França S C, Wackers F, Andreasson E, Stenberg J A. 2022. Bee-Vectored Aureobasidium Pullulans for Biological Control of Gray Mold in Strawberry. Phytopathology 112 (2): 232–37. https://doi.org/10.1094/PHYTO-05-21-0205-R.
11. Joshi N K, Ngugi H K, Biddinger, D J. 2020. Bee Vectoring: Development of the Japanese Orchard Bee as a Targeted Delivery System of Biological Control Agents for Fire Blight Management. Pathogens 9 (1). https://doi.org/10.3390/pathogens9010041.
12. Meeus I, Parmentier L, Billiet A, Maebe K, Nieuwerburgh F V, Deforce D, Wäckers F, Vandamme P, Smagghe G. 2015. 16S RRNA Amplicon Sequencing Demonstrates That Indoor-Reared Bumblebees (Bombus Terrestris) Harbor a Core Subset of Bacteria Normally Associated with the Wild Host. PLoS ONE 10 (4): e0125152. https://doi.org/10.1371/journal.pone.0125152.
13. Legein M, Smets W, Wuyts K, Bosmans L, Samson R, Lebeer S. 2022. The Greenhouse Phyllosphere Microbiome and Associations with Introduced Bumblebees and Predatory Mites. Microbiology Spectrum 10 (4). https://doi.org/10.1128/spectrum.01755-22.
14. Lebeer S, Vanderleyden J, De Keersmaecker S C J. 2008. Genes and Molecules of Lactobacilli Supporting Probiotic Action. Microbiology and Molecular Biology Reviews 72 (4): 728–64. https://doi.org/10.1128/mmbr.00017-08.
15. Morella N M, zhang X, Koskella B. 2019. Tomato Seed-Associated Bacteria Confer Protection of Seedlings against Foliar Disease Caused by Pseudomonas Syringae. Phytobiomes Journal 3 (3): 177–90. https://doi.org/10.1094/PBIOMES-01-19-0007-R.
16. Redford A J, Fierer N. 2009. Bacterial Succession on the Leaf Surface: A Novel System for Studying Successional Dynamics. Microbial Ecology 58 (1): 189–98. https://doi.org/10.1007/s00248-009-9495-y.
17. Zhao Y, Vlasselaer L, Ribeiro B, Terzoudis K, Van den Ende W, Hertog M, Nicolaï B and De Coninck B. 2022. Constitutive Defense Mechanisms Have a Major Role in the Resistance of Woodland Strawberry Leaves Against Botrytis cinerea. Front. Plant Sci. 13:912667. doi: 10.3389/fpls.2022.912667
18. Mommaerts V, Put K, Smagghe G. 2011. Bombus Terrestris as Pollinator-and-Vector to Suppress Botrytis Cinerea in Greenhouse Strawberry. Pest Management Science 67 (9): 1069–75. https://doi.org/10.1002/ps.2147.
19. Smagghe G, Boecking O, Maccagnani B, Mänd M, Kevan P G. 2020. Entomovectoring for Precision Biocontrol and Enhanced Pollination of Crops. Entomovectoring for Precision Biocontrol and Enhanced Pollination of Crops. https://doi.org/10.1007/978-3-030-18917-4.
20. Thomson S V 1992. Dissemination of Bacteria Antagonistic to Erwinia Amylovora by Honey Bees . Plant Disease 76 (10): 1052. https://doi.org/10.1094/PD-76-1052.
21. Ngugi H K, Delaplane S D K S, Savelle A T, Scherm H. 2005. Effect of Flower-Applied Serenade Biofungicide (Bacillus Subtilis) on Pollination-Related Variables in Rabbiteye Blueberry. Biological Control 33 (1): 32–38. https://doi.org/10.1016/J.BIOCONTROL.2005.01.002.
22. Kapongo J P, Shipp L, Kevan P G, Sutton J C. 2008. Co-Vectoring of Beauveria Bassiana and Clonostachys Rosea by Bumble Bees (Bombus Impatiens) for Control of Insect Pests and Suppression of Grey Mould in Greenhouse Tomato and Sweet Pepper. Biological Control 46 (3): 508–14. https://doi.org/10.1016/J.BIOCONTROL.2008.05.008.

592 23. Mommaerts V, Put K, Vandeven J, Jans K, Sterk G, Hoffmann L, Smagghe G. 2010. Development of
593 a New Dispenser for Microbiological Control Agents and Evaluation of Dissemination by
594 Bumblebees in Greenhouse Strawberries. Pest Management Science 66 (11): 1199–1207.
595 https://doi.org/10.1002/ps.1995.
596 24. De Simone N, Capozzi V, de Chiara M L V, Amodio M L, Brahimi S, Colelli G, Drider D, Spano D,
597 Russo P. 2021. Screening of Lactic Acid Bacteria for the Bio-Control of Botrytis Cinerea and the
598 Potential of Lactiplantibacillus Plantarum for Eco-Friendly Preservation of Fresh-Cut Kiwifruit.
599 Microorganisms 9 (4). https://doi.org/10.3390/microorganisms9040773.
600 25. Cabo M L, Braber A F, Koenraad P M F J. 2002. Apparent Antifungal Activity of Several Lactic Acid
601 Bacteria against Penicillium Discolor Is Due to Acetic Acid in the Medium. Journal of Food
602 Protection 65 (8): 1309–16.
603 26. Dalié D K D, Deschamps A M, Richard-Forget F. 2010. Lactic Acid Bacteria - Potential for Control of
604 Mould Growth and Mycotoxins: A Review. Food Control 21 (4): 370–80.
605 https://doi.org/10.1016/J.FOODCONT.2009.07.011.
606 27. Magnusson J, Ström K, Roos S, Sjögren J, Schnürer J. 2003. Broad and Complex Antifungal Activity
607 among Environmental Isolates of Lactic Acid Bacteria. FEMS Microbiology Letters 219 (1): 129–35.
608 https://doi.org/10.1016/S0378-1097(02)01207-7.
609 28. Allonsius C N, Vandenheuvel D, Oerlemans E F M, Petrova M I, Donders G G G, Cos P, Delputte P,
610 Lebeer S. 2019. Inhibition of Candida Albicans Morphogenesis by Chitinase from Lactobacillus
611 Rhamnosus GG. Scientific Reports 9 (1). https://doi.org/10.1038/s41598-019-39625-0.
612 29. Bejarano A, Puopolo G. 2020. Bioformulation of Microbial Biocontrol Agents for a Sustainable
613 Agriculture. Progress in Biological Control 21: 275–93. https://doi.org/10.1007/978-3-030-53238-
614 3_16.
615 30. Broeckx G, Vandenheuvel D, Claes I J J, Lebeer S, Kiekens F. 2016. Drying Techniques of Probiotic
616 Bacteria as an Important Step towards the Development of Novel Pharmabiotics. International
617 Journal of Pharmaceutics. Elsevier. https://doi.org/10.1016/j.ijpharm.2016.04.002.
618 31. Burgess E C, Schaeffer R N. 2022. The Floral Microbiome and Its Management in Agroecosystems:
619 A Perspective. Journal of Agricultural and Food Chemistry 70 (32): 9819–25.
620 https://doi.org/10.1021/acs.jafc.2c02037.
621 32. Debray, Herbert R A, Jaffe A L, Crits-Christoph A, Power M E, Koskella B. 2021. Priority Effects in
622 Microbiome Assembly. Nature Reviews Microbiology. Springer US.
623 https://doi.org/10.1038/s41579-021-00604-w.

624