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Gut bacteria of *Spodoptera frugiperda* establish endophytic association and affect the interactions of their host herbivore with maize plants

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Abstract

Insects and plants established long-lasting associations with microbes, whose role on insect–plant associations remains largely unknown. We hypothesized that both plant endophytes and insect symbionts benefit their hosts during insect–plant interactions. In the present study, we used the maize-*Spodoptera frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae) system and five bacterial symbionts: a maize endophyte (*Rhizobium larrymoorei* IILzm-Idp03), two residents (*Enterococcus spodopteracolus* IIL-Sfm05 and *E. entomosocium* IILSfc-sus01) and one transient (*Bacillus* sp. IIL-Sfb05) gut bacteria of *S. frugiperda*. The ant-associated actinobacterium *Streptomyces novaecaesareae* IIL-ASP45 was also tested for not sharing any interactions with the maize-herbivore system studied. Bacteria associated with maize and/or *S. frugiperda* promoted plant growth depending on the inoculation strategy used. The tested bacteria colonized roots and leaves of plants regardless of their original host. Mortality and/or *S. frugiperda* larval efficiency of food utilization was affected in plants inoculated with IILSfc-sus01, IILSfb05, IILzm-Idp03 and IILASP45, but not with IILSfm05. The expression of selected maize-defensive genes and the volatile organic compounds (VOCs) profile was altered in all inoculated plants. Changes in VOCs did not affect adult *S. frugiperda* female preference for oviposition, but significantly affected the number of eggs laid/plant.

Keywords Insect–plant interaction · Plant growth-promoting bacteria · Pest control · Symbiosis

Introduction

The evolutionary history of plants and herbivorous insects has been marked by their continuous arms race (Fraenkel 1959; Schuman and Baldwin 2016), with their own evolution being particularly influenced by their associations with microbes (Jason et al. 2008; Hansen and Moran 2014). Plants and insects have been exposed to each other microbiome during their own coevolutionary history, and despite the importance of their microbial associations to their adaptive success, the tripartite association insect–plant–microbes is rarely approached as a three-way interaction system (Frago et al. 2012).

Several insects are host and vector microbes, some pathogenic, to their host plants (Casteel and Hansen 2014; Pirttilä et al. 2023). Plant pathogens vectored by insects are required to live a "double life" and have their molecular machinery adapted to exploit two different hosts, the plant and the insect (Chatterjee et al. 2008). Several plant pathogens directly alter the insect vector behavior (Martini et al. 2015), while others indirectly change the vector behavior manipulating the host plant physiology (Praer et al. 2015). Entomopathogenic microorganisms have also been reported as plant endophytes and are certainly required to promote the needed adaptations to live a double life. These entomopathogens have been shown to improve host plant growth and immunity when associated with plants as endophytes (Jaber and Enkerli 2017; Jaber and Ownley 2018).

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The interactions between plants and insects with microbes capable of living double lives pose interesting ecological questions and call for a more comprehensive investigation of their evolutionary histories and type of associations they establish with their hosts—insects and plants (Bressan et al. 2012; Bressan 2014; Flórez et al. 2017). This becomes particularly important with the biotechnological and economic potential for exploitation of such microbes as plant growth promoters, plant defense inducers, insect entomopathogens and drivers of new technologies for pest control (e.g., as a source of dsRNA molecules) (Berasategui et al. 2016; Whitten et al. 2016).

The potential contribution of microbial symbionts to facilitate or restrict the exploitation of host plants by their host herbivores has been argued to be dependent on symbiont location (intracellular, gut or environmental) and on the fidelity of symbiont–insect association. Gut symbionts were considered more likely to interfere with the interactions of insects and their host plants (Hansen and Moran 2014). Lepidopteran insects are highly adapted to plant exploitation, but as far as we know no information is available about lepidopterans acting as vectors of plant pathogens during their plant tissue feeding stage, although several bacteria in the oral secretions and feces of larval lepidopterans were shown to interact and induce plant responses (Ray et al. 2015; Acevedo et al. 2017; Wang et al. 2018).

The gut microbiota of lepidopteran larvae is highly affected by changes in the host diet (Colman et al. 2012; Mason et al. 2020), and core members have been identified in the gut microbiota of several lepidopteran species (Higuita Palacio et al. 2021; Paniagua et al. 2018; Shao et al. 2024; but see Hammer et al. 2017 for contradictory data). The core community of the microbiome of lepidopterans has been shown to be beneficial to the host insect, particularly by supporting food digestion and metabolization of food-associated xenobiotics (Paniagua et al. 2018; Shao et al. 2024). Nonetheless, lepidopteran larvae also carry gut symbionts that trigger plant-defensive mechanisms, affecting their successful exploitation of plants (Ray et al. 2015; Acevedo et al. 2017; Wang et al. 2018). This is counter-adaptive to the lepidopteran host, as these symbionts benefit the plant the host insect is exploiting, snitching the herbivore activity. However, lepidopterans also carry gut symbionts that promote plant growth (Indiragandhi et al. 2008).

The genus *Spodoptera* (Lepidoptera: Noctuidae) is native to Africa and is composed of 31 species that evolved in the New and Old World (Kergoat et al. 2021). Several *Spodoptera* species are highly polyphagous and have become invasive to new continents, where they cause serious threats to food security due to their destructive potential and resilience to the control tactics available (Hilliou et al. 2021). *Spodoptera frugiperda* is currently the most widespread *Spodoptera* species (Tay et al. 2023), and it has been shown to carry a variety of gut symbionts capable of metabolizing different types of insecticides (Almeida et al. 2017; Gomes et al. 2020). *Enterococcus* sp. are the major core members of *S. frugiperda* gut microbial community, similarly to other *Spodoptera* species (Chen et al. 2016; Xia et al. 2020; Oliveira et al. 2023). Comparative genomic analyses of the gut-associated *Enterococcus* species of *S. frugiperda* demonstrated their potential to contribute to their host in several ways, but also pointed out that they carry several virulence factors common to plant and insect pathogens (Gomes et al. 2023). However, there is no information on whether these *Enterococcus* species interfere with the host plant and could affect the interactions between maize plants and *S. frugiperda* larvae.

In this study, we aimed to understand how the core (resident–microbes that are constantly associated with its host) *Enterococcus* species of the gut microbiota of *S. frugiperda* interact with the host plant and affect the interactions between the plant and the *S. frugiperda* larvae. We hypothesized that *S. frugiperda* gut-resident symbionts would induce alterations in maize plants to benefit the host larval survival and development, as opposed to a transient, temporarily associated gut symbiont of *S. frugiperda* larvae and a maize endophyte. We infected plants with these gut symbionts and compared several plant and larval biological traits to control plants colonized with a maize endophyte and an actinomycete bacterium. Finally, we also investigated the emission of volatile organic compounds (VOCs) of maize plants colonized with such microbes and their subsequent effect on *S. frugiperda* female ovipositional preference.

Material and methods

Bacterial strains

We selected three of the most common isolates from the gut of *S. frugiperda* to investigate whether their interactions with *Zea mays* (maize) plants would interfere with the response plants produced against herbivore attack. Two of these isolates belong to *Enterococcus*, *E. spodopteracolus* (IIL-Sfm05) and *E. entomosocium* (IILSfc-sus01), and one belongs to *Bacillus* (IIL-Sfb05—closest hit GenBank accession no.: KX280776). Two additional isolates were selected as controls. The maize endophyte identified as *Rhizobium larrymoorei* IILzm-Idp03 (Preto 2018) and the ant-associated *Streptomyces novaecaesareae* IIL-Asp45 (Martinez et al. 2017).

The selected isolates were grown in tryptic soy broth (TSB) at 28 °C under constant agitation (120 rpm) to reach a turbidity of OD_{600} from 0.9 to 1.4. Samples were then used in micro-colony assays to determine the number of colony-forming units (CFU) (Green and Goldman 2021). Cells were

pelleted by centrifugation (3000 g for 5 min), washed in minimum medium 9 (MM9) and re-suspended in MM9 to a final concentration of 10^8 colony-forming units (CFU).mL^{-1}.

Effects of selected bacteria in maize plants

Assessing the role of selected bacteria on maize growth

The effects of the selected bacteria on plant growth were assessed through (1) seed inoculation, (2) soil application and (3) leaf application. All experiments were conducted using the commercial maize hybrid 30F53 Pioneer (Pioneer Co.). Seeds were surface sterilized twice in 70% ethanol (4 min), followed by 5% sodium hypochlorite (4 min), and two final rinses in sterile water (4 min each).

Seed inoculation followed Müller and Berg (2008). Briefly, 150 seeds were incubated in 250-mL bacterial suspensions (10^8 CFU.mL^{-1}) under constant agitation (20 °C × 12 h × 100 rpm) and dried on filter paper at 20 °C for 24 h. The soil application of bacteria was done by irrigating potted seeds in 1.5 L of substrate with 150 mL bacterial solutions (2.9×10^7 CFU.mL^{-1}). Bacterial inoculation by leaf application followed Ray et al. (2015) after sanding a 15 mm in diameter surface area of the last fully expanded leaf with sandpaper and treating it with 50 µL 10^8 CFU.mL^{-1} bacterial solutions. Controls were obtained by reproducing each inoculation procedure using sterile MM9.

Plants were grown in 1.5-L plastic pots (12 × 14 × 8 cm) containing autoclaved vegetable soil (Fert Solo, Brazil) under greenhouse conditions. Plants were equally irrigated and treated on a weekly basis with Hoagland and Snyder (1933) micro and macronutrient solutions [100 mL 0.83 g/L $MgSO_4$, 0.36 g/L KNO_3, 0.14 g/L $NH_4H_2PO_4$, 0.02 g/L $ZnSO_4.H_2O$, 0.03 g/L $CuSO_4.5H_2O$ and 0.09 g/L $C_{18}H_{18}FeN_2O_6^{+2}$; and 200 mL 0.08 g/L NH_4NO_3 and 0.91 g/L $Ca(NO_3)_2$]. The effects of plant inoculation with the selected bacterial isolates were verified at the V8 stage 30 days after emergence (DAE) by assessing the plant height (cm), stem diameter (mm) and the number of expanded leaves. Afterward, plants were unpotted and stem and leaves (aerial part) were separated from roots and washed under running water to remove any debris. The aerial part and the roots were separated in paper bags and dried to a constant weight (65 °C) before determination of the dry mass.

Experiments were conducted with a completely randomized factorial design with bacterial isolates (six levels = *Bacillus* sp., *E. spodopteracolus, E. entomosocium, R. larrymoorei, S. novaecaesareae* and control treatment) and type of bacterial inoculation (three levels = seed inoculation, leaf treatment and soil treatment) as factors. Control treatments were represented by plants that were not subject to inoculation with bacteria. The experiment was done with eight replicates per treatment. Each replicate consisted of a pool of three plants, with each of them seeded in individual pots.

Assessing the role of selected bacteria on the expression of targeted maize genes

The role of selected bacteria in priming the expression of a set of maize genes involved in plant response to stressors was assessed by RT-qPCR (*ETR2*—ethylene receptor 2; *GDPS*—geranylgeranyl-diphosphate synthase; *MPI*—maize protease inhibitor; *RIP2*—ribosome-inactivating protein; *TBP1*—TATA-box-binding protein 1; *WRKY1*—WRKY transcription factors) (Online Supplementary Material 1—Table S1). Plant samples (2.5 × 2.5 cm) were collected from the median region of the last fully expanded leaf and placed directly into liquid nitrogen, homogenized in TissueLyser (Qiagen) at 20 Hz.s^{-1} for 1 min and subjected to total RNA extraction using TRIzol reagent (Life Technologies, USA). One microgram of total RNA was used for single-strand cDNA (sscDNA) synthesis using the GoScript Reverse Transcription System (Promega) with 0.5 µg.μL^{-1} oligo-dT. sscDNA was used as a template to analyze the expression the six selected genes involved in maize stress response (Online Supplementary Material 1—Table S1). β-Tubulin was selected as the reference gene. Gene-specific primers were designed with Primer Express 3.0.1 (Life Technologies) (Online Supplementary Material 1—Table S1) based on *Z. mays* sequences. RT-qPCRs were performed with SYBR Green Master Mix (Life Technologies) on a ViiA™ 7 Real-Time PCR System (Applied Biosystems®, Life Technologies©) set with a pre-incubation at 95 °C (10 min), followed by 45 cycles consisting of denaturation at 95 °C for 10 s, followed by annealing at 60 °C for 10 s. Samples were analyzed using three biological independent replicates (1 replicate = 1 tissue sample), and each biological replicate was run in technical triplicates. Differential gene expression among samples was calculated according to Pfaffl (2001).

Assessment of maize colonization by selected bacterial symbionts

The colonization of maize plants with the selected bacterial symbionts was confirmed after pGFP transformation (Clontech, Takara) of each bacterial isolate investigated, using the method of Friesenegger et al. (1991) with few adaptations for each bacterial isolate. Shortly, cells of the selected isolates were cultivated in TSB at 28 °C under constant agitation (120 rpm), harvested after centrifugation (15 min; 5000 g) and washed to obtain 10^{10} CFU.mL^{-1}. Cells of *E. entomosocium* IILSfc-sus01 were serially washed with different volumes of 10% glycerol solution (1 L, 500 mL, 200 mL, 20 mL and 2 mL); *E. spodopteracolus* IIL-Sfm05 were washed 3x-1 L, 3x-500 mL,

3x-200 mL and 1 × with 20 mL and 2 mL); *Bacillus* IIL-Sfb05 were washed once with 1 L, 500 mL and 200 mL of 1 mM Hepes (pH = 7.0), followed by washes in milliQ-water and 10% glycerol in 1 mM Hepes. Cells of *R. larrymoorei* IIL-Idp03 and *S. novaecaesareae* IIL-ASP45 were washed in the same solutions used for *Bacillus,* but with two washes of each volume of water and in 10% glycerol in 1 mM Hepes. Cells were stored in 100 µL aliquots at -80° C for later transformation. Cell transformation used 50 µL of cell suspension and 200 ng.µL^{-1} of *pGFP* plasmid for *E. entomosocium*, *E. spodopteracolus*, and *Bacillus*, 500 ng.µL^{-1} for *R. larrymoorei* and 1 µg.µL^{-1} for *S. novaecaesareae* transformation in a 2 mm (Pulse M1) electroporation cuvette exposed to an instant pulse at 1.8 kV (Bio-Rad, USA). The cells were immediately transferred to 1 mL super optimum broth with catabolite repression (SOC) and incubated (1 h) under continuous shaking (30 °C × 120 rpm). Finally, the bacteria were plated on tryptic soy agar (TSA) medium containing 100 mg.L^{-1} ampicillin. Transformants were detected up to 36 h after plating.

The selected transformants were cultivated (28 °C × 24 h × 120 rpm) in 300 mL TSB containing ampicillin to the concentration of 10^8 CFU. The number of CFU was determined by micro-colony assays (Green and Goldman 2021). Then, each isolate was individually inoculated in maize seeds (150 seeds) and sown as described above. Plants were grown in a greenhouse in separate plots to avoid transmission from plant to plant. Samples of roots and leaves were taken six days after plant emergence and immediately processed for visualization under a Nikon C2 + laser scanning confocal at an excitation wavelength of 488 nm. Part of the samples (100 mg of roots and leaves) were subjected to qPCR for confirmation and quantification of *gfp*-transformed bacteria. Total DNA extraction from leaves and roots of maize plants followed Doyle and Doyle (1987).

The quantification of *gfp*-transformed bacteria was done by targeting the *gfp* gene using the set of primers GFPII (F- GTCAGTGGAGAGGGTGAAGG and R- CCTGTACAT AACCTTCGGGC) and the SYBR Green Master Mix (Life technologies). qPCRs were set at 95 °C (10 min–1x), followed by 40 cycles consisting of denaturation at 95 °C for 10 s and annealing at 62 °C for 10 s in a ViiA™ 7 Real-Time PCR System. Samples were analyzed in biological replicates (3), and each biological replicate was run in technical triplicates. The number of bacterial cells in plant tissues was determined using the absolute quantification procedure of Whelan et al. (2003), using a standard curve constructed with known number of copies of the target gene (*gfp*) (= number of bacterial cells) using serial dilutions of *pGFP* plasmids. The determination of the number of bacteria in maize samples assumed that each p*GFP*-transformed bacterium cell carried a single copy of the *GFP* gene.

Effects of seed inoculation with selected microbial isolates on *Spodoptera frugiperda*

Based on our data on the effects of the inoculation methods on the plant growth and development, and on the colonization of the roots and leaves of maize plants by all tested bacteria, all further experiments were conducted only with seed-inoculated bacteria.

Effects on larval survival

Seed inoculation and plant cultivation were done as earlier described. Newly molted second instars of *S. frugiperda* were individually reared in plastic containers (50 mL) lined with moistened cotton pads and fed leaf disks (22 mm in diameter) ad libitum under controlled conditions (24 ± 1 °C; 60 ± 10% RH; 14:10 L:D photoperiod). Food availability was check on a daily basis, and larval mortality was assessed at the onset of the prepupa stage. The experimental design was completely randomized, with 30 replicates (1 larva/container = 1 replicate) per treatment.

Effects on larval nutritional indices

The nutritional indices relative consumption rate (RCR), relative growth rate (RGR), relative metabolic rate (RMR), conversion efficiency of ingested food (ECI), conversion efficiency of digested food (ECD), approximate digestibility (AD) and metabolic cost (MC) of *S. frugiperda* larvae were assessed from early second to late fourth instar feeding leaf disks (22 mm in diameter) from the middle part of leaves from seed-inoculated maize plants following Waldbauer (1968), modified by Scriber and Slansky (1981) (Online Supplementary Material 1—Table S2). The experimental design was completely randomized with 20 replicates/treatment (1 larva per container = 1 replicate).

Effects on adult oviposition preference

The effects of the association plant–bacteria–*S. frugiperda* were assessed under laboratory and semi-field experiments. In laboratory assays, newly emerged (0–24 h-old) moths were coupled and maintained for 24 h in PVC cylinders (10 × 22 cm) for mating. Afterward, ventilated cages (45 × 55 × 45 cm) containing one inoculated and one control maize plant were infested with one *S. frugiperda* couple (24–48 h-old). Adults remained in contact with plants for 72 h, when they were removed and the number and distribution of the eggs laid were assessed. Each cage containing two plants was considered a replicate, and 24 replicates were used for each treatment.

Mated moths were also used in semi-field experiments. The effects of seed inoculation with selected bacteria on

oviposition preference of *S. frugiperda* were assessed in free-choice assays using 6 m^3 cages (2.0 m long × 2.0 m wide × 1.5 m high) set under field conditions. Each cage received three of each inoculated and control plants at the V8 stage, totaling 18 plants per cage. Seeds were inoculated as before. Five mated *S. frugiperda* couples were released in each cage, and females were allowed to oviposit for 72 h, when the number of egg masses and the total number of eggs per egg mass were recorded. Seven replicates used with each cage in which five *S. frugiperda* couples were released were considered a replicate.

Effects of microbes isolated from insects on volatile emission of inoculated maize plants

Maize seeds were inoculated with the selected insect-associated bacteria (IILSfm05, IILSfc-sus01, IIL-Sfb05 and IIL-ASP45) as described previously. Subsequently, they were sown in 500-mL pots filled with autoclaved soil. Plants were maintained individually in cultivation cages and irrigated whenever necessary. At 20 DAE, plants were individually placed in glass chambers of a volatile collection system. Each glass chamber was connected with Tenax® TA tubes (Supelco) to a compressed breathing air cylinder (medical grade) equipped with a valve and a pressure gauge. Each glass chamber had an entry point for ventilation laterally located at its base and an exit opening at the top for volatile collection. Chambers were ventilated with 1 L breathing air/min for 5 h, and volatiles released during ventilation were collected using Tenax® TA tubes (6.35 mm wide × 8.89 cm long, stainless steel TD tube, unconditioned, 35–60 mesh; Supelco cat# 30,131-U, USA) connected to the exit opening at the top of the chambers. Then, the cartridges containing the collected volatiles were subjected to GC/MS analysis in a Shimadzu GC/MS 2010 Plus. Analyses were carried in the split mode, the injector chamber was set at 250 °C, and a BP-1 capillary column (SGE, USA) (0.25 mm × 30 m × 0.25 µm, 100% polydimethylsiloxane) was used for compound separation. Helium was used as the carrier gas at an internal pressure of 15 psi. The GC oven temperature was programmed to operate from 50 to 180 °C, starting at 50 °C for 2 min and then increasing to 180 °C at 4 °C.min^{-1}. The detector temperature was set at 280 °C. Mass spectra were acquired over a mass range of 50–600 m/*z*, with the electron impact ionization chamber set at 70 eV. Compounds were identified by comparing experimental mass spectra with those from the NIST62.LIB mass spectra library, and chemical nomenclature was confirmed following ACD/MS (ACD/Labs, Toronto, Canada). Only metabolites with scores of 700 or higher were considered. The intensity of each metabolite was normalized by the total ion count (TIC) of each sample.

Statistical analysis

Data were first tested for normality (Shapiro and Wilk 1965) and homoscedasticity (Hartley 1950). Data on plant height, stem width, number of fully expanded leaves, total dry mass, dry mass of the aerial part and roots, and the root-to-shoot ratio (R/S) of plants inoculated with selected bacterial isolates using different techniques were then subjected to 2-way ANOVA (factors "treatment" and "application" and their interaction), followed by post hoc analyses using Tukey test ($p < 0.05$).

Regarding *S. frugiperda* immature mortality when feeding on plants inoculated with different bacterial isolates, some treatments lead to no mortality. Hence, these data were analyzed by a so-called quasi-logistic regression for binomial data. This corresponds to a logistic regression using a quasi-logit instead of a standard link function (MacCullagh and Nelder 1989). Statistical comparisons were made using standard likelihood ratio tests, followed by Tukey tests ($p < 0.05$). The nutritional indices obtained for *S. frugiperda* and the differential expression of maize genes were subjected to ANOVA followed by post hoc analyses using Tukey test ($p < 0.05$). Data collected on plant growth and development, nutritional indices and mortality obtained for *S. frugiperda* larvae, and gene expression levels in maize plants inoculated with selected isolates were used to perform a principal component analysis (PCA) and hierarchical grouping using the Ward's method.

Oviposition preference data for *S. frugiperda* adults in laboratory assays were analyzed with a polytomous regression (a logistic regression for multinomial data) using standard likelihood ratio tests followed by Tukey tests ($p < 0.05$) testing both global contrasts between treatments and contrasts between treated plants, control plants and cages. Oviposition preference data for *S. frugiperda* adults in the semi-field were analyzed using a Generalized Linear Mixed Model (GLMM) for Poisson-distributed data with cages as a random effect and treatments as a fixed effect, followed by Tukey tests ($p < 0.05$). All statistical analyses were conducted using the R program (R Core Team 2023).

Analysis of the VOCs collected from inoculated and control maize plants was conducted using the web-based pipeline for metabolomic analysis MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) (Chong et al. 2019). The peak intensity data obtained from the GC–MS analysis were normalized and subjected to $\log_2$-transformation and Pareto scaling, using the mean-centered divided by the square root of the standard deviation to scale each variable. Partial least square discriminant analysis (PLS-DA) was used to describe the grouping of the different volatile profiles observed. Pairwise analysis was performed by individually comparing each treatment against the control using t-tests as implemented in MetaboAnalyst.

Only the volatiles with a $\log_2$ fold-change (FC) ≥|2.0| and a false discovery ratio (FDR) of $p < 0.05$ were considered significantly different. We also report the variable importance in projection (VIP) scores obtained as a measure of importance in PLS-DA analysis for volatiles that were significantly different among treatments.

Results

Effects of bacterial application in the initial growth of maize plants

The bacterial isolates investigated affected plant height ($F_{5, 143} = 23.24$; $p < 0.001$), stem diameter ($F_{5, 143} = 19.35$; $p = 0.001$) and the number of leaves ($F_{5, 143} = 6.33$; $p = 0.004$). The procedure used to inoculate the bacterial isolates tested also affected plant height ($F_{2, 143} = 3.95$; $p = 0.0215$), stem diameter ($F_{2, 143} = 12.24$; $p = 0.001$) and the number of leaves ($F_{2, 143} = 15.47$; $p < 0.001$). The bacterial isolate interacted with the inoculation procedure used to affect plant height ($F_{10, 143} = 10.13$; $p < 0.001$), stem diameter ($F_{10, 143} = 10.32$; $p < 0.001$) and the number of leaves ($F_{10, 143} = 6.34$; $p < 0.001$) (Table 1; Online Supplementary Material 1—Table S3). The gut-resident symbionts, *E. spodopteracolus* (IIL-Sfm05) and *E. entomosocium* (IILSfc-sus01), and the gut-transient symbiont of *S. frugiperda, Bacillus* (IIL-Sfb-05) stimulated the growth of one or more of the maize traits assessed (Table 1). The plant growth-promoting capacity of these bacteria was similar or better than that observed for the maize endophyte *R. larrymoorei* IILzm-Idp03 (Table 1).

The total dry mass (TDM), aerial dry mass (ADM) and root dry mass (RDM) were affected by the bacterial isolate (TDM: $F_{5, 143} = 5.97$; $p < 0.001$; ADM: $F_{5, 143} = 5.17$; $p = 0.029$; RDM: $F_{5, 143} = 8.57$; $p < 0.001$), the mode of inoculation (TDM: $F_{2, 143} = 15.48$; $p < 0.0001$; ADM: $F_{2, 143} = 13.22$; $p < 0.0001$; RDM: $F_{2, 143} = 67.81$; $p < 0.0001$) and the interaction between these two factors (TDM: $F_{10, 143} = 9.24$; $p < 0.0001$; ADM: $F_{10, 143} = 8.51$; $p < 0.0001$; RDM: $F_{10, 143} = 5.94$; $p < 0.0001$) (Table 2; Online Supplementary Material 1—Table S4). The mode of inoculation affected the total and the aerial dry weight of plants for each bacterial treatment in a way very similar to what was observed for the maize traits reported earlier (Table 2).

The dry mass root-to-shoot (R:S) ratio was also affected by the bacterial isolate ($F_{5, 143} = 5.52$; $p = 0.014$) and the application method ($F_{2, 143} = 21.87$; $p < 0.0001$), but not by the interaction between these two factors ($F_{10, 143} = 3.18$; $p = 0.119$) (Table 2, Online Supplementary Material 1—Table S4). R:S ratio was positively affected only when plants had their seeds inoculated with *Bacillus* IIL-Sfb05 or had it inoculated by leaf application (Table 2).

Table 1 Height (cm), stem diameter (mm) and leaf number of maize plants with 30 DAE (mean ± SD), inoculated with selected bacterial isolates using three types of inoculation: seed inoculation, soil and leaf application (IIL-Sfm05 = *E. spodopteracolus*; IILSfc-sus01 = *E. entomosocium*; IIL-Sfb05 = *Bacillus* sp.; IILzm-Idp03 = *R. larrymoorei*; IIL-ASP45 = *S. novaecaesareae*; Control = non-inoculated plants)

	Seed inoculation	Soil application	Leaf application
Height (cm)			
IIL-Sfm05	67.45 ± 0.80 ABab	69.85 ± 0.97 Aa	64.53 ± 0.92 Bab
IILSfc-sus01	63.94 ± 0.73 Bb	65.55 ± 0.61 ABab	69.24 ± 0.71 Aa
IIL-Sfb05	58.03 ± 1.37 Bc	65.85 ± 0.68 Aab	62.90 ± 1.25 Ab
IILzm-Idp03	71.36 ± 0.72 Aa	65.00 ± 0.70 Bab	60.05 ± 0.63 Cb
IIL-ASP45	50.54 ± 1.09 Bd	58.93 ± 1.07 Ac	61.90 ± 0.38 Ab
Control	63.10 ± 0.46 Abc	62.23 ± 0.55 Abc	62.04 ± 0.71 Ab
Stem diameter (mm)			
IIL-Sfm05	5.05 ± 0.05 Aab	4.88 ± 0.06 Aa	4.39 ± 0.06 Bab
IILSfc-sus01	4.61 ± 0.07 Bbc	5.16 ± 0.05 Aa	4.76 ± 0.06 Ba
IIL-Sfb05	4.06 ± 0.06 Bde	4.91 ± 0.10 Aa	4.53 ± 0.08 Aa
IILzm-Idp03	5.30 ± 0.03 Aa	4.84 ± 0.09 Bab	4.16 ± 0.06 Cbc
IIL-ASP45	3.84 ± 0.10 Be	4.11 ± 0.09 ABc	4.42 ± 0.03 Aa
Control	4.51 ± 0.08 Acd	4.37 ± 0.05 ABbc	4.01 ± 0.07 Bc
Leaf number			
IIL-Sfm05	7.50 ± 0.13 Aa	7.25 ± 0.08 Aab	6.43 ± 0.11 Ba
IILSfc-sus01	7.50 ± 0.11 Aa	7.12 ± 0.05 ABab	6.68 ± 0.10 Ba
IIL-Sfb05	6.50 ± 0.10 Bbc	7.50 ± 0.11 Aa	6.75 ± 0.10 Ba
IILzm-Idp03	7.56 ± 0.11 Aa	7.00 ± 0.00 Aab	6.25 ± 0.08 Ba
IIL-ASP45	6.31 ± 0.23 Ac	6.62 ± 0.09 Abc	6.68 ± 0.10 Aa
Control	7.06 ± 0.03 Aab	6.12 ± 0.05 Bc	6.37 ± 0.15 Ba

Means followed by the same uppercase letters in rows, or lowercase letters in columns, do not differ statistically from each other by the Tukey test ($p > 0.05$)

Effects of seed inoculation on larval survival of *Spodoptera frugiperda*

Nearly 30% of larval mortality of *S. frugiperda* was observed in control plants (Fig. 1A). Similar values were observed for larvae feeding on maize plants inoculated with *E. spodopteracolus* or *E. entomosocium*. However, very high mortalities were observed for larvae feeding on plants inoculated with *Bacillus* sp. or *S. novaecaesareae* (Fig. 1A). Plants inoculated with *R. larrymoorei* induced intermediate levels of larval mortality, but not different from that obtained in *E. spodopteracolus* or *E. entomosocium* inoculated plants (Fig. 1A, Online Supplementary Material 1—Table S5).

Table 2 Dry weight (g) of whole plants, the aerial part, the root system and dry mass root-to-shoot (R/S) ratio of maize plants 30 DAE (mean ± SD), inoculated with selected bacterial isolates using three types of inoculation: seed inoculation, soil and leaf application (IIL-Sfm05 = *E. spodopteracolus*; IILSfc-sus01 = *E. entomosocium*; IIL-Sfb05 = *Bacillus* sp.; IILzm-Idp03 = *R. larrymoorei*; IIL-ASP45 = *S. novaecaesareae*; Control = non-inoculated plants)

	Seed inoculation	Soil application	Leaf application
Total dry weight (g)			
IIL-Sfm05	6.4 ± 0.20 Aab	5.4 ± 0.27 Abc	5.3 ± 0.09 Abc
IILSfc-sus01	6.1 ± 0.18 Ab	6.3 ± 0.32 Aab	6.3 ± 0.06 Aa
IIL-Sfb05	4.4 ± 0.27 Bc	7.2 ± 0.27 Aa	4.2 ± 0.05 Bc
IILzm-Idp03	7.5 ± 0.09 Aa	5.0 ± 0.22 Bbc	5.6 ± 0.10 Bab
IIL-ASP45	5.8 ± 0.38 Ab	5.1 ± 0.17 Abc	4.7 ± 0.10 Abc
Control	6.6 ± 0.25 Aab	4.7 ± 0.21 Bc	4.1 ± 0.04 Bc
Aerial part dry weight (g)			
IIL-Sfm05	5.6 ± 0.20 Aab	4.9 ± 0.25 Abc	4.6 ± 0.09 Abc
IILSfc-sus01	5.3 ± 0.17 Ab	5.6 ± 0.23 Aab	5.7 ± 0.17 Aa
IIL-Sfb05	3.7 ± 0.27 Bc	6.6 ± 0.31 Aa	3.5 ± 0.14 Bc
IILzm-Idp03	6.7 ± 0.09 Aa	4.6 ± 0.22 Bbc	4.9 ± 0.14 Bab
IIL-ASP45	5.1 ± 0.39 Ab	4.6 ± 0.14 Abc	4.1 ± 0.10 Abc
Control	5.8 ± 0.26 Aab	4.3 ± 0.22 Bc	3.6 ± 0.05 Bbc
Root system dry weight (g)			
IIL-Sfm05	0.81 ± 0.01 Aa	0.52 ± 0.02 Cbc	0.68 ± 0.010 Ba
IILSfc-sus01	0.79 ± 0.01 Aa	0.66 ± 0.02 Ba	0.64 ± 0.004 Bab
IIL-Sfb05	0.66 ± 0.01 Abc	0.60 ± 0.04 Ab	0.68 ± 0.006 Aa
IILzm-Idp03	0.81 ± 0.02 Aa	0.41 ± 0.02 Cc	0.63 ± 0.005 Bab
IIL-ASP45	0.60 ± 0.02 Ac	0.50 ± 0.03 Abc	0.57 ± 0.009 Aab
Control	0.76 ± 0.01 Aab	0.47 ± 0.02 Bc	0.53 ± 0.006 Bb
Root-to-shoot (R/S) ratio			
IIL-Sfm05	0.14 ± 0.004 Ab	0.10 ± 0.004 Aa	0.14 ± 0.004 Aab
IILSfc-sus01	0.15 ± 0.004 Aab	0.12 ± 0.009 Aa	0.11 ± 0.005 Bb
IIL-Sfb05	0.19 ± 0.017 Aa	0.09 ± 0.008 Ba	0.19 ± 0.006 Aa
IILzm-Idp03	0.12 ± 0.003 Ab	0.09 ± 0.005 Aa	0.12 ± 0.004 Ab
IIL-ASP45	0.11 ± 0.012 Ab	0.10 ± 0.005 Aa	0.14 ± 0.004 Ab
Control	0.13 ± 0.007 Ab	0.11 ± 0.009 Aa	0.14 ± 0.003 Aab

Means followed by the same uppercase letters in rows or lowercase letters in columns do not differ from each other by the Tukey test ($p > 0.05$)

Effects of seed inoculation on larval nutritional indices of *Spodoptera frugiperda*

All seed-inoculated maize plants affected the nutritional indices of *S. frugiperda* larvae, except those inoculated with *E. spodopteracolus*. The nutritional indices of larvae feeding on *Bacillus*-inoculated plants could not be calculated because all larvae died before completing the larval growth period (Table 3). The relative consumption rate (RCR) was the least affected nutritional index. Only *S. novaecaesareae*-inoculated plants reduced the RCR of *S. frugiperda* larvae, with plants inoculated with *E. spodopteracolus*, *E. entomosocium* and *Rhizobium* resulting in intermediate RCR values (Table 3). Larvae fed on plants inoculated with *E. entomosocium*, the maize endophyte *Rhizobium* and *S. novaecaesareae* had the highest metabolic costs (Table 3). The efficiency of conversion of the ingested (ECI) and digested (ECD) food, the approximate digestibility (AD) and the relative growth rate (RGR) of larvae fed on plants inoculated with these bacteria were the most affected (Table 3).

Effects of seed inoculation on maize gene expression

Differences in *MPI* gene expression were much higher in *Bacillus* when compared to *Rhizobium*, and *S. novaecaesareae*-inoculated plants (Fig. 1B). *RIP2* expression in *Bacillus*-, *Rhizobium*- and *S. novaecaesareae* was down-regulated when compared to control and *E. entomosocium*-inoculated plants (Fig. 1C). *GDPS* expression was drastically reduced in all inoculated when compared to control plants (Fig. 1D). *ETR* expression was only affected in plants inoculated with *E. entomosocium*, *Bacillus* or *R. larrymoorei* when compared to control plants (Fig. 1G). No differences in *WRKY1* and *EIN3* expression were observed (Fig. 1E, H).

Maize colonization after seed inoculation

GFP-transformed cells of all isolates tested were observed colonizing roots of maize plants by confocal microscopy, but not stems and leaves (Online Supplementary Material 1—Figure S1). Nevertheless, all GFP-transformed isolates were detected and quantified in roots and leaves by qPCR. The density of all tested isolated was higher in roots than in leaves at day 6 after maize plant emergence (Online Supplementary Material 1—Figure S2).

Effects of seed inoculation on *S. frugiperda* adult oviposition preference

Laboratory cage experiments demonstrated bacterial seed inoculation altered *S. frugiperda* adult females' behavioral decisions regarding both the number of egg masses ($\chi^2 = 43.0$; $df = 8$; $p < 0.001$) and the number of eggs laid ($\chi^2 = 3449.7$; $df = 8$; $p < 0.001$) in inoculated or control plants or in the cage structure. The highest number of egg masses was laid in the *E. entomosocium* and *E. spodopteracolus* treatments, with the lowest number being observed in *S. novaecaesareae* and *Bacillus* treatments. Intermediate values of egg masses were observed in *R. larrymoorei*-treated plants (Fig. 2A). Nevertheless, the highest number of eggs was laid in *E. entomosocium* and the lowest in *R. larrymoorei* treated plants (Fig. 2A).

Comparisons of the number of egg masses and eggs laid on inoculated or control plants or on the cage identified significant differences among treatments. The number of egg

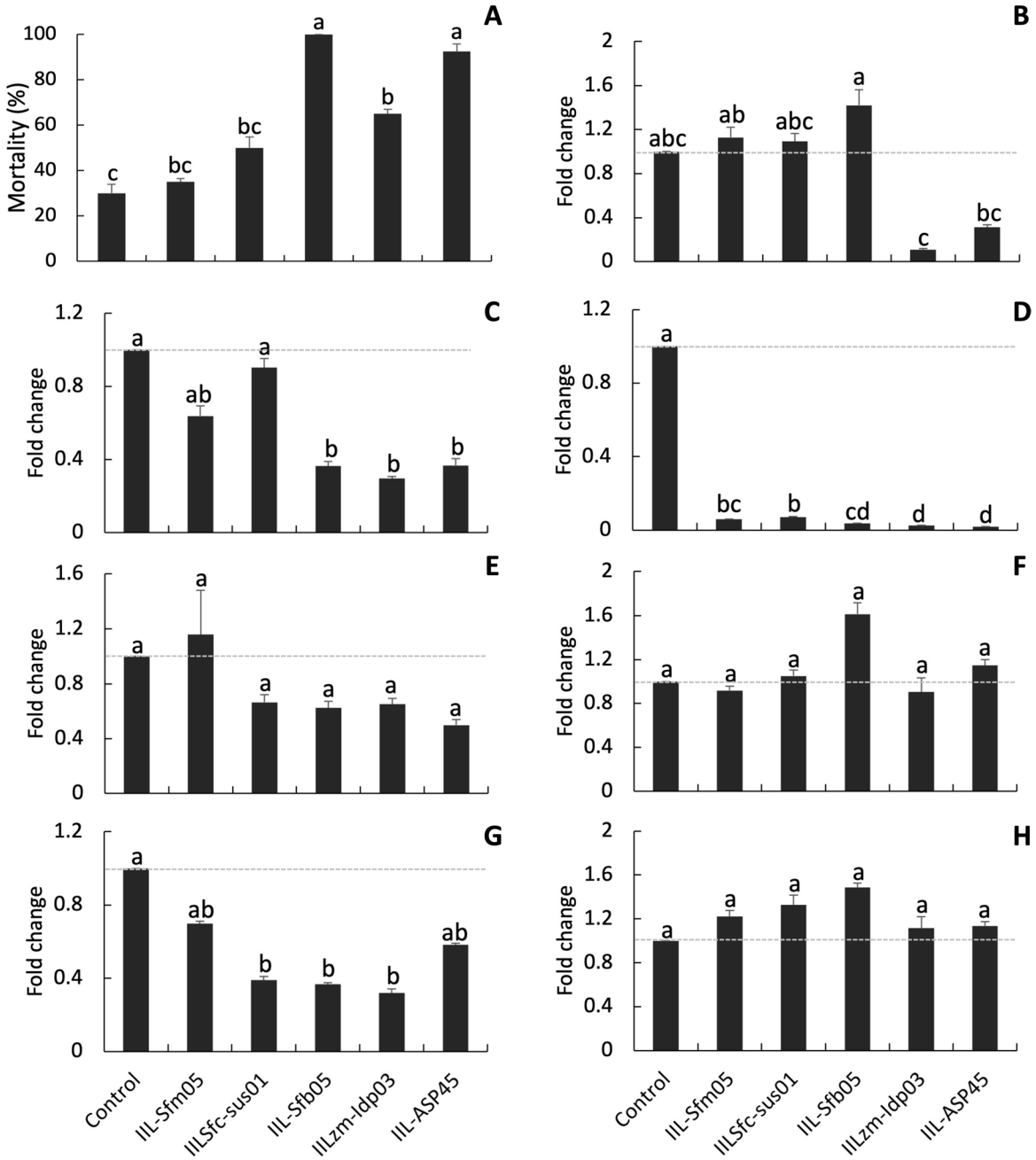

Fig. 1 Mortality of *Spodoptera frugiperda* and analysis of expression of genes of maize plants. **A** Mortality (%) of *S. frugiperda* larvae fed leaves of maize plants inoculated with different bacteria. Relative expression of genes analyzed by RT-qPCR of maize plants inoculated with different bacteria (IIL-Sfm05 = *E. spodopteracolus*; IILSfc-sus01 = *E. entomosocium*; IIL-Sfb05 = *Bacillus* sp.*;* IILzm-Idp03 = *R. larrymoorei*; IIL-ASP45 = *S. novaecaesareae*) compared to control plants (non-inoculated plants). The genes analyzed were: **B** Proteinase inhibitor in maize (MPI), **C** Ribosomal inactivation protein (RIP2), **D** Truncated geranylgeranyl-diphosphate synthase (GDPS), **E** WRKY1-transcription factor (WRKY1), **F** TATA-box-binding protein (TBP1), **G** ethylene receptor (ETR2) and **H** ET-insensitive-3-like (EIN3). The data represented are mean values with error bars (mean ± SD). (Tukey's test, $p < 0.05$)

masses laid on plants inoculated with *E. entomosocium* did not differ from that laid on plants inoculated with *E. spodopteracolus*, *R. larrymoorei* or *S. novaecaesareae*, but the total number of eggs laid on *E. entomosocium* was significantly higher than the number of eggs laid in the remaining inoculated plants. The lower number of egg masses and eggs was laid on *Bacillus*-inoculated plants. The majority of egg masses and eggs in *Bacillus*, *S. novaecaesareae* and *R. larrymoorei*-treated plants were laid on the cage (Fig. 2A; Online Supplementary Material 1—Table S6).

Table 3 Relative consumption rate (RCR), relative growth rate (RGR) and relative metabolic rate (RMR) in mg/mg/day, conversion efficiency of ingested food (ECI), conversion efficiency of digested food (ECD), approximate digestibility (AD) and metabolic cost (MC) for larvae *Spodoptera frugiperda* from second to fourth instar, fed with leaves of maize plants inoculated with different bacteria (mean ± SD) (IIL-Sfm05 = *E. spodopteracolus*; IILSfc-sus01 = *E. entomosocium*; IILzm-Idp03 = *R. larrymoorei*; IIL-ASP45 = *S. novaecaesareae*; Control = non-inoculated plants). (Tukey's test, $p < 0.05$)

Treatments	RCR mg/mg/d	RGR	RMR	ECI %	ECD	AD	MC
IIL-Sfm05	0.2553 ± 0.009 ab	0.1610 ± 0.007 b	0.0514 ± 0.007 c	63.68 ± 2.58 a	76.44 ± 3.05 a	72.54 ± 2.39 ab	23.55 ± 3.05 c
IILSfc-sus01	0.2568 ± 0.009 ab	0.1088 ± 0.005 c	0.1125 ± 0.007 b	42.79 ± 1.80 b	49.90 ± 2.23 b	66.18 ± 1.76 b	50.09 ± 2.30 b
IILzm-Idp03	0.2757 ± 0.015 ab	0.0626 ± 0.006 d	0.1802 ± 0.016 a	24.06 ± 2.67 c	28.01 ± 3.35 c	40.77 ± 4.54 c	71.98 ± 3.35 a
IIL-ASP45	0.2418 ± 0.010 b	0.0357 ± 0.002 d	0.1778 ± 0.007 a	14.93 ± 0.79 c	16.99 ± 1.01 c	22.04 ± 3.64 d	83.00 ± 1.01 a
Control	0.3152 ± 0.012 a	0.2190 ± 0.005 a	0.0598 ± 0.009 bc	70.62 ± 2.12 a	79.99 ± 2.48 a	83.34 ± 0.77 a	20.01 ± 2.48 c

In free-choice field-cage experiments, female moths laid a similar number of egg masses in inoculated and control plants. However, the number of eggs laid on each plant was highly different. A very strong ovipositional stimulatory effect was observed in *E. entomosocium*-inoculated plants, while an ovipositional deterrent effect was seen for the remaining inoculated plants when compared to control plants (Fig. 2B). *Rhizobium*- and *Bacillus*-inoculated plants were the least stimulant to female moths to lay eggs (Fig. 2B; Online Supplementary Material 1—Table S7).

Effects of seed inoculation on maize plant volatiles

The qualitative and quantitative changes in the volatile organic compounds (VOCs) released by maize plants were dependent on the bacterium species used (Fig. 3B; Online Supplementary Material 2—Table S1). The highest VOCs diversity (34) was identified in plants inoculated with *Bacillus*, while control plants had the least VOCs diversity (16). Plants inoculated with *E. spodopteracolus* released 11, *E. entomosocium* 22, *Bacillus* 31 and *S. novaecaesareae* 29 VOCs that were not detected in the volatile profile collected from control plants (Fig. 3A). Clustering analyses of VOCs resulted in well-separated clusters, with plants inoculated with *E. spodopteracolus* resolving in a clade with control plants. VOCs from *S. novaecaesareae*-inoculated plants were the most diverse, resolving alone in a branch (Online Supplementary Material 1—Figure S3).

Partial least squares discriminant analysis (PLS-DA) produced similar clusters as PCA (Fig. 3C). Sixteen VOCs were found to hold VIP scores higher than 1.25, showing potential use as biological markers for the samples analyzed. Abundance of 2,3-dimethyl-4-decenal (VIP = 2.10), (Z,14R)-14-methylhexadec-8-en-1-ol (VIP = 1.62), 3,5,24-trimethyltetracontane (VIP = 1.59) and 1-octoxyoctane (VIP = 1.48) was always higher in the VOC profiles of inoculated than in the control plants (Online Supplementary Material 1—Figure S4). Pairwise comparisons demonstrated plants inoculated with *S. novaecaesareae* (22 VOCs) or *Bacillus* (18 VOCs) had higher numbers of differentially abundant VOCs when compared to control plants, while the least number of VOCs differently abundant was recorded in plants inoculated with *E. spodopteracolus* (6) and *E. entomosocium* (7) primed plants (Online Supplementary Material 2—Table S1).

Grouped PCA

A final PCA including all data generated was produced to compare the effects of the selected bacteria in the maize-*S. frugiperda* system, resulting in four well-defined groups (Online Supplementary Material 1—Figure S5). Maize plants inoculated with *E. spodopteracolus* resolved in the same quadrant and very close to control plants (Online Supplementary Material 1—Figure S5). Plants inoculated with *E. entomosocium* resolved alone in a quadrant but close to that of control and *E. spodopteracolus*-inoculated plants (Online Supplementary Material 1—Figure S5). Two major variables (larval mortality and metabolic costs) grouped the other inoculated plants away from the remaining treatments. Plants inoculated with *Bacillus* sp. resolved alone in a quadrant, while plants inoculated with *S. novaecaesareae* or *R. larrymoorei* resolved together in the same quadrant (Online Supplementary Material 1—Figure S5).

Discussion

All tested bacteria induced physiological changes in maize plants despite their history of association with *S. frugiperda* and/or maize. Their effects as plant growth promoters ranged from neutral to positive depending on the method of inoculation used. Only *Bacillus* IIL-Sfb05 affected maize biomass partitioning when inoculated through seed treatment, diverging disproportionally more nutrients to roots than to shoot development. Changes in the root-to-shoot (RS) ratio

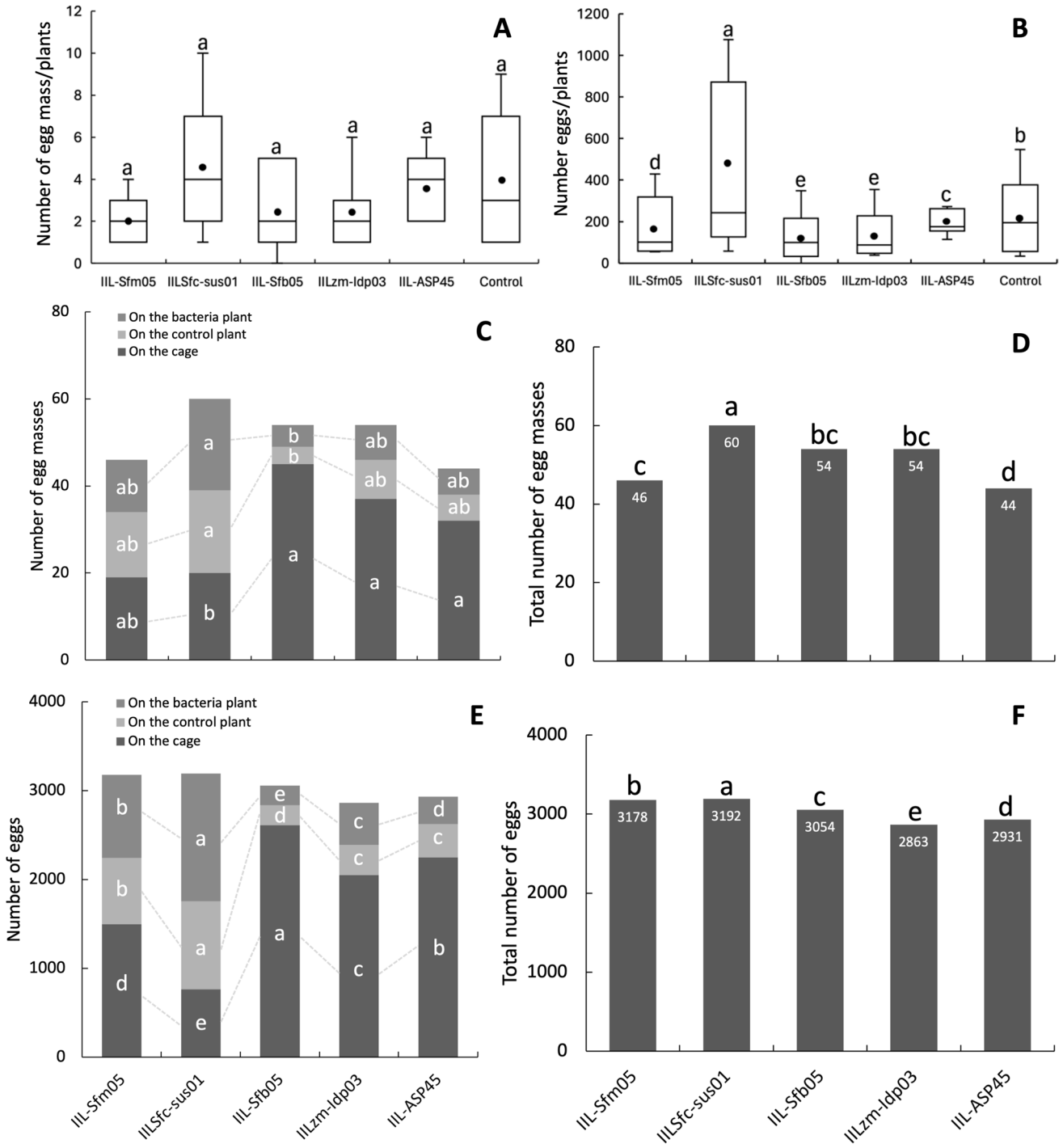

Fig. 2 Number of eggs and egg mass laid by *Spodoptera frugiperda* in semi-field and laboratory cages. **A** Number of egg mass and of **B** number eggs (± SD) laid by *S. frugiperda* on maize plants inoculated with different bacteria (IIL-Sfm05 = *E. spodopteracolus*; IILSfc-sus01 = *E. entomosocium*; IIL-Sfb05 = *Bacillus* sp.*;* IILzm-Idp03 = *R. larrymoorei*; IIL-ASP45 = *S. novaecaesareae*) compared to control plants (non-inoculated plants), based on experiment using semi-field cages with free choice. **C** Number of egg masses, **D** total number of egg masses, **E** number of eggs and **F** total number of eggs laid by *S. frugiperda* on maize plants inoculated with different bacteria (IIL-Sfm05, IILSfc-sus01, IIL-Sfb05, IILzm-Idp03 and IIL-ASP45) compared to control plants, based on experiment using cages in laboratory. •Mean; — Median. (GLMM, Tukey's test, $p < 0.05$)

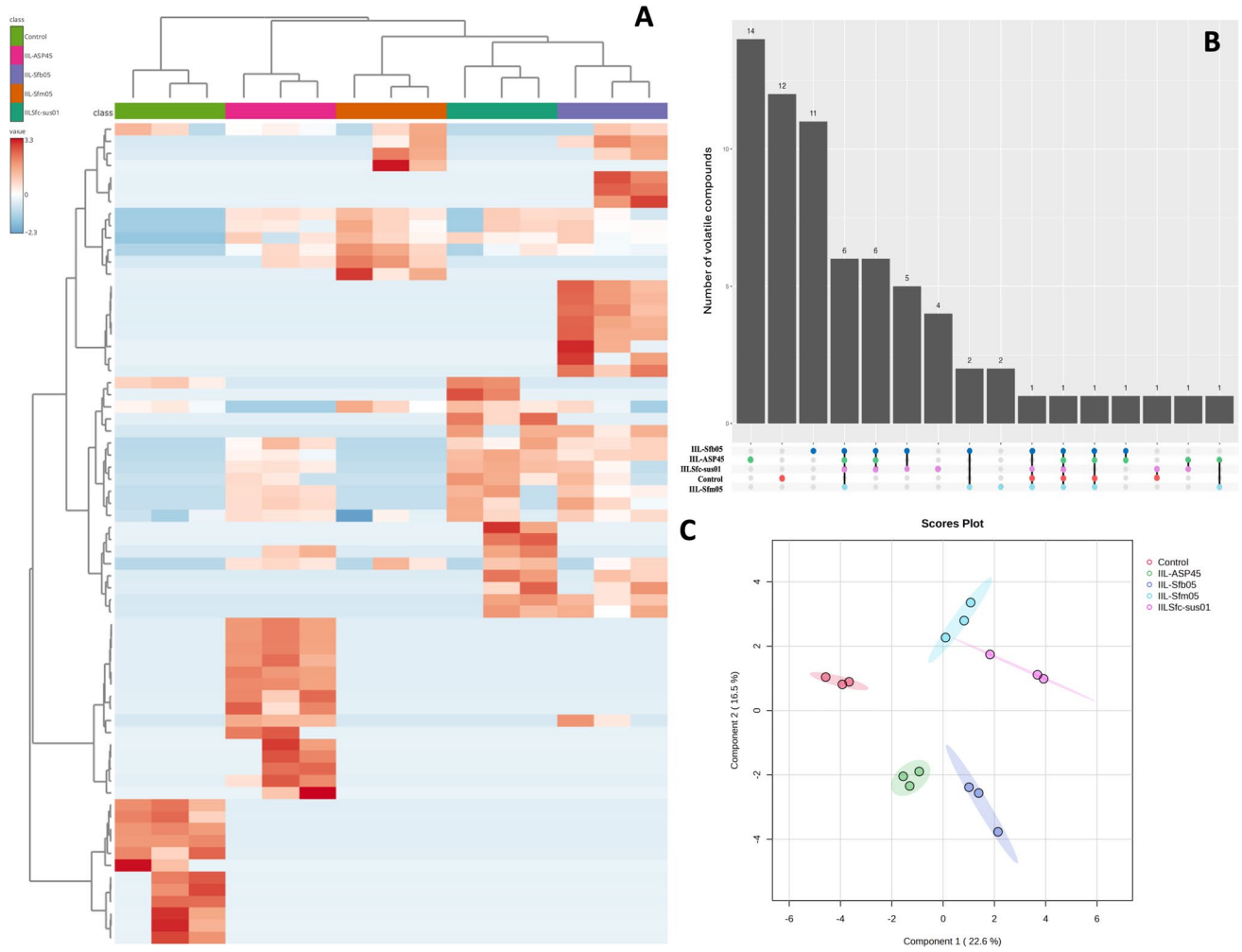

Fig. 3 **A** Heatmap with volatile organic compounds (VOCs) profile, **B** number of unique and shared VOCs among samples and **C** PLS-DA of VOCs of non-inoculated (Control) and inoculated maize plants with the insect-associated bacteria (IIL-Sfm05 = *E. spodopteracolus*; IILSfc-sus01 = *E. entomosocium*; IIL-Sfb05 = *Bacillus* sp.*;* IIL-ASP45 = *S. novaecaesareae*)

are a plastic response of plants to stress conditions, and the reported effects of endophytes on RS ratio have been variable (Henning et al. 2016; Labcanca et al. 2020; Lopez et al. 2023). But RS ratio has been demonstrated to enhance under nutrient deficiency, particularly of nitrogen (Lopez et al. 2023), leading us to hypothesize that the inoculation of *Bacillus* IIL-Sfb05 impairs nutrient uptake in maize, contrarily to what would be the expected contribution of an endophyte (Garcia-Latorre et al. 2021).

The plant growth-promoting (PGP) abilities observed for the maize endophyte *R. larrymoorei* IILzm-Idp03 were expected, as *Rhizobium* are well-known PGP bacteria (Vargas et al. 2017). The PGP capacity of *Enterococcus* is less commonly reported, but there are records for environmental and rhizospheric species (Mussa et al. 2018; Kumawat et al. 2024). *Enterococcus faecium* LKE12 promoted rice growth through gibberellin and indole-3-acetic acid secretion (Lee et al. 2015), while PGP *E. casseliflavus* induced grass pea growth through phosphate solubilization (Mussa et al. 2018). However, as far as we are aware, there are no reports of PGP *Enterococcus* species associated with insects. Our predictions that the resident *Enterococcus* species of the gut microbiota of *S. frugiperda* would positively stimulate host plant growth to benefit its host insect were satisfied. Nevertheless, it was a surprise to verify that all insect-associated bacteria tested established rhizospheric and endophytic interactions with maize plants, including the *Enterococcus* core gut symbionts of *S. frugiperda*. The core *Enterococcus* species associated with *S. frugiperda* were recently recognized as new taxonomic entities (*E. entomosocium* and *E. spodopteracolus*) after comparative genomics and phylogenetic analysis (Gomes et al. 2023). Their rhizospheric and endophytic associations with maize demonstrate they are well adapted to infect and exploit both hosts—maize and *S. frugiperda*—and

engage in different interactions with each one of them. The fact that *E. spodopteracolus* did not increase larval mortality nor affect *S. frugiperda* food utilization led us to hypothesize that it may share a longer evolutionary history with the maize-*S. frugiperda* system than *E. entomosocium,* since plants inoculated with *E. entomosocium* reduced the food utilization abilities of *S. frugiperda* larvae.

Plants inoculated with *Bacillus* IIL-Sfb05 were fully protected from *S. frugiperda* attack. Many species of endophytic *Bacillus* were shown to have antagonistic activity against maize pathogens or to induce defense mechanisms in maize (Gond et al. 2015; Pal et al. 2022), while others are entomotoxic to herbivores (Kebede et al. 2020). However, *Bacillus* IIL-Sfb05 did not display direct pathogenicity to *S. frugiperda* larvae (data not shown), leading us to hypothesize this bacterium induce alterations to enhance maize defense responses against herbivores.

Our data demonstrate that most inoculated plants reduced *S. frugiperda* food utilization efficiency, mainly by increasing the metabolic costs of *S. frugiperda* larvae to digest and/or metabolize maize leaves. The very high metabolic costs detected in larvae feeding on plants inoculated with *Rhizobium* or *S. novaecaesareae* could explain the larval mortality observed in these treatments. The metabolic costs to feed on plants inoculated with *E. entomosocium* were also high, but with much lower negative effects on the other nutritional indices than larvae fed on plants inoculated with *Rhizobium* or *S. novaecaesareae*.

The metabolic costs observed for *S. frugiperda* fed on plants inoculated with *Rhizobium* or *S. novaecaesareae* were mainly associated with a reduction in their digestive capacity and/or increased costs of food metabolization, as indicated by drastic reductions in approximate digestibility (AD), and efficiency to convert the ingested (ECI) and the digested food (ECD). The lower digestion efficiency of *S. frugiperda* feeding on bacteria-inoculated plants suggests plants produced defensive substances, such as proteins and/or other metabolites that affect the efficiency of food digestion and nutrient assimilation in *S. frugiperda.* These alterations were elicited by the larval feeding activity, as we did not observe changes in the constitutive expression of several selected genes known to participate in the plant response against herbivory (*MPI*, *RIP2, WRKY* and *TATA* -box) (Dowd et al. 2012; Chuang et al. 2014a, Chuang et al. 2014b; Tamayo et al. 2000; Wong et al. 2020; Singh et al. 2021; Tang et al. 2021). Expression of these genes is enhanced under herbivory (Tamayo et al. 2000), but the lack of negative effects on larval feeding on control plants (plants not inoculated with bacteria) demonstrates they do not have a significant effect in the anti-herbivory response of the maize genotype we used. However, the down-regulation of *GDPS* plants inoculated with bacteria points for the negative regulation of plant-produced defensive phytoalexins. Phytoalexins are produced in response to herbivory and microbial infections (Block et al. 2019) and are highly toxic to bacteria (Christensen et al. 2018). Phytoalexins regulation seems a defensive strategy used by the tested bacteria in the process of plant tissue colonization, which could also diminish plant-defensive capacity to herbivores. The negative effects of herbivore-associated bacteria (*Enterobacter, Pseudomonas* and *Stenotrophomonas*) on plant defense have been reported for those eliciting the salicylic acid pathway (SA). SA elicitation will inhibit the jasmonic acid pathway (JA) due to the negative crosstalk regulation between JA and SA, affecting plant response to herbivore attack (Chung et al. 2013; Sorokan et al. 2020).

Fatty alcohols in the epicuticular waxes of leaves were demonstrated to negatively affect the growth and development of generalist and specialist herbivores (Negin et al. 2024). All inoculated plants that affected larval food utilization had increased abundance of the fatty alcohol (Z,14R)-14-methylhexadec-8-en-1-ol. *Bacillus* IIL-Sfb05-inoculated plants carried 18-fold more of another fatty alcohol, 4-methym-2-propylpentan-1-ol, than control plants, turning into a candidate to explain the high mortality observed in this treatment. Nevertheless, the nearly 220 specialized metabolites maize plants produced to resist to herbivore- and pathogen-induced stress (Zhou et al. 2023) require further secondary metabolomics analysis to provide a more comprehensive understanding of the changes inoculated maize plants have in their anti-herbivory responses.

Differences in the outcome of maize-bacteria and of maize-bacteria-*S. frugiperda* larval interactions may occur from differences in microbial recognition patterns and microbial virulence factors among the tested bacteria, and how maize plants respond to these virulence factors (Boller and He 2009; Segonzac and Zipfel 2011; Yu et al. 2017; Saijo et al. 2018). The way plants recognize and respond to associated microbes is also dependent on the evolutionary history shared among them. The gut microbes of *S. frugiperda, E. spodopteracolus* and *E. entomosocium,* did stimulate plant growth, but plants inoculated with *E. entomosocium* did affect biological and behavioral traits of *S. frugiperda*, while plants inoculated with *E. entomosocium* did not.

Laboratory experiments on moth oviposition preference demonstrated plants inoculated with *Bacillus* IIL-Sfb05, *R. larrymoorei* and *S. novaecaesareae* were avoided as substrates for oviposition since most of the eggs were laid on the screen of the cages. Field-cage free-choice experiments did not result in differences in the oviposition preference of *S. frugiperda*, but clear differences were observed in the number of eggs laid. These results demonstrate the selected bacteria produced specific changes in maize plants that alter the ovipositional stimulation perception by *S. frugiperda*.

Chemical cues affect the initial steps of the host selection process (Honda 1995), and the chemical-oriented female oviposition decisions of *S. frugiperda* have been demonstrated to depend on the maize genotype (Yactayo-Chang et al. 2021). Thus, the differential effects of microbes on the VOCs released by maize plants add another layer of complexity to the complex nature of the chemical interactions of plants and herbivores and also likely with the third trophic level. VOCs we analyzed did not carry the oviposition attractants and repellents of *S. frugiperda* identified in maize (Yactayo-Chang et al. 2021; Wang et al. 2023), which could explain the absence of any preference response in our experiments.

Host selection and ovipositional stimulation in Lepidoptera can also rely on short-range chemicals and physical cues perceived by sensory receptors in the antennae, body surface, tarsi and ovipositor of females (Anderson and Hallberg 1990; Renwick and Chew 1994; Maher and Thiery 2004). Yet, many leaf surface chemicals (monoterpenes, epicuticular waxes) can act as oviposition stimulants or deterrents (Udayagiri and Mason 1997; Cervantes et al. 2002). The only n-alkane (4-methyltetradecane) identified in the VOCs analyzed had similar abundance among inoculated plants and a much higher abundance in control than in inoculated plants.

The reduction in the total abundance of VOCs release by plants inoculated with bacteria has been suggested to reduce the oviposition by the European corn borer moths (Disi et al. 2018), but all inoculated maize plants we tested had their VOCs enriched when compared to control plants. Since our quantitative and qualitative analysis of VOCs did not provide any correlation of volatiles with the differential ovipositional activity observed for *S. frugiperda*, we hypothesize that the differences in the number of eggs laid by *S. frugiperda* would be associated with changes in contact chemical cues acting upon female contact with the host plant, which is supported by the identification of odorant receptors in the ovipositor of a closely related species, *S. littoralis* (Seada et al. 2016) and the need of the active odorant co-receptor (*orco*) to allow oviposition to occur in *S. frugiperda* (Sun et al. 2023). The large number of compounds that have been demonstrated to elicit the oviposition of lepidopterans, most of the times acting synergistically (Honda 1995) calls for additional research to identify the chemical cues that would explain the stimulatory or the inhibitory ovipositional activity observed in inoculated maize plants. The higher oviposition activity obtained in plants inoculated with *E. entomosocium* seems negative for a species that turns to cannibalism as the larvae grow (Andow et al. 2015).

In conclusion, plants inoculated with resident (*E. spodopteracolus* and *E. entomosocium*) and transient (*Bacillus* sp. IILSfb05) gut symbionts of *S. frugiperda* resulted in different alterations in maize-*S. frugiperda* interactions. *Enterococcus spodopteracolus* IIL-Sfm05 do not affect maize suitability to *S. frugiperda* larvae and the reduction in female oviposition activity could be associated with cues that inhibit moth selection of infested plants (Wang et al. 2023). *Enterococcus entomosocium* IILSf-sus01 reduce the suitability of maize leaves to *S. frugiperda* larvae, but enhance the oviposition activity of adult moths. *Bacillus* sp. IIL-Sfb05 induced severe larval mortality and reduced egg laying of *S. frugiperda*, similarly to *S. novaecaesareae* and *R. larrymoorei*.

These results generate puzzling questions on the history of association and on the role gut-associated bacteria may have on maize and/or on *S. frugiperda*. Both *Enterococcus* species did not appear to have fitness costs to the host plant according to the traits we measured, but *E. spodopteracolus* has an amenable effect on the *S. frugiperda* -maize system when compared to *E. entomosocium* and *Bacillus* sp. Its strong association with *S. frugiperda* and its ability to colonize host plant tissues and insect tissues also open new opportunities for its exploitation as a delivery system of desired molecules to affect the *S. frugiperda* larval development. In fact, all bacteria tested are amenable for exploitation as endophytes to deliver different technologies for the management of *S. frugiperda*. Nonetheless, additional experiments for the identification of the mechanisms involved in plant regulation, insect toxicity and oviposition inhibition must be conducted for the safe exploitation of such symbionts in alternative management strategies of insect pests.

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Author's contributions D.A. helped in investigation, data curation, formal analysis, methodology, visualization, writing—review; E.W. contributed to statistical analyses and review—editing; F.LC. was involved in conceptualization, funding acquisition, methodology, project administration, supervision, writing—review and editing. All authors read and approved the final version of the manuscript.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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