Ecol Res (2017) 32: 435–444
DOI 10.1007/s11284-017-1454-3

ORIGINAL ARTICLE

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Efficacy of botanical extracts from Brazilian savannah against *Diabrotica speciosa* and associated bacteria

Received: 26 April 2016 / Accepted: 3 April 2017 / Published online: 11 April 2017

Abstract Botanical extracts are a plentiful resource of molecules with different biological activities, such as insecticides and antimicrobial pesticides. In this context, the aim of this work was to evaluate the efficacy of botanical extracts from the Brazilian savannah against *Diabrotica speciosa* and bacterial strains isolated from the gut of this insect under aseptic conditions. The bacterial isolates were identified by genomic and proteomic approaches, and bioassayed against eighteen botanical extracts in vitro. The best results of bacterial inhibitions were obtained for the extracts of *Casearia sylvestris* and *Psidium laruotteanum*. Fractions of *C. sylvestris* and *P. laruotteanum,* quantitatively evaluated by chromatographic analyses, showed a relationship between the bactericidal activity and phytochemical profile. In vivo assays showed that *P. laruotteanum* was also effective for the control of *D. speciosa*. Those results show that selected natural products can have both antimicrobial and insecticidal activities.

Keywords *Diabrotica speciosa* · Gut microorganisms · Brazilian savannah plants · *Casearia sylvestris* · *Psidium laruotteanum*

Introduction

Insects are the main competitors of humans for food and cause economic losses to agricultural products. Their evolutionary success has been attributed to the fact that they occupy areas limited in nutrients, with unbalanced food rich in certain nutrients but poor in others (Feldhaar and Gross 2009). For example, herbivorous insects may feed on an unbalanced diet with excessive carbohydrates compared to nitrogen compounds by modifying their eating habits and lifestyle (Denno and Fagan 2003). The use of different food sources such as plants and prey requires physiological and morphological specializations (Coll and Guershon 2002; Azevedo et al. 2007).

Diabrotica speciosa (Germar, 1824; Coleoptera: Chrysomelidae), an important Brazilian pest, damages plants of several botanical families such as sugarcane, maize, wheat, potatoes, oats and soybeans (Fouad et al. 2014). Its larvae consume plant tubers and roots, causing the death of young plants. The root-damaging larvae can reduce the plant's ability to absorb water and nutrients, thus decreasing its production. It might also cause the plant to become curved (gooseneck) and lower its weight and, consequently, productivity (Khaler et al. 1985; Marques et al. 1999). A recent study suggests an active role of the microbiota of *D. virgifera* in adaptation of this insect against control methods, indicating the importance of gut-associated microorganisms to the insect fitness and survival (Chu et al. 2013).

Electronic supplementary material The online version of this article (doi:10.1007/s11284-017-1454-3) contains supplementary material, which is available to authorized users.

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Natural products are an alternative source of new pesticides. Secondary metabolites from plants, such as azadirachtin, pyrethrin, rotenone, ryanodine, cevadine, nicotine, capsaicin, and piperine, may affect various organisms in the environment (Almeida et al. 2010; Gonzalez-Coloma et al. 2013). The *cerrado* (a vast tropical savannah ecoregion of Brazil) is an important source of species for biorational studies on the control of insect pests. This biome occupies approximately 25% of Brazilian territory, with almost 44% of the vascular plants being endemic (Brannstrom et al. 2008). Medicinal uses of the Brazilian savannah species and their efficacy in controlling mosquitoes, important vectors of human diseases, have been developed; however, the development of new pesticides from these plants has not been extensively studied. Brazilian savannah plant extracts showed efficient activity against *Zabrotes subfasciatus*, *Acanthoscelides obtectus*, *Spodoptera frugiperda* and *Sitotroga cerealella* (Silva et al. 2009; Santos et al. 2013).

This study had two parts: (1) the isolation and identification of bacteria from the gut of *D. speciosa* and (2) the investigation of the biological activities of extracts and fractions of Brazilian savannah plants against bacteria isolated from *D. speciosa* and the insect itself.

Method

Insect collection and aseptic rearing

Adults of *D. speciosa* were obtained from *Phaseolus vulgaris* L. crops (common beans, ball-type cultivate) and reared in the Laboratory of Plant Resistance to Insects, Department of Plant Protection, Center of Agricultural Sciences and Veterinary, São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil.

The insects used in the experiments were reared in a glass cage (40 cm × 30 cm × 30 cm) at 25 ± 2 °C with a relative air humidity of 70 ± 10%, and germicidal UV light with photoperiod of 12 h for two complete generations. This glass cage was previously sterilized using ethanol 70% v/v and germicidal UV-radiation (TUV Philips 15 W, 30 min). The medium was prepared from sieved clay soil and vermiculite (3:1 v/v) of average granularity and previously sterilized in an autoclave (3-fold at 120 °C, for 20 min). As a food supply, corn seeds (*Zea mays*) were planted and grown inside the captive areas. These seeds were previously washed in $CuSO_4$ solution (1 mol L^{-1}) and Vitavax® Thiran (Chemtura Ind. Quím. Brasil Ltda., Brazil) using a dose of 275 mL 100 kg^{-1} of seeds.

After oviposition, the eggs were washed in $CuSO_4$ solution (1 mol L^{-1}, Sigma-Aldrich, St. Louis, USA), to sterilize their surface (Cooksey 1990; Raudales et al. 2014), and then with wash water. After hatching, the larvae were reared in captivity in a sterile medium inside a laminar flow hood (HBaldin Industrial, Ribeirão Preto, Brazil) at 25 ± 2 °C with an air relative humidity of 70 ± 10% and photoperiod of 12 h. At specific times, the larvae and adults were killed by dropping them into $N_{2(liq)}$ and subsequently storing them in an ultrafreezer (Sanyo MDF–U56VC, Sanyo Electric Co., Japan) at −80 °C.

Some insects were also kept for the evaluation of their behavior under aseptic and natural conditions (sterilized environment as described above or non-sterilized environment, respectively). To confirm the sterilization of the eggs' surface, 100 µL aliquots of the sterile wash water that was used to wash them were plated onto potato dextrose agar (PDA), nutrient agar (NA) and Czapek in order to verify the effectiveness of the process. In addition, some sterilized eggs were rolled inside Petri dishes containing nutrient-agar medium, also to confirm the asepsis.

The data obtained were subjected to analysis of variance, and the means were compared with Student's t test at a 5% probability level using STATISTICA® software, version 7.0. Statistical analyses were performed on the larval-adult period, weight, longevity, reproductive rate, total emergence, and viability of the insects. Data were transformed into $(x + 0.5)^{1/2}$ or arcsine $(x/100)^{1/2}$ as necessary, where x represents the experimental data used to obtain the averages and perform the statistical analyses. Equations were used to adjust the data to a normal distribution and to ensure the homoscedasticity of the variances.

Bacterial strains isolation from the *D. speciosa* gut

The larvae and adults of *D. speciosa* were rinsed serially for 1 min using the following solutions: ethanol 70% (v/v), sodium hypochlorite 2% (w/v) and sterile distilled water (3 times). 100-µL aliquots of the last sterile distilled water washes were plated onto potato dextrose agar (PDA), nutrient agar (NA) and Czapek as controls to show the effectiveness of the surface sterilization process. This protocol was adopted to ensure that external microorganisms were not isolated. Afterwards, the larvae were macerated with a pestle in sterile plastic tubes (Eppendorf®, Hamburg, Germany) containing sterile distilled water. The resulting suspensions were plated onto NA, Czapek and PDA and streaked using an inoculation loop. The adult insects had their elytra, legs and heads removed. Subsequently, a longitudinal cut on the ventral abdomen of the insect allowed the removal of the digestive tract, which was divided and distributed onto NA, PDA, and Czapek to allow the growth and development of bacterial colonies. Petri dishes were incubated at 28 °C for 48 h. When bacterial colonies were observable, they were re-isolated using streak plate method until only one phenotype was observed. Single colonies of the successfully grown axenic cultures were transferred to liquid nutrient broth, and overnight cultures were preserved in 25% glycerol at −80 °C.

Identification of bacterial isolates—deoxyribonucleic acid analysis

The cryopreserved isolates were cultured on nutrient agar plates until vigorous growth was observed. Single colonies were used to inoculate 5 mL of nutrient broth, which was kept for 16 h at 28 °C. Deoxyribonucleic acid (DNA) was extracted, amplified using the universal primers 27f and 1525r, and sequenced (Weisburg et al. 1991). Details of the experimental procedures are presented in the Electronic Supplementary material, Materials and methods, and in Perlatti et al. (2017). Partial 16S rRNA gene sequences (~500 bp) were assembled with Bioedit and then compared to the NCBI database using the BLASTn algorithm (Altschul et al. 1990; Blibech et al. 2012). The partial 16S rRNA gene sequences used for identification were deposited in the GenBank database under accession numbers KP036890–KP036896.

Identification of bacterial isolates—matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry analysis (MALDI-TOF MS)

Bacterial isolates were plated on NA and then transferred to nutrient broth (NB) and incubated for 24 h at 28 °C. Afterwards, bacterial suspensions were streaked onto at least 2 different NA plates and incubated for 24 h at 28 °C. Each individual Petri dish had four colonies transferred using a sterilized toothpick onto a target spot of an MTP 384 ground steel TF target plate (Bruker Daltonics GmbH, Germany) in triplicate, giving a total of 24 samples for each isolate. Samples were overlaid with 1 µL of matrix solution containing 20 mg mL^{-1} 4-hydroxy-α-cyanocinnamic acid (HCCA; Sigma-Aldrich, St. Louis, USA) in acetonitrile:water (1:1) with 0.2% (v/v) trifluoroacetic acid and left to dry under a laminar flow hood. All spots were then analyzed using the Autoflex speed MALDI-TOF/TOF MS spectrometer (Bruker Daltonics GmbH, Germany). Details of the MS parameters are given in the Electronic Supplementary material, Materials and Methods.

Collection of plants, extract preparations, and phytochemical steps

All plants were collected in the Brazilian savannah of the Federal University of São Carlos in May 2014, after 20 days without rain (Table 1). The leaves were botanically identified, and classified reference vouchers were deposited at the Herbarium of the Department of Botany at UFSCar (SCSP-UFSCar). Plant material was extracted using ethanol/water maceration followed by liquid–liquid extraction. The leaves were washed with distilled water and dried in a forced air circulation oven (Tecnal, TE–394/3, Piracicaba, Brazil) for 7 days at 40 °C and subsequently powdered (Tecnal TE-631, Brazil). Plant extracts were prepared using 100 g of leaf powder and 500 mL of 96% ethanol. The extraction was carried out by turbolysis (RW-20 Basic Ultraturrax®IKA, Guangzhou, China), in two steps of 5 min. Subsequently, the material was packed in a container and subjected to the maceration process for 12 h. These processes of extraction were carried out 6 times. Afterwards, the solvent was distilled under reduced pressure in a rotary evaporator (Büchi RW-215, Switzerland) until the extracts became completely dry. Crude extracts (30 g) of *P. laruotteanum* and *C. sylvestris* were partitioned by liquid–liquid extraction to obtain fractions of dichloromethane, ethyl acetate, and ethanol/water.

P. laruotteanum and *C. sylvestris* extracts were fractionated to evaluate them for the presence of compounds that could show specific bacterial activities against the microorganisms from *D. speciosa*.

Table 1 Botanical materials collected from the Brazilian savannah of UFSCar

Species	Family	Location
Eriotheca gracilipes	Malvaceae	−21°58′16″S; −47°52′59″W
Bauhinia rufa	Fabaceae	−21°58′15″S; −47°52′59″W
Nectandra sp.	Lauraceae	−21°58′15″S; −47°52′59″W
Campomanesia pubescens	Myrtaceae	−21°58′16″S; −47°52′59″W
Annona coriaceae	Annonaceae	−21°58′15″S; −47°52′59″W
Symplocos pubescens	Symplocaceae	−21°58′14″S; −47°53′00″W
Schefflera vinosa	Araliaceae	−21°58′16″S; −47°52′59″W
Anadenanthera falcata	Mimosoideae	−21°58′16″S; −47°52′59″W
Roupala montana	Proteaceae	−21°58′15″S; −47°52′59″W
Xylopia aromatica	Annonaceae	−21°58′14″S; −47°53′01″W
Ananas ananassoides	Bromeliaceae	−21°58′14″S; −47°52′58″W
Solanum lycocarpum	Solanaceae	−21°58′19″S; −47°53′01″W
Caryocar brasiliensis	Caryocaraceae	−21°58′23″S; −47°53′02″W
Psidium laruotteanum	Myrtaceae	−21°58′14″S; −47°52′58″W
Eugenia aurata	Myrtaceae	−27°53′60″S; −47°52′56″W
Davilla elliptica	Dilleniaceae	−27°58′14″S; −47°52′57″W
Miconia ligustroides	Melastomataceae	−27°53′16″S; −47°13′12″W
Casearia sylvestris	Flacourtiaceae	−21°58′00″S; −47°51′55″W

UFSCar: Federal University of São Carlos

Quantitative analyses of botanical extracts and fractions by high-performance liquid chromatography (HPLC)

Chromatographic analyses of the *C. sylvestris* and *P. laruotteanum* extracts and fractions were carried out by high-performance liquid chromatography (HPLC), with method details provided in the Electronic Supplementary material, Materials and Methods. Standard solutions (1.00 mg mL^{-1}) of clerodane diterpene and *β*'-chalcanone were used after being isolated beforehand from *C. sylvestris* and *P. laruotteanum*, respectively, and were applied as quality control markers. The purpose of these markers was to ensure the reproducibility of the biological activity of the botanical extracts investigated. Calibration curves ranging from 5.00 to 100 µg mL^{-1}

were prepared by diluting these solutions. For each sample, an extract of a fraction, a quantity of 10.00 mg was diluted in 10.0 mL of HPLC-grade methanol (J.T. Baker, Ecatepec, Mexico) and filtered through a 0.22 μm cellulose acetate membrane filter (Chromafil, Düren, Germany).

Antimicrobial assays of botanical extracts

Initially, thawed stock bacterial suspensions were transferred to tubes containing 10 mL of Mueller–Hinton broth (MHB) and pre-cultured at 28 °C for 24 h. Each bacterial isolate was then inoculated at 10^3 colony-forming units (CFU) mL^{-1} in MHB tubes and cultured at 27 °C until it reached a turbidity equal to that of 0.5 McFarland standard (1.5×10^8 CFU mL^{-1}). Serial dilutions of these bacterial suspensions were performed with NaCl solution (0.15 mol L^{-1}) to 5×10^6 CFU mL^{-1} and were afterwards used as inoculums in antimicrobial tests (Mytilinaios et al. 2012).

The botanical sample concentrations (1.00 mg mL^{-1}) were prepared using a solution of dimethyl sulfoxide (DMSO) 2.5% (v/v; Synth, Diadema, Brazil) diluted in MHB. Growth controls were prepared with DMSO 2.5% (v/v) diluted in MHB containing no antibiotic, tetracycline solution (500 μg mL^{-1}), and only MHB in each plate. Each plate had 5 wells filled with only MHB as the sterility control.

Qualitative antimicrobial assays were carried out by mixing 100 μL of the botanical extracts, 90 μL of MHB and 10 μL of each bacterial suspension in 96-well polystyrene round-bottomed sterile plates (TPP, Switzerland) and incubating for 24 h at 28 °C. After the addition of all components into a 96-well plate, the final cell concentration was approximately 5×10^5 CFU mL^{-1}. The microplates were covered and wrapped loosely with cling film to ensure that the bacteria did not become dehydrated.

Afterwards, 20 μL of a 1.00 mg mL^{-1} aqueous solution of resazurin was added (Sigma-Aldrich, St. Louis, USA) to each well as an indicator of bacterial growth, and the plates were re-incubated at 28 °C for a further 1 h. The color change was assessed visually, where changes from blue to pink indicated positive cell viability; any modifications in color were positive for antibacterial activities. To control the staining of the plates, two other series were carried out in 96-well plates using 100 μL of the sample solutions, 80 μL of MHB and (1) 20 μL of oxidized resazurin or (2) reduced resazurin. The plates were prepared in duplicate, and each sample was analyzed in five wells per plate.

The quantitative antimicrobial method was adapted from Hussain et al. (2011) and Sarker et al. (2007). For quantitative assays, a 96-well black flat-bottomed plate was used (Whatman, Florham Park, USA). In this assay, the bacterial growth inhibition was analyzed by using a spectrofluorometer (SpectraMax® Paradigm® Multi-Mode reader, Sunnyvale, USA). The fluorescence intensity was measured by excitation at 530 nm and emission at 590 nm. The calibration curves for resazurin were prepared in concentrations ranging from 26.0 to 165 ng mL^{-1} and were used during the quantitative analyses. Samples without any bactericide and with tetracycline (500 μg mL^{-1}) were used as negative and positive controls, respectively (Clinical and Laboratory Standards Institute 2005). Extracts and fractions were quantified by comparing the intensity of the fluorescence of the samples with the linear relationship obtained by an external standard calibration curve of resazurin (Figure S1—Electronic Supplementary material).

Effect of *P. laruotteanum* extract on *D. speciosa*

Experimental assays with *D. speciosa* insects and *P. laruotteanum* extracts were also carried out in the Laboratory of Plant Resistance to Insects at UNESP at 25 ± 2 °C with an air relative humidity of 70 ± 10% and a photoperiod of 12 h.

The experiment was carried out in 300 mL plastic containers filled with 25 g of sieved clay soil and vermiculite (3:1 v/v). The soil was previously sterilized in an oven (Model AS200S, Quimis, Diadema, SP, Brazil) at 110 ± 2 °C for 48 h. The insects were reared within an environment that was either previously exposed, or not exposed to samples of *P. laruotteanum*. The botanical extract was solubilized (2.0%; w/v) in a water:dimethyl sulfoxide (DMSO) solution (2.5%, v/v), and 1 mL of the resulting solution was applied into furrows (1.5 cm) that were previously made in the soil, near the roots. One common bean plant (*P. vulgaris*) was subsequently added at 15 days post-emergence, with the roots covered with soil. Afterwards, the plants and soil were watered with 15 mL of deionized water and 3-day old *D. speciosa* larvae were inoculated into each plastic container, which was then covered with another one and sealed with adhesive tape to form a symmetrical cage. Ten plastic containers were prepared, resulting in 30 insects per treatment. The insects were released onto the soil surface, and the beetle emergence was assessed through the total number of emerged insects and the survival percentage. The dry weights of the shoot and root systems were also determined to evaluate the effects of the *P. laruotteanum* extracts on common bean plants (*P. vulgaris*) and to determine if the difference in the development of the insects could result in different performance of the plant. The data obtained were subjected to analysis of variance, and the averages were compared by the Student t test at a 5% probability using STATISTICA software, version 7.0®.

Results and discussion

Bacterial isolates from the gut of the *D. speciosa*

The aseptic condition of eggs and larvae were confirmed, as no culturable microorganisms were observed in the

Table 2 Bacterial strains isolated from *Diabrotica speciosa*

Insect	Medium	DNA identification	MALDI identification
Larvae	NA	*Serratia* sp.	*Serratia marcescens*
Larvae	Czapek	*Acinetobacter* sp.	*Acinetobacter pittii*
Larvae	NA	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
Larvae	NA	*Pseudomonas* sp.	*Pseudomonas mosselli*
Larvae	NA	*Pseudomonas* sp.	*Pseudomonas mosselii*
Larvae	NA	*Pseudomonas* sp.	*Pseudomonas chlororaphis*
Larvae	NA	*Enterobacter* sp.	*Enterobacter cloacae*

NA nutrient agar, *MALDI* matrix-assisted laser desorption/ionization

three different evaluated growth media after 5-day incubation of the wash waters used in the sterilization processes at 28 °C. Seventy-three bacterial isolates were obtained from *D. speciosa* and identified, and seven of them were used in this study (Table 2). They were selected according to the main genera identified in this work. The bacterial isolates were identified by partial 16S rDNA gene sequencing and MALDI-TOF MS profiling (Fig. 1).

Each bacterial isolate produced a characteristic and easily distinguishable MS spectrum (Fig. 1). The most intense m/z ions of the species in Fig. 1d, e were also observed in the spectra of Fig. 1f; all of them were assigned as *Pseudomonas*, and good similarity could be observed among the three spectra overall. Genetic analyses also confirmed that all three strains belonged to the *Pseudomonas* genus, but the species could not be identified using only partial 16S sequencing. However, minor differences in low-intensity ions allowed distinguishing *P. mosselli* (strains Fig. 1d, e) and *P. chlororaphis* (strain Fig. 1f) based on MALDI-TOF spectra and the instrument database.

Most bacterial genera observed in this study have known associations with other species of *Diabrotica*. *E.*

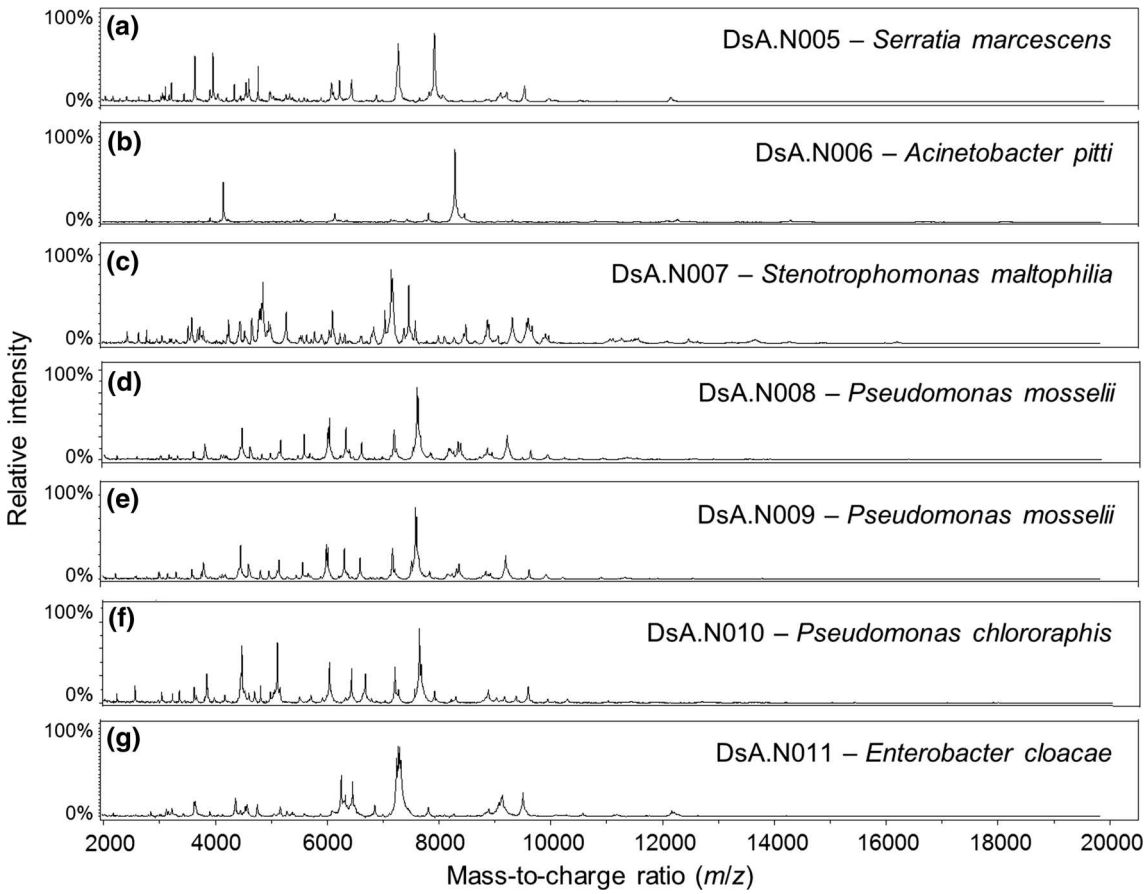

Fig. 1 Whole cell matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectra of bacterial strains isolated from the gut of *Diabrotica speciosa*. **a** DsA.N005; **b** DsA.N006; **c** DsA.N007; **d** DsA.N008; **e** DsA.N009; **f** DsA.N010; **g** DsA.N011. The x axis represents mass-to-charge ratio (m/z), and y is the relative signal intensity

cloacae, Serratia sp., and *Pseudomonas* sp. were isolated from *Diabrotica undecimpunctata howardi* (Tran and Marrone, 1988, Prischmann et al. 2008), while *Enterobacter, Serratia* and *Pseudomonas* were isolated from *Diabrotica balteata* (Schalk et al. 1987). These microorganisms are involved in the ability of insects to survive for longer periods in the field, overcoming crop rotation methods for controlling insects. Wild-type insects harbored *Enterobacter*, *Serratia* and *Pseudomonas* species and trace amounts of *Stenotrophomonas*, while the rotation-resistant varieties exhibited the disappearance of the *Pseudomonas* species, lower levels of *Enterobacter,* and rising levels of the *Stenotrophomonas, Acinetobacter* and other *Pseudomonas* strains (Chu et al. 2013). A comparison between the *D. virgifera* and *D. speciosa* microbiota and feeding habits indicated a direct correlation between the abundance of species in the gut and polyphagy. These bacteria have been reported to inhabit the midgut of several *Diabrotica* spp., suggesting that they might be important in the lifecycle of these insects.

Phytochemical investigation of Brazilian savannah of UFSCar

Eighteen botanical specimens were randomly collected in the Brazilian savannah of UFSCar and identified and classified within 14 families (Table 1). The extract yields ranged from 7.2 to 21.4% (w/w). The extraction steps provided raw samples comprising a broad chemical profile. The extracts of plants from the families Myrtaceae (*C. pubescens*, *P. laruotteanum*, and *E. aurata*), Annonaceae (*Annona coriacea*, *Xylopia aromatica*), Lauraceae (*Nectandra* sp.), Mimosoideae (*A. falcata*), Dilleniaceae (*D. elliptica*), Melastomataceae (*M. ligustroides*), and Flacourtiaceae (*C. sylvestris*) showed antimicrobial results in at least four of the seven isolates of assayed bacterial isolates (Table 3).

Extracts from the Fabaceae and Solanaceae families, represented by *B. rufa* and *S. lycocarpum*, respectively, which include important crops such as beans, soybeans, peas, lentils, potatoes, tomatoes, and peppers that are commonly attacked by *D. speciosa*, did not show antibacterial activity. These results corroborate a relationship between the feeding preference of *D. speciosa* and its gastrointestinal flora. *C. sylvestris* and *P. laruotteanum* were selected for further studies.

Quantitative analysis and biological activity of *P. laruotteanum and C. sylvestris* on *Enterobacter* sp.

The linear dynamic range of the curve from 5.00 to 100 μg mL^{-1} was assessed through a regression equation ($y = 0.4912x \pm 0.0044$), correlation coefficient ($r^2 = 0.9601$), method of least squares, and analysis of variance (ANOVA). Analysis of variance of the linearity for both the slope and the intercept gave P values of $\leq 9.330 \times 10^{-15}$ and 0.0696, respectively, at 95% confidence level. This indicates a strong relationship between each pair of y and x values in the regression equations, high sensitivity for the method, and an intercept close to zero (Table S1—Electronic Supplementary material).

The crude extracts and fractions at 500 μg mL^{-1} of *C. sylvestris* and *P. laruotteanum* inhibited bacterial growth between 22.3 and 97.0% (Fig. 2). The inhibition percentages of the crude extract and fractions of *C. sylvestris* were similar, but it was possible to identify some variation among the fractions. The crude extract of *C. sylvestris* inhibited 69.0% of bacterial growth, whereas the ethyl acetate, dichloromethane, and hydroalcoholic fractions showed values of 75.0, 60.2, and 50.2%, respectively. This showed that the *C. sylvestris* extracts contained compounds of different polarities inhibiting bacterial growth, as found for clerodane diterpene compounds in *C. sylvestris* with several different functional groups such as hydroxyls, acetyls, and esters, (Carvalho et al. 1998; Oberlies et al. 2002).

The crude extract and fractions of *P. laruotteanum* had different activities, with the former inhibiting 84.0% of microbial growth. The inhibition for the ethyl acetate fraction was 97.0%, while the values for the dichloromethane and hydroalcoholic fractions were only 22.3 and 42.7%, respectively, indicating specific activity of one molecule or one class of compounds. Liquid–liquid partitioning was able to concentrate the active compound(s) responsible for the antimicrobial activity by removing interfering substances. The ethyl acetate fraction of *P. laruotteanum* was as active as the tetracycline control (93.0%) in inhibiting bacterial growth.

C. sylvestris is widely known for its antitumor (Carvalho et al. 1998; Ferreira et al. 2010) and antimicrobial

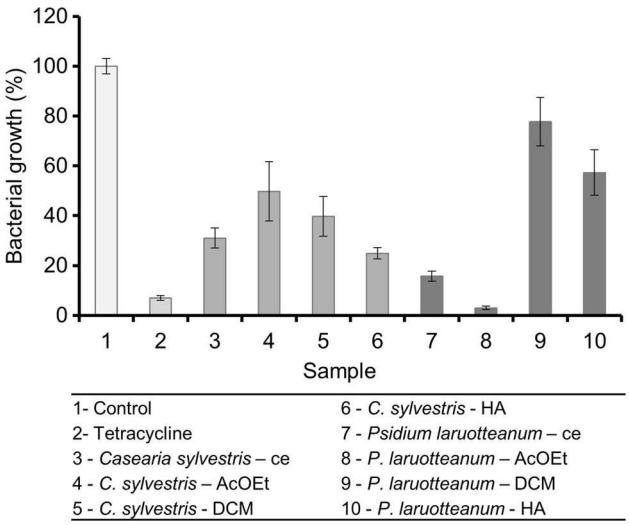

Fig. 2 Antimicrobial activities of *Casearia sylvestris* and *Psidium laruotteanum* extracts and fractions against *Enterobacter* sp. *Cs C. sylvestris*, *Pl P. laruotteanum, ce* crude extract, *AcOEt* ethyl acetate fraction, *DCM* dichloromethane fraction *HA* ethanol/water fraction. *Error bars* represent standard deviations ($n = 3$)

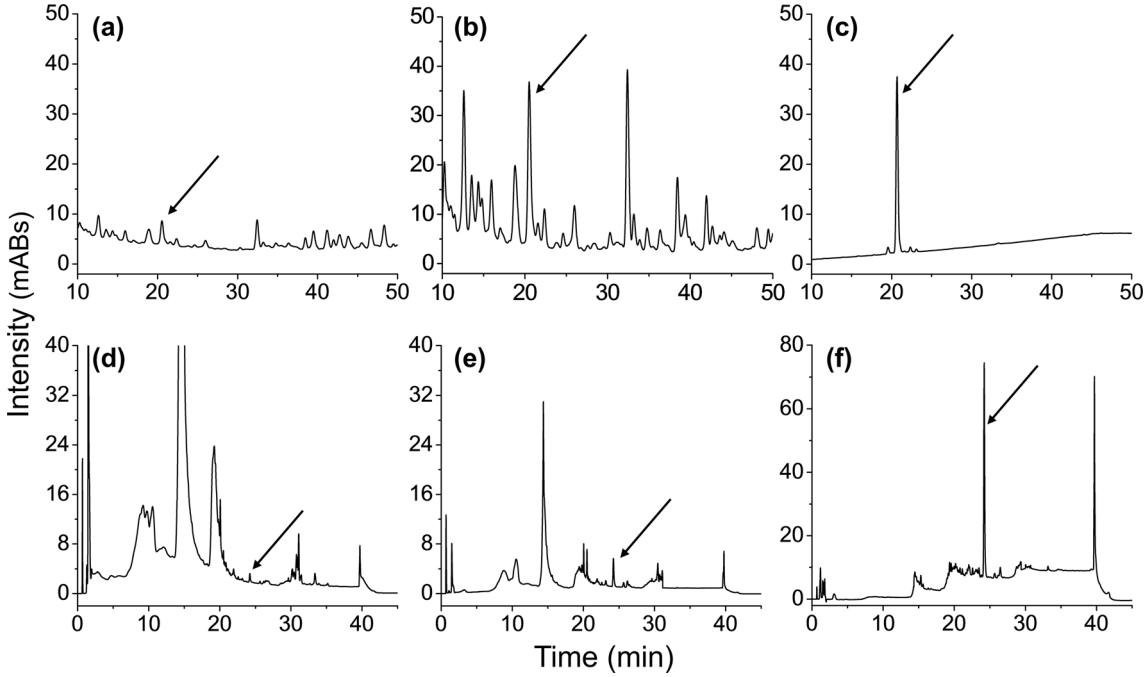

Fig. 3 Analytical chromatograms of **a** crude extract of *Casearia sylvestris*; **b** ethyl acetate fraction of *C. sylvestris*; **c** clerodane diterpene isolated from *C. sylvestris* **d** crude extract of *Psidium laruotteanum*; **e** ethyl acetate fraction of *P. laruotteanum*; and **f** *β*′-chalcanone isolated from *P. laruotteanum*. *Arrows* in **a**–**c** indicate peaks corresponding to clerodane diterpene, and *arrows* in **d**–**f** indicate peaks corresponding to *β*′-chalcanone

(Silva et al. 2008) activities. On the other hand, few phytochemical studies have been performed on *P. laruotteanum*, but *Psidium guajava*, a close relative, has known antimicrobial activity (Fernandes et al. 2014).

P. laruotteanum inhibited bacterial growth to a greater extent than *C. sylvestris*. There are no citations in the literature or experimental data indicating that these species are populated or used as food by *D. speciosa*. *C. sylvestris* has several compounds that can inhibit microbial growth. *P. laruotteanum* showed a more specific relationship between the bactericidal action and specific polarity (or class) of the secondary metabolites.

Quantitative analyses of crude extracts and fractions of *C. sylvestris* and *P. laruotteanum*

The crude extract and ethyl acetate fraction of *C. sylvestris* (Fig. 3a, b, respectively) have compounds of increased concentration, including a secondary metabolite identified as a clerodane diterpene (Fig. 3c). Similar results were found for *P. laruotteanum*, with a higher level of the secondary metabolite *β*′-chalcanone found in its ethyl acetate fraction than in its crude extract (Fig. 3d–f).

The HPLC–UV analyses allowed quantifying clerodane diterpene in the crude extract and in the acetate fraction from *C. sylvestris*, yielding values of 0.42 and 6.53% (w/w), respectively. The contents of *β*′-chalcanone in the crude extract and in the acetate fraction from *P. laruotteanum* were 0.13 and 2.30% (w/w), respectively. The two compounds, clerodane diterpene and *β*′-chalcanone, were isolated and used as markers to ensure the reproducibility of the observed biological activity due to the difficulties with obtaining analytical standards for the total quality control of the botanical extracts.

The analytical validation data that were used in the HPLC analyses are given in Table S2 of the Electronic Supplementary material. Products with a large quantity of the highlighted markers had high antimicrobial efficacy. All antimicrobial assays used the same quantity of sample (500 μg mL^{-1}), but they yielded different quantities of the markers clerodane diterpene and *β*′-chalcanone. The latter might be associated with resistance mechanisms of apples as an antibacterial and antioxidant compound used in defense strategies against both biotic and abiotic stress (Gaucher et al. 2013). Antibacterial, antileishmanial, and anti-inflammatory activities were also reported for *β*′-chalcanone (Hermoso et al. 2003; Nowakowska 2007). Clerodane diterpene from *C. sylvestris* has shown cytotoxic, anti-fungal, and anti-parasitic activities (Prieto et al. 2013; Bou et al. 2014).

Effects of sterile environment on *D. speciosa* and of *P. laruotteanum* extract on *D speciosa* and *P. vulgaris*

The reproductive differences of *D. speciosa* in an aseptic environment and under regular conditions (non-sterilized environment) permitted the comparison of the results in these situations (Table 4). Insects reared in an

Table 3 Antibacterial activity of botanical extracts from Brazilian savannah plants against bacterial strains isolated from the gut of *Diabrotica speciosa*

Species	*S. marcescens*	*A. pitii*	*S. maltophila*	*P. mosselli*	*P. mosselli*	*P. chlororaphis*	*E. cloacae*
Eriotheca gracilipes	–	–	–	–	–	–	–
Bauhinia rufa	–	–	–	–	–	–	–
Nectandra sp.	–	+	+	–	+	+	–
Campomanesia pubescens	+	–	+	+	+	+	–
Annona coriaceae	–	–	+	+	+	+	–
Symplocos pubescens	–	–	–	–	–	–	–
Schefflera vinosa	–	–	–	–	–	–	–
Anadenanthera falcata	+	+	+	+	+	+	+
Roupala montana	–	–	–	–	–	–	–
Xylopia aromatica	+	+	–	+	+	+	–
Ananas ananassoides	–	–	–	–	–	–	–
Solanum lycocarpum	–	–	–	–	–	–	–
Caryocar brasiliensis	–	–	–	–	–	–	–
Psidium laruotteanum	–	+	+	+	+	+	+
Eugenia aurata	+	+	+	+	+	+	–
Davilla elliptica	+	–	–	+	+	+	–
Miconia ligustroides	–	+	+	+	+	+	–
Casearia sylvestris	–	+	–	+	+	+	+

The "+" sign indicates antibacterial activity. All experiments were carried out in two batches with 5 replicates each (total n = 10)
S. marcescens: *Serratia marcescens*; *A. pitii*: *Acinetobacter pittii*; *S. maltophila*: *Stenotrophomonas maltophilia*; *P. mosselli*: *Pseudomonas mosselli*; *P. chlororaphis*: *Pseudomonas chlororaphis*; *E. cloacae*: *Enterobacter cloacae*

Table 4 Evaluation of the biological development of *Diabrotica speciosa* reared under aseptic and regular conditions

Total	Treatments (reared)		*F* (treatments)	*R.S.D.* (%)	*P*
	Aseptic	Regular			
Larvae-adult period (days)[a]	27.71 ± 0.20 a	29.10 ± 0.25 b	19.08***	2.3	< 0.001
Weight (mg)[a]	7.88 ± 0.35 b	6.36 ± 0.46 a	6.70**	18.0	< 0.0117
Longevity (days)[a]	3.54 ± 0.17 b	2.32 ± 0.19 a	22.45***	16.3	< 0.001
Sexual rate[a]	0.44 ± 0.08 a	0.48 ± 0.09 a	0.14[ns]	27.7	0.7101
Emerged insect number[a]	49.00 ± 4.38 b	36.00 ± 4.48 a	4.30*	80.3	< 0.0397
Viability of insect (%)[b]	61.25 ± 5.48 b	45.00 ± 5.60 a	4.30**	93.3	< 0.0397

Averages that have the same letter in two columns did not show significant difference between them (Student t test at 5% probability level)
[a] Data transformed into$(x + 0.5)^{1/2}$
[b] Data transformed into arcsine $(x/100)^{½}$
[ns] Not significant at 5% probability level by the F test; * Significant at 5% probability level by the F test; ** significant at 1% probability level by the F test; *** significant at < 1% probability level by the F test

aseptic environment had a shorter larval to adult period, larger weight, longer longevity, larger number of emerged insects and greater viability. Only the reproductive rate was similar between treatments, as is characteristic of microorganisms that are vertically transmitted (Scarborough et al. 2005). These results suggest two hypotheses: first, these insects were born with all the symbiotic microorganisms necessary for their life cycle, and second, other environmental microorganisms (in the soil) can disturb insect development.

The biological activity of *P. laruotteanum* extract against *D. speciosa* (Table 5) manifested itself through a lower number of emerged insects and a reduction in their survival to approximately 50%. This result may be due to the direct effects of the plant extracts on the insect as induced defenses, This supports the hypothesis that *P. laruotteanum* produces compounds such as *β′*-chalcanone as chemical defense products against insects, which may be the first report describing the microbial and insecticidal activities of this plant.

The weights of the shoots and roots of the plants used as food were similar between plant groups (Table 6). This result suggests that *P. laruotteanum* was incipient to the plant (common bean) while effective for control of *D. speciosa*.

The ethanolic extracts of ten Brazilian savannah plants showed antimicrobial activity, especially *Anadenanthera falcata*, *Eugenia aurata*, *Psidium laruotteanum*, and *Xylopia aromatic*. They inhibited microbial growth in at least 70% of bacterial species isolated from the gut of *D speciosa*. Furthermore, the botanical extract of *P. laruotteanum* was active for the bacterial isolates assessed and could control the development and population of *D. speciose*, showing both bactericidal and insecticidal activities.

Insects can exploit the potential of symbiotic microorganisms in their gastrointestinal tract. This

Table 5 Survival assays of *Diabrotica speciosa* exposed to extracts of *Psidium laruotteanum*

Variables	Treatments		*F*	*P*
	P. laruotteanum	Control		
Number of emerged insects[a]	9.00 ± 2.55 b	17.00 ± 2.76 a	4.53*	0.0376
Survival (%)[b]	30.00 ± 8.51 b	56.67 ± 9.20 a	4.53*	0.0376

Averages that have the same letter in both columns did not show a significant difference between them (Student's t-test at 5% probability level)
[a] Data transformed into $(x + 0.5)^{1/2}$
[b] Data transformed into $\text{arcsine}(x/100)^{1/2}$
* $P < 0.05$

Table 6 Effects of the *Psidium laruotteanum* extract on common bean plants used in control experiments with *Diabrotica speciosa*

Weight (mg)	Treatments		*F* (treatments)	*P*
	P. laruotteanum	Control[b]		
Aerial part[a]	77.11 ± 11.23 a	97.40 ± 12.98 a	1.28[ns]	0.2735
Root system[a]	678.90 ± 94,12 a	630.80 ± 39.63 a	0.09[ns]	> 0.050

Averages that have the same letter in both columns did not show a significant difference between them (Student's t-test at 5% probability level)
[a] Data transformed into $(x + 0.5)^{1/2}$
[b] Without larval infestation
[ns] Not significant at 5% probability level by the F test

makes it possible for them to use unbalanced food sources that are rich in certain nutrients but poor in others, and also to avoid or manipulate toxic molecules (Denno and Fagan 2003; Brannstrom et al. 2008; Oliver and Martinez 2014). The exploitation of the insect-microorganism symbiosis could be a powerful tool for insect pest management. Insects requiring enzymatic or nutritional complements from microorganisms are vulnerable to interventions targeting their symbionts, or their interactions with the host (Douglas 2007). Chemical compounds capable of disrupting or blocking the symbiosis between the extracellular gut bacteria and pest insects may lead to nutritional and hormonal deficiency, paving the road for the exploration of this interaction as a tool for insect control.

Acknowledgements We would like to thank the following Brazilian agencies for financial support: the São Paulo Research Foundation (FAPESP: 2011/11860-5); the National Council for Scientific and Technological Development (CNPq: 477051/2012-0; 301427/2012-7; SISBIOTA 563063/2010-6); Professor Maria Fátima das Graças Fernandes da Silva, coordinator of INCT-Biorational Control of Plague Insects (CNPq/INCT: 573742/2008-1 and FAPESP/INCT: 2008/57859-5), who helped with the structure and other materials; and CAPES—Higher Education Improvement Coordination.

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