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Supplemental Information

Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota

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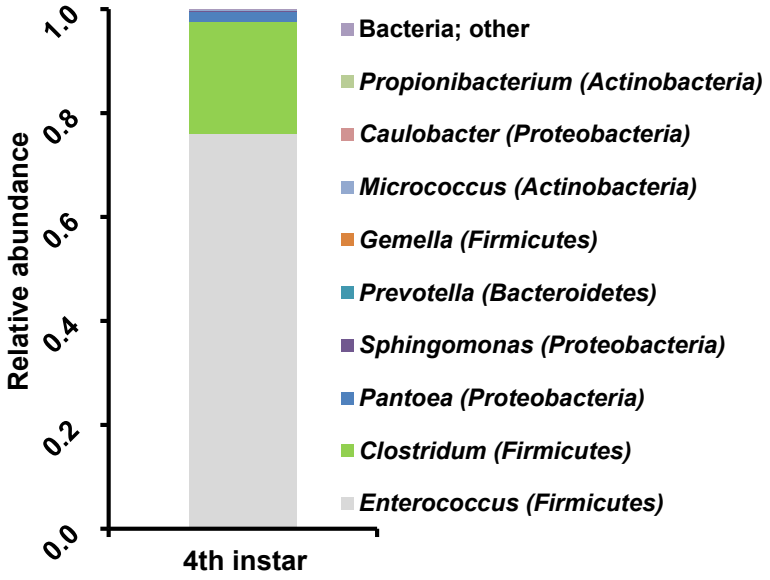
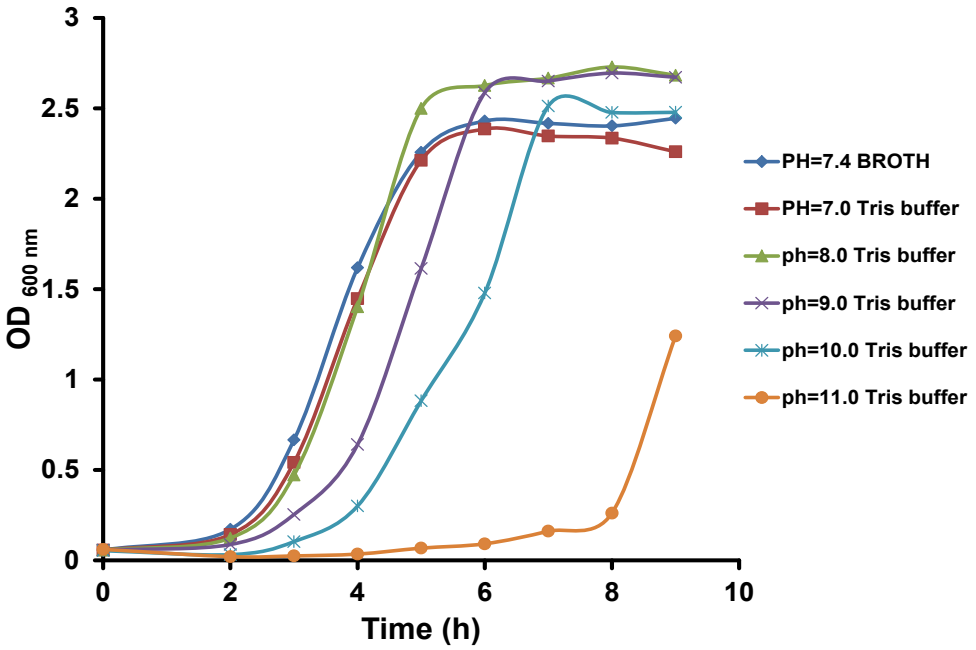


Figure S1, related to Figure 1. Sequencing analysis of the gut microbiota of fourth-instar *S. littoralis* larvae.

A



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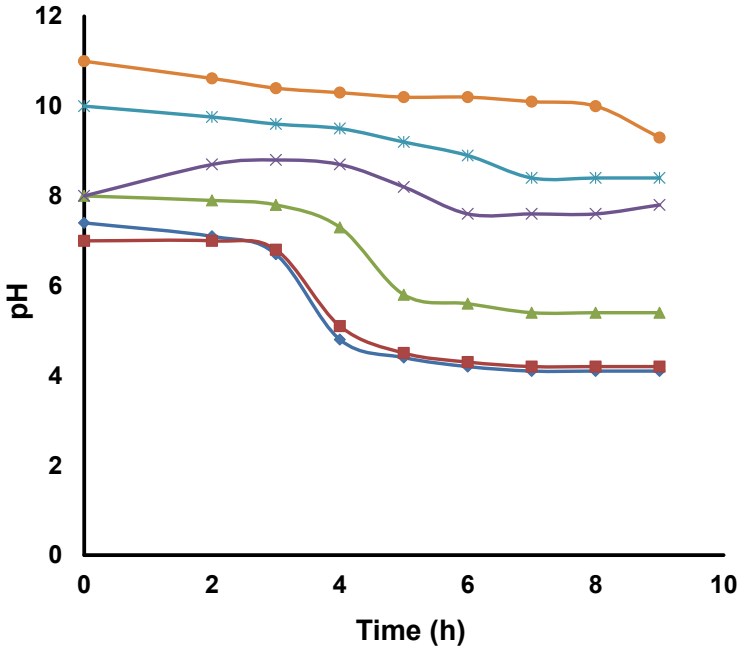

Figure S2, related to Figure 3. *E. mundtii* SL grows well in alkaline pH condition. (*A*) Growth curves of *E. mundtii* SL strain inoculated under pH-buffered conditions. (*B*) Change of pH during growing.

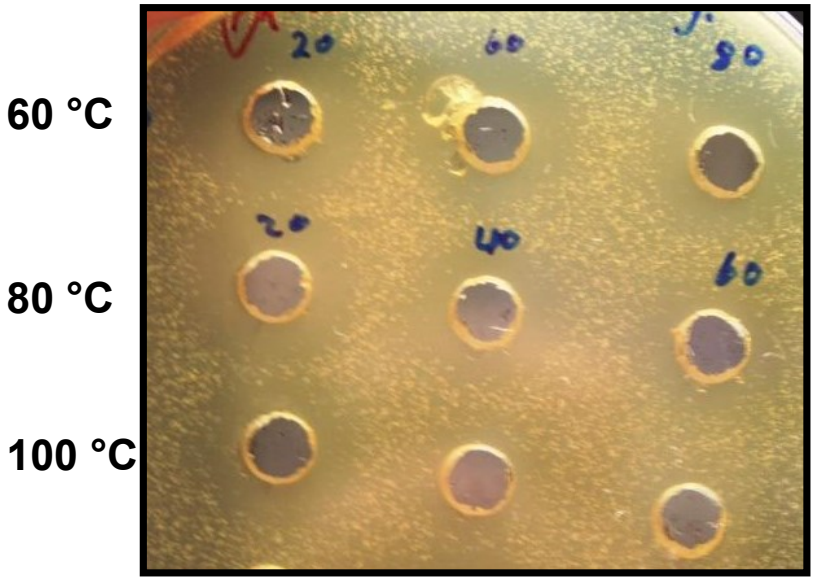

Figure S3, related to Figure 3. The effect of temperature on the inhibitory substance was tested by heating the supernatant at 60, 80 and 100°C, respectively.

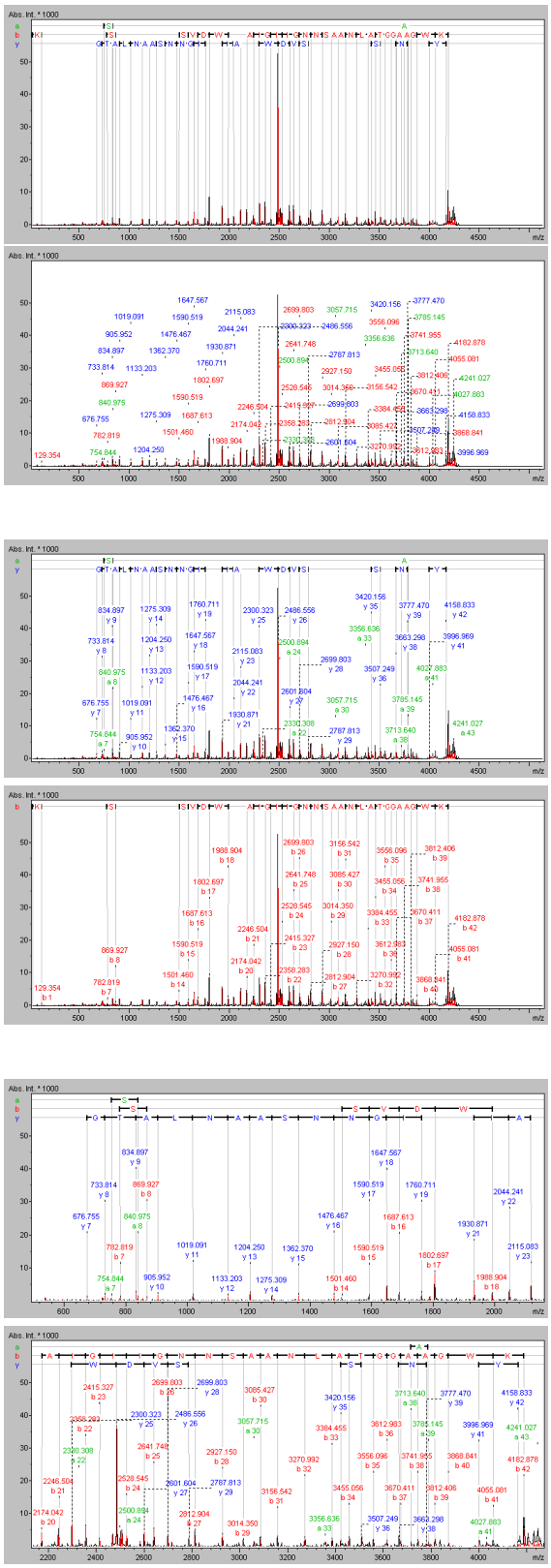

Figure S4, related to Figure 4. The fragmentation pattern obtained from the high resolution tandem mass spectrometry (MS/MS) analysis of the purified peptide.

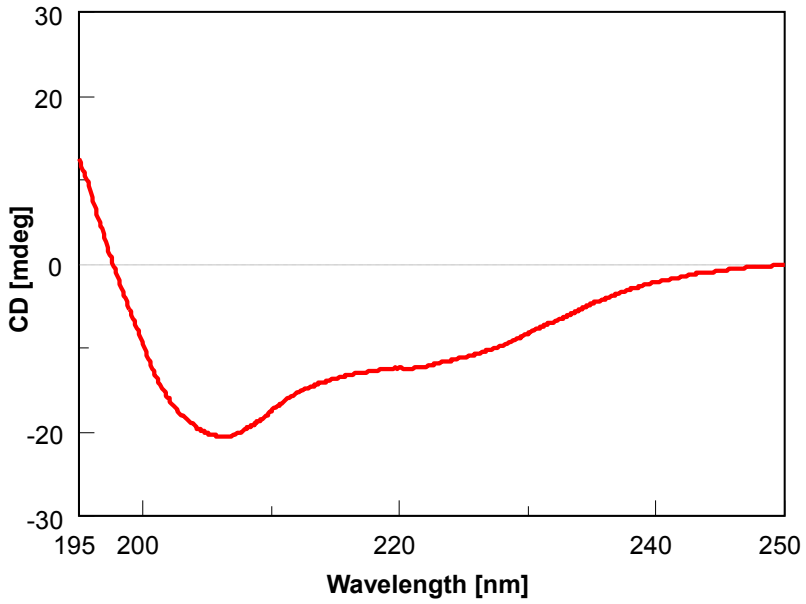

Figure S5, related to Figure 5. CD spectrum of mundticin KS in 90% 2, 2, 2-trifluoroethanol (0.1% trifluoroacetic acid final concentration, pH 2.5) at 20°C.

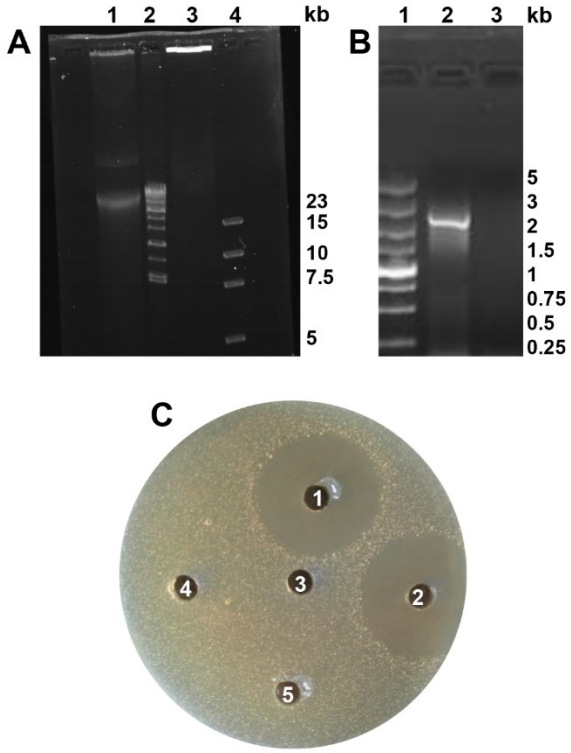

Figure S6, related to Figure 6. Curing of plasmids from *E. mundtii* SL. (*A*) Plasmid profiles. 1, plasmid DNA isolated from the native *E. mundtii* SL strain; 2, Lambda Mix marker; 3, no plasmid DNA was isolated from the novobiocin-treated *E. mundtii* SL colonies (*E. mundtii* SL *Mun*$^-$ strain); 4, Takara DL15000 DNA marker. (*B*) Amplification of the mundticin gene. 1, Takara DL5000 DNA marker; 2, *E. mundtii* SL genomic DNA as template; 3, *E. mundtii* SL *Mun*$^-$ genomic DNA as template. (*C*) Detection of antimicrobial activity against *E. faecalis* SL. 1 and 2, *E. mundtii* SL strains; 3-5, *E. mundtii* SL *Mun*$^-$ strains.

Table S1, related to Figure 2. Antimicrobial spectrum of mundticin KS against indicator organisms. Symbols: [a], pathogenicity was reported in at least one insect species. [b], -, no inhibition was observed or inhibition zone was less than 2 mm diameter; +, inhibition zone larger than 2 mm diameter. Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen.

Organisms	Strain designation	Natural occurrence	Insect pathogen[a]	Inhibition in the agar diffusion assay[b]
Enterococcus faecalis	DSM 20478	Soil, ground water, plants, animals	+	+
Enterococcus faecium	DSM 20477	Soil, ground water, plants, animals	+	+
Enterococcus hirae	DSM 20160			+
Listeria monocytogenes	DSM 20600	Soil, ground water, plants, animals	+	+
Listeria innocua	DSM 20649	Soil, ground water, plants, animals	+	+
Lactobacillus pentosus	DSM 20314			+
Lactobacillus plantarum	DSM 20174			+
Lactobacillus brevis	DSM 1267			-
Lactococcus lactis subsp. lactis	DSM 20481			+
Leuconostoc mesenteroides subsp. mesenteroides	DSM 20343			+
Streptococcus thermophilus	DSM 20617			+
Pediococcus acidilactici	DSM 20336			+
Pediococcus pentosaceus	DSM 20284			+
Clostridium cellulolyticum	DSM 5812			-
Escherichia coli	DSM 10198			-
Pseudomonas fluorescens	DSM 50090	Soil, ground water, plants	+	-

Table S2, related to experimental procedures. Primers used to characterize bacterial taxa, virulence gene and mundticin production.

Primer	Sequence (5'-3')	Target	Use
27f	AGAGTTTGATCCTGGCTCAG	Eubacteria	16s rRNA gene amplification
1492r	GGTTACCTTGTTACGACTT	Eubacteria	16s rRNA gene amplification
M13f	GTAAAACGACGGCCAG	Plasmid	Sequencing
M13r	CAGGAAACAGCTATGAC	Plasmid	Sequencing
Gray28f	GAGTTTGATCNTGGCTCAG	Eubacteria	454 sequencing
Gray519r	GTNTTACNGCGGCKGCTG	Eubacteria	455 sequencing
gelE f	TATGACAATGCTTTTTGGGAT	*Enterococcus* genus	Virulence factor
gelE r	AGATGCACCCGAAATAATATA	*Enterococcus* genus	Virulence factor
Mnt-1f	TGAGAGAAGGTTTAAGTTTTGAAGAA	Mundticin KS	PCR
Mnt-1r	TCCACTGAAATCCATGAATGA	Mundticin KS	PCR
CRL35-f	GCAAACCGATAAGAATGTGGGAT	Enterocin CRL35	PCR
CRL35-r	TATACATTGTCCCCACAACC	Enterocin CRL35	PCR

Supplemental Experimental Procedures

Insects rearing

Spodoptera littoralis was maintained in the laboratory at 23 – 25°C under a long-day regimen of 16 h of light and 8 h of dark. The larvae hatched from eggs and were reared on a sterile artificial diet according to a standard protocol (Shao et al., 2014).

Bacterial isolation and identification

Enterococcus bacteria were isolated from normal *S. littoralis* larvae using the *Enterococcus* Selective Agar. 2, 3, 5-Triphenyltetrazolium chloride (TTC) in the medium is reduced to insoluble formazan inside the bacterial cells, which gives pink or red coloration to colonies. Prior to be dissected, the larvae were washed and sedated on ice for at least 1 h to anesthetize them. The whole gut sections were dissected from larvae using a fine Vannas scissor and forceps under a binocular microscopy following the procedure described by Shao and colleagues (Shao et al., 2013). The insect tissues were immediately subjected to bacterial isolation or were stored in the freezer (-80°C) until nucleic acids were extracted.

For bacteria isolation, the fresh gut tissues and egg clutches consisting of 200 to 800 eggs (5-9 replicates for each sample) were put into phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and 2.7 mM KCl) and homogenized by hand with a sterile pestle. After homogenization, each sample was shaken well and briefly centrifuged at 800 x *g* to pellet the debris. The up bacterial suspensions were plated on *Enterococcus* selective agar. The isolated enterococcal colonies were randomly picked from the agar plate and subcultured at least three times before being examined. The purified isolates were used for broth culture and later were subjected to the biochemical tests. The enterococcal isolates were first screened by phenotypic methods as described by Albert Manero and Anicet R. Blanch et al. (1999). The related biochemical tests were listed in Table 1. Carbohydrate fermentations were performed with the basal medium phenol red broth (Proteose peptone 10.0 g, beef extract 1.0 g, sodium chloride 5.0 g and phenol red 0.018 g per liter, final pH 7.4 +/- 0.2 at 25°C). L-Arabinose (A3256, Sigma) and methyl-α-D glucopyranoside (M9376, Sigma) were added at 1% concentrations to phenol red broth. The inoculated carbohydrate broths were incubated at 37°C for 24 h. The appearance of a yellow color indicated a positive result. The production of yellow pigment was determined by growing the strains overnight at 37°C in brain heart infusion broth (53286, Fluka). The bacterial cells were pelleted, washed by PBS buffer and observed. The representative enterococcus isolates, designated strain SL, were stored as glycerol stocks at −80°C for further analysis.

For the cloning and sequencing of the bacterial 16S rRNA gene of the isolated strains, a colony PCR was performed with each purified representative strain and a 1.5 kb segment of bacterial 16S rRNA gene was amplified with the general primer 27f/1492r (Table S2). PCR reactions were conducted with Taq DNA polymerase (10342020, Life Technologies) and the supplemented buffer system, under the following temperature profile: 95°C for 5 min, followed by 35 cycles consisting of 95°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. The obtained PCR products with correct size were eluted from the agarose gel using QIAquick gel extraction kit (28704, Qiagen) and ligated into the pCR®4-TOPO® vector (K4575-01, Life Technologies) for sequencing. The recombinant plasmids were isolated from the positive colonies using a plasmid Miniprep kit (K0502, Thermo Scientific) and commercially sequenced by using the M13 primers, at Eurofins MWG Operon, Ebersberg, Germany. DNA sequences were cleaned and assembled with DNASTAR Lasergene software package (DNASTAR, Inc., Madison, WI, USA). The obtained sequences were subjected to the BLASTN search in GenBank and Greengenes.

For the 454 pyrosequencing analysis of the gut microbiota, the total genomic DNA was first extracted from the gut tissue using the MasterPure DNA Purification Kit (MC85200, Epicentre Technologies) according to the manufacturer's instructions. The quantity and quality of the purified DNA were measured with a NanoVue spectrophotometer (GE Healthcare Europe GmbH, Freiburg, Germany). The hypervariable V1–V3 region of the 16S rRNA gene was amplified from the gut genomic DNA using a Gray28F/519r primer pair (Table S2) and sequenced using the Roche 454 FLX Titanium based strategy as previously described (Shao et al., 2014). The software package "Quantitative Insight into Microbial Ecology" (QIIME, 1.4.0 version) was used to process the obtained sequencing data (Caporaso et al., 2010). Briefly, all low quality reads (quality cutoff = 25) and short sequences (read < 200 bp) were first removed from the raw data. Next UCLUST with 97% similarity cut-offs were employed in OTU picking to cluster the high-quality sequences into operational taxonomic units (OTUs), from which one representative sequence was chosen and the RDP classifier was used to determine the highest

resolution of taxonomy. Finally, an OTU table was created describing the occurrence of bacterial phylotypes within the sample.

For the molecular phylogenetic analysis, multiple alignments of nucleotide sequences were generated by using the program package MEGA, version 5.05 (Tamura et al., 2011). A model-test was used to estimate the best-fit substitution model (ML) for phylogenetic analyses and phylogenetic tree was constructed with 1000 bootstrap resamplings.

Determination of inhibitory spectrum

Enterococcus strains, including the isolated *E. faecalis* SL, *E. casseliflavus* SL, *E. mundtii* SL strain and *E. mundtii* type strain, were cultured in de Man-Rogosa-Sharpe (MRS) broth (X925.1, Carl Roth) at 30°C without shaking. The overnight culture supernatant was adjusted to pH 7.0 with 1 M NaOH and filtered through 0.22 µm PVDF membrane for testing. The indicator strains were grown in the media (BHI or MRS) recommended by culture collections. Antimicrobial activity was evaluated by using a well diffusion assay as described by Sammer et al. (Sammer et al., 2009). After the overnight broth culture of the bacterial indicator strain was adjusted to an optical density of 1 at 600 nm (OD600), a portion (0.5 mL) was mixed with 20 mL of melted LB agar medium (19344, Fluka) at 50°C, and the inoculated agar was poured into Petri dishes. Holes (6-mm) were cut out with sterile Pasteur pipettes in the inoculated agar plate and filled with 80 µL of the test culture filtrates. The inhibition zones were measured after 24 to 48 h incubation at 30°C. The diameters of clear growth inhibition zones around the well were recorded. All assays were repeated at least three times and were compared with equally prepared type strain controls. The antimicrobial spectrum of the purified compound was analyzed according to the same procedure described above.

Growth test at high pH and the antimicrobial production

To better characterize the effects of pH on the *E. mundtii* strain isolated from the larval gut of *S. littoralis*, cultures were inoculated under pH-buffered conditions and growths were monitored. A portion (1 mL) was removed at appropriate intervals for measuring biomass by absorbance at 600 nm and the antimicrobial activity calculated from the result of the agar diffusion test. The activity titer was expressed in arbitrary units (AU) per mL of bacteriocin preparation, which was calculated from the reciprocal of the highest dilution that produced an inhibition zone of at least 2 mm in diameter as described elsewhere (Line et al., 2008). The effect of pH on the inhibitory substance was tested by adjusting each of the cell-free supernatants of *E. mundtii* SL strain to pH 5.0 to 10.0 (at intervals of 1 pH unit) with 1 M HCl or 1 M NaOH. After 20 min of incubation, the samples were readjusted to pH 7.0 and tested for antimicrobial activity by using the agar well diffusion method. The effect of temperature was tested by heating the supernatant at 60, 80 and 100°C for 1 h, respectively, and the residual activity was measured. To investigate the mode of action, the exponential phase cells of the indicator *E. faecalis* SL strain were exposed to the purified antimicrobials. As control, samples without inhibitory substance were used. After incubation over time, the viable cells were counted on agar plates.

Purification and characterization of the antimicrobial substance

Five liters of MRS medium were inoculated with 50 mL of *E. mundtii* SL strain preculture and incubated for 24 h at 30°C without shaking. The broth culture was clarified by centrifugation at 8000 rpm for 10 min. Mundticin KS was purified from the cell-free culture supernatant by a three-step procedure consisting of cation exchange chromatography (TSK SP-650, Tosoh Bioscience LLC), absorbent interaction (Amberlite XAD-16, Sigma-Aldrich), and reverse-phase high performance liquid chromatography (HPLC) equipped with a C18 semi-preparative column (5 µm, 250 x 10 mm) or a C18 analytic column (5 µm, 250 x 4.6 mm). The culture supernatant was directly applied to a column containing 100 mL cation exchange media and was washed with 500 mL of 10 mM PBS (pH 7.0). The active fraction was eluted with 200 mL of 1 M NaCl and further extracted twice with 20 g XAD-16 resin which was equilibrated with water. The resin was first washed with 100 mL of water and subsequently with 100 mL of 30% (v/v) ethanol. The active fraction was eluted with 100 mL of 70% ethanol containing 0.1% trifluoroacetic acid (TFA) and lyophilized for further purification. HPLC separations were conducted with an Agilent 1200 HPLC system with diode array detection under the following LC conditions: 1–10 min, 20% B; 10–40 min, 20–50% B; 40–50 min, 50%–100% B; 50–51 min, 20% B (solvent A: water with 0.1% TFA; solvent B: acetonitrile); the flow rate was set to 5 or 1 mL min^{-1} according to the column used. The purified compound was stored at −20°C. Mass spectrometry (MS) analyses were conducted with an Agilent 6530 Accurate-Mass Q-TOF MS. The MALDI–TOF/TOF (PSD; post source decay) spectrum was obtained by Bruker UltrafleXtreme and analyzed by BioTools. The LC-HRMS measurement was carried out on a Thermo Fisher Scientific Exactive Orbitrap with an electrospray ion source using a Betasil 100-3 C_{18} column (150 × 2.1 mm)

and an elution gradient (solvent A: water with 0.1% formic acid, solvent B: acetonitrile; gradient: 5% B for 1 min, 5% to 98% B in 15 min, 98% B for 3 min, flow rate: 0.2 mL min^{-1}, injection: 5 µL).

Identification of the bacteriocin coding gene and plasmid-curing experiments

The total genomic DNA from *E. mundtii* SL strain was extracted using a Genomic DNA Purification Kit (K0512, Thermo Scientific) according to the manufacturer's instructions. Several primer pairs, designed for screening enteriocins (Table S2), were used for amplifying possible gene cluster from the *E. mundtii* SL genomic DNA template. PCR was performed under the conditions described elsewhere (Kawamoto et al., 2002). The amplified product with the expected size was purified from the agarose gel, cloned and then sequenced as described above. Database searches for the DNA sequences were performed using the BLAST program of the National Center for Biotechnology Information, which was further analyzed using the software Geneious Pro v5.5.7 (Biomatters) (Kearse et al., 2012). Amino acid sequences were aligned with MUSCLE or CLUSTALW algorithm. The peptide secondary and tertiary structure was predicted by Phyre2 using default parameters and structure templates extracted from Protein Data Base (PDB) (Kelley and Sternberg, 2009). The α-helix domain was determined by performing the circular dichroism (CD) spectroscopy (Kaur et al., 2004). Plasmid curing was carried out by a procedure combining three conditions known to lead to plasmid loss: the use of novobiocin and growth at 42°C in the medium lacking its buffering component (Floriano et al., 1998). *E. mundtii* SL was cultured for 96 h under these conditions and the procedure was repeated several times. Cultures were plated on LB agar plates to yield individual colonies. After 18 h at 30°C, MRS soft agar (0.7% agar) containing the indicator strain *E. faecalis* SL was poured onto the plates, which were incubated for an additional 24 h. Colonies without clear zones of inhibition were purified and repeatedly transferred into MRS broth, and their ability to inhibit the growth of *E. faecalis* SL was determined.

Quantification of expression by Real-Time RT-PCR and clearance of *E. faecalis* in the infection assay

The abundance of mundticin KS transcripts was determined by quantitative RT-PCR using the absolute quantification method (Herrmann et al., 2012). Total RNA was first extracted from fresh gut tissues using the MasterPure RNA Purification Kit (MCR85102, Epicentre Technologies), according to the manufacturer's instructions. Isolated RNA was treated with DNAse (D9905K, Epicentre Technologies) to remove contaminating DNA and quantified by the Nanovue spectrophotometer. 500 ng of pure RNA was used to make cDNA using a cDNA Synthesis Kit (205311, Qiagen) with random primers. The reactions were conducted in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) with 5 µL Brilliant II SYBR Green PCR master mix (600828, Agilent Technologies), 2.0 µL 2.5 µM primers and 1 µL of cDNA template in 10 µL volume, with no RT samples, *E. mundtii* cured samples, and the *E. mundtii* DSM4838 strain as negative controls. The thermal profile was 95°C for 3 min, 40 amplification cycles of 95°C for 5 s, 62°C for 5 s, and dissociation cycle of 95°C for 1 min, 65°C for 5 s then brought back to 95°C. The primer efficiency was calculated in the software Bio-Rad CFX Manager (2.1) and the dissociation curve confirmed that every reaction yielded a single PCR product with the predicted Tm. Standard curves were constructed using plasmids with cloned *munA* gene from *E. mundtii* SL strain and were linear from 1×10^8 to 10 copies with R^2 values > 0.99. Based on the standard curves, the transcript number could be calculated for each individual gut sample from the qPCR threshold values (Ct). The experiment was done in triplicates and repeated at least three times.

For the infection assay, the same batch of larvae was fed with a low amount of antibiotic (10 µg erythromycin/g artificial diet) to eliminate native *E. mundtii* and starved 2 days before the tests. Half of the treated larvae were re-introduced of *E. mundtii* SL and another half larvae, as the control group, were re-introduced of *E. mundtii Mun*$^-$ which lacks the mundticin gene. Then the larvae in both groups were intensively fed artificial diet treated with *E. faecalis* SL one time and the gut enterococcal population was analyzed as described above. The whole infection assay was performed in triplicate under the same rearing condition. The bacteriocin feeding experiment was performed by methods described elsewhere (Dabour et al., 2009; Salvucci et al., 2012). *E. mundtii* SL-cured larvae were infected with *E. faecalis* SL and subsequently separated as six groups including five mundticin treatments and one control. In the treatment group, mundticin was administered at five concentrations (1, 2.5, 5, 10 and 20 µM made up in PBS). PBS only was used as placebo in the control group. At least three larvae were used per group. After 24 h feeding on the amended diet, *E. faecalis* SL CFUs were determined for each case. Analysis of variance (ANOVA) was performed using the SPSS Statistics 17.0 software and a *post hoc* Tukey's honestly significantly different (HSD) test was used to evaluate differences.

Supplemental References

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. Nat Methods *7*, 335-336.

Dabour, N., Zihler, A., Kheadr, E., Lacroix, C., and Fliss, I. (2009). In vivo study on the effectiveness of pediocin PA-1 and Pediococcus acidilactici UL5 at inhibiting Listeria monocytogenes. Int J Food Microbiol *133*, 225-233.

Floriano, B., Ruiz-Barba, J.L., and Jimenez-Diaz, R. (1998). Purification and genetic characterization of enterocin I from Enterococcus faecium 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl Environ Microbiol *64*, 4883-4890.

Herrmann, M., Hadrich, A., and Kusel, K. (2012). Predominance of thaumarchaeal ammonia oxidizer abundance and transcriptional activity in an acidic fen. Environmental microbiology *14*, 3013-3025.

Kaur, K., Andrew, L.C., Wishart, D.S., and Vederas, J.C. (2004). Dynamic relationships among type IIa bacteriocins: temperature effects on antimicrobial activity and on structure of the C-terminal amphipathic alpha helix as a receptor-binding region. Biochemistry *43*, 9009-9020.

Kawamoto, S., Shima, J., Sato, R., Eguchi, T., Ohmomo, S., Shibato, J., Horikoshi, N., Takeshita, K., and Sameshima, T. (2002). Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by Enterococcus mundtii NFRI 7393. Appl Environ Microbiol *68*, 3830-3840.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., *et al.* (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics *28*, 1647-1649.

Kelley, L.A., and Sternberg, M.J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc *4*, 363-371.

Line, J.E., Svetoch, E.A., Eruslanov, B.V., Perelygin, V.V., Mitsevich, E.V., Mitsevich, I.P., Levchuk, V.P., Svetoch, O.E., Seal, B.S., Siragusa, G.R., *et al.* (2008). Isolation and purification of enterocin E-760 with broad antimicrobial activity against gram-positive and gram-negative bacteria. Antimicrob Agents Chemother *52*, 1094-1100.

Salvucci, E., Saavedra, L., Hebert, E.M., Haro, C., and Sesma, F. (2012). Enterocin CRL35 inhibits Listeria monocytogenes in a murine model. Foodborne pathogens and disease *9*, 68-74.

Sammer, U.F., Volksch, B., Mollmann, U., Schmidtke, M., Spiteller, P., Spiteller, M., and Spiteller, D. (2009). 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, an effective peptide antibiotic from the epiphyte Pantoea agglomerans 48b/90. Appl Environ Microbiol *75*, 7710-7717.

Shao, Y., Arias-Cordero, E., Guo, H., Bartram, S., and Boland, W. (2014). In vivo Pyro-SIP assessing active gut microbiota of the cotton leafworm, Spodoptera littoralis. PLoS ONE *9*, e85948.

Shao, Y., Arias-Cordero, E.M., and Boland, W. (2013). Identification of metabolically active bacteria in the gut of the generalist Spodoptera littoralis via DNA stable isotope probing using 13C-glucose. Journal of visualized experiments : JoVE, e50734.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution *28*, 2731-2739.