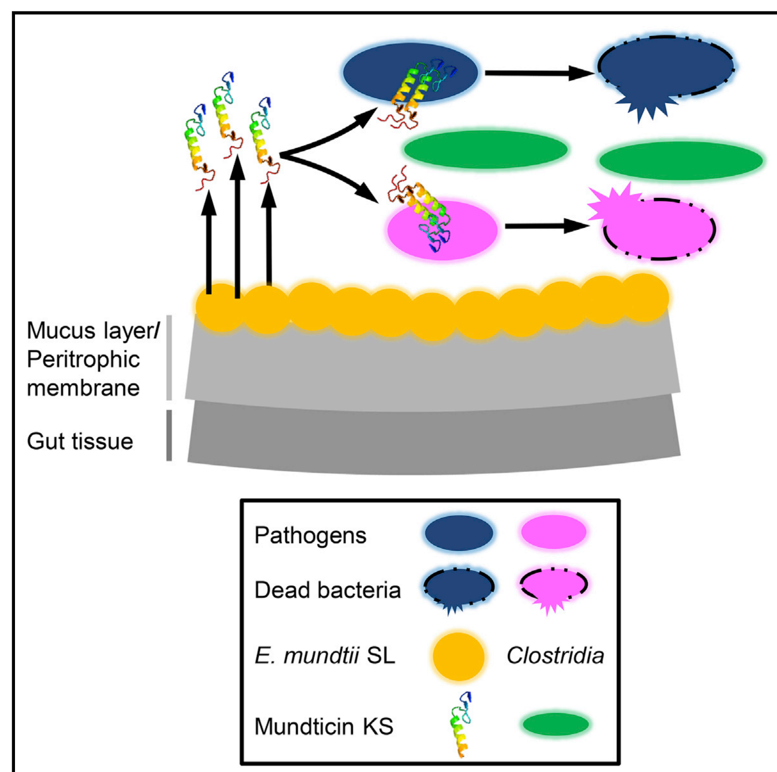
Article

Cell Chemical Biology

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

Graphical Abstract



Authors

Yongqi Shao, Bosheng Chen,
Chao Sun, Keishi Ishida,
Christian Hertweck, Wilhelm Boland

Correspondence

yshao@zju.edu.cn (Y.S.),
boland@ice.mpg.de (W.B.)

In Brief

Shao et al. show that the extracellular symbiont *E. mundtii* of the cotton leafworm *S. littoralis* selectively clears pathobionts from the host gut lumen by secreting a stable antimicrobial peptide, which facilitates the normal development of gut microbiota and reduces risks of infection via the gut.

Highlights

- The gut bacterium *E. mundtii* of *S. littoralis* produces an antimicrobial peptide
- Mundticin KS strongly inhibits some of the competing and potentially pathogenic organisms
- Mundticin KS expresses in vivo and can impair pathogen colonization

Shao et al., 2017, Cell Chemical Biology *24*, 66–75
January 19, 2017 © 2017 Elsevier Ltd.
http://dx.doi.org/10.1016/j.chembiol.2016.11.015

CellPress

Please cite this article as: Shao et al., Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota, Cell Chemical Biology (2017), http://dx.doi.org/10.1016/j.chembiol.2016.11.015

CellPress

Cell Chemical Biology
Article

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

Yongqi Shao,[1,*] Bosheng Chen,[1] Chao Sun,[3] Keishi Ishida,[4] Christian Hertweck,[4,5] and Wilhelm Boland[2,6,*]
[1]College of Animal Sciences, Zhejiang University, Hangzhou 310058, China
[2]Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Beutenberg Campus, 07745 Jena, Germany
[3]Analysis Center of Agrobiology and Environmental Sciences, Zhejiang University, Hangzhou 310058, China
[4]Department of Biomolecular Chemistry, Leibniz Institute for Natural Product Research and Infection Biology–Hans Knöll Institute, 07745 Jena, Germany
[5]Friedrich Schiller University Jena, 07745 Jena, Germany
[6]Lead Contact
*Correspondence: yshao@zju.edu.cn (Y.S.), boland@ice.mpg.de (W.B.)
http://dx.doi.org/10.1016/j.chembiol.2016.11.015

SUMMARY

Insects develop efficient antimicrobial strategies to flourish in a bacterial world. It has long been proposed that native gut microbiota is an important component of host defense; however, the responsible species have rarely been isolated to elucidate the mechanism of action. Here we show that the dominant symbiotic bacterium *Enterococcus mundtii* associated with the generalist herbivore *Spodoptera littoralis* actively secretes a stable class IIa bacteriocin (mundticin KS) against invading bacteria, but not against other gut residents, facilitating the normal development of host gut microbiota. A mundticin-defective strain lost inhibitory activity. Furthermore, purified mundticin cures infected larvae. Thus, the constitutively produced antimicrobials by native extracellular symbionts create a significant chemical barrier inside limiting invader expansion. This unique property also benefits *E. mundtii* itself by providing a competitive advantage, contributing to its dominance within complex microbial settings and its prevalence across Lepidoptera, and probably promotes the long-term cooperative symbiosis between both parties.

INTRODUCTION

Insects, the largest group of animals on Earth, comprise over a million described species. As nearly half of insects feed on plants, they are the most significant herbivores in the world (Mithoefer and Boland, 2012). Herbivorous insects inhabit diverse niches and typically consume large amounts of plant material. Owing to their immoderate foraging behavior, herbivorous insects also ingest a wide variety of potentially harmful microorganisms from either the food resources that contain abundant microbial flora or the surrounding environment associated with microbes (Herzner et al., 2013; Lindow and Brandl, 2003; Ruffner et al., 2013). Such invading microbes compete for limited resources inside the host, as well as increase the risk of infection via the gut, which is probably the main route of insect infection in nature (Vallet-Gely et al., 2008). Although they face repeated challenges from nonnative microorganisms, herbivorous insects are notably resistant to microbial infections and almost always flourish in microbe-rich environments (Zasloff, 2002). It is not surprising that they have evolved effective antimicrobial strategies, including the immune system as well as the physical and chemical barriers created in the gut, such as the strongly alkaline pH in lepidopteran larvae (Funke et al., 2008). In addition, the native gut microbiota (or indigenous microbiota) is increasingly recognized as an important component of host defense against microbial invaders in many animals, from invertebrates to vertebrates (Donia et al., 2014; Iatsenko et al., 2014). For example, the microbial community of bumblebees was shown to protect against a common intestine parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011). Experiments on mice have demonstrated that a normal microbiota is necessary and sufficient for mediating pathogen clearance from the gut lumen in primary infections with *Salmonella typhimurium* (Endt et al., 2010). Despite these accumulating observations that the native gut microbiota is involved in combating infections, the species which exert this protective effect have rarely been either identified from the microbial consortia or cultured outside the host (Servin, 2004), and the molecular mechanisms of action are poorly understood (Endt et al., 2010; Hudault et al., 2001; Koch and Schmid-Hempel, 2011; Servin, 2004; Stecher et al., 2013; Vasquez et al., 2012).

Here, we experimentally address the antimicrobial activity of a culturable gut symbiotic bacterium in the cotton leafworm *Spodoptera littoralis* (Lepidoptera, Noctuidae), which represents one of the most polyphagous and widespread insect herbivores in the temperate regions, causing substantial economic losses in crop production. Like most lepidopteran larvae, cotton leafworms possess a simple alimentary canal, shaped like a tube without any specialized substructures; however, a large bacterial population, exceeding 10^7 colony-forming units (CFU)/mL, is evident in the gut (Funke et al., 2008). Recent surveys of the taxonomic composition of this gut microbiota reveal a relatively simple but specific community, dominated by *Enterococcus* and *Clostridium* spp. (Tang et al., 2012). In particular, *E. mundtii* is repeatedly found in normal individuals regardless of diet and

CrossMark

Table 1. General Features of *Enterococcus* Bacteria Isolated from *S. littoralis*

	Fermentation Pattern					
Enterococcus sp.	L-Arabinose	Methyl-α-D-glucopyranoside	Pigment	16s rDNA Identity (%)	Virulence Factor (gelE)	Insect Mortality (%)[a]
E. faecalis SL	+	–	–	100	+	47(a) ± 10
E. mundtii SL	+	–	+	99.78	–	0(b) ± 0
E. casseliflavus SL	+	+	+	100	–	40(a) ± 14

SL, strains isolated from the larval gut of *S. littoralis*; +, positive results; –, negative results; gelE, enterococcal gelatinase.
[a]Accumulative mortality until adult stage, ANOVA analysis showed significant differences.

persists in the gut across host developmental stages. Our previous study using stable isotope labeling indicates that *E. mundtii* is also the most metabolically active member in the gut microbiota (Shao et al., 2014), which suggests that this symbiotic species plays important roles.

We found that, although *E. mundtii* is a major constituent of the gut microbiota across generations, suggesting the vertical transmission mode of this symbiont, other ubiquitous environmental enterococci, such as *E. faecalis* and *E. casseliflavus*, often started out at high density in early instar when *E. mundtii* had not yet established abundant populations inside the gut (Tang et al., 2012). *Enterococcus* bacteria not only occur in a wide variety of habitats including plants, soil, and water, but also are well known for their ability to adapt to the gastrointestinal tracts of animals and could cause serious infections under certain conditions (Holt et al., 2015). Especially, *E. faecalis* and *E. casseliflavus* have developed many mechanisms to invade the host that allow them to immediately fill empty niches early in life and are generally considered opportunistic pathogens in many animals, whereas *E. mundtii* is rarely documented as a pathogen. It is known that *E. faecalis* poorly supported larval development in the housefly (Ghosh et al., 2014). Certain strains of *E. faecalis* have also been shown to cause lethal infection in Lepidoptera (Doane and Redys, 1970). Since enterococci strains carrying diverse virulence factors have emerged in many habitats and are isolated with alarming regularity (Palmer et al., 2012), these possible pathogens that are often orally acquired by *S. littoralis* nymphs from their diet (first contaminated eggshell, then plants) and the surrounding environment, represent a critical challenge to the host. But, notably, they are quickly depleted from the microbiota concomitant with an increase of the *E. mundtii* population, which subsequently stabilizes in the gut together with clostridia as core components of the flora. It seems likely that the indigenous gut-resident *E. mundtii* has an inherent advantage over those closely related but potentially pathogenic bacteria, although they have already blocked the gut niche (highly adherent to gut epithelium) at an early stage, and thus potentially confers invasion resistance benefit on its host. These observations prompted us to isolate and identify the enterococci in *S. littoralis*, ask whether *E. mundtii* could specifically inhibit nonindigenous invading microbes, and investigate the mechanism of action.

RESULTS

Survey and Characteristics of *Enterococcus* Isolated from *S. littoralis*

To study the antagonism between enterococci, we isolated different species from *S. littoralis* larvae and eggs using a selective differential medium on which enterococci grow well and produce a pink pigmentation that distinguishes species of this genus from others. Using three-step phenotyping (Table 1), we were able to discriminate among purified bacterial strains (Manero and Blanch, 1999). Carbohydrate fermentation tests with L-arabinose or methyl-α-D-glucopyranoside give a yellow color for a positive reaction and a red color for a negative reaction. The production of pigment is considered positive when the strain presents a strong yellow color. Based on those phenotypic characteristics, three *Enterococcus* species, *E. mundtii*, *E. casseliflavus*, and *E. faecalis*, were identified from both the larval gut and eggs. We focused on representative strains of the three species, designated strain SL, for further analysis. Their DNA was extracted and the nearly full-length 16S rRNA genes were amplified and sequenced. The obtained 16S rRNA sequence of each of the three representative strains showed >99% similarity with a published sequence in GenBank, and the closest relatives were selected to construct a phylogenetic tree (Figure 1A), which revealed that the isolates were phylogenetically placed within three clades and enterococci from the larval gut were closely related to that from eggs, suggesting eggs as a source for *Enterococcus* to larvae.

The gut enterococcal population analysis revealed a high proportion of *E. faecalis* and *E. casseliflavus* in the first instar (Figure 1B). *E. faecalis* was on average ten times more prevalent than *E. mundtii* in the gut, but these pathogenic enterococci were completely eradicated in the second instar. In contrast, although *E. mundtii* was the minority member at a very early stage, making up only 6% of the consortium, it accounted for a large percentage of the second-instar population and subsequently colonized the gut until pupation without any significant changes. The level of *E. mundtii* ranged from 5.7×10^6 to 2.1×10^8 CFU per larva, and it was consistently recovered from different batches of samples (n = 9). Culture-independent pyrosequencing further confirmed that *E. mundtii* was the major constituent of the gut microbiota in mature larvae and also revealed that *Clostridium* species was another dominant gut bacterium (Figure S1). Symbiotic clostridia resist cultivation outside the host.

Because *E. faecalis* and *E. casseliflavus* are commonly described as opportunistic pathogens, we screened related virulence factors in isolates. A major virulence gene, *gelE*, which encodes a lethal protein to lepidopterans (Gaspar et al., 2009), was detected in *E. faecalis* isolates, but not in *E. mundtii* SL (Table 1). Infection assays of cured (*E. mundtii*-free) larvae with *E. faecalis* SL and *E. casseliflavus* SL resulted in similar disease symptoms (Figure 1C) and high mortality (Table 1) as described in other lepidopterans (Doane and Redys, 1970).

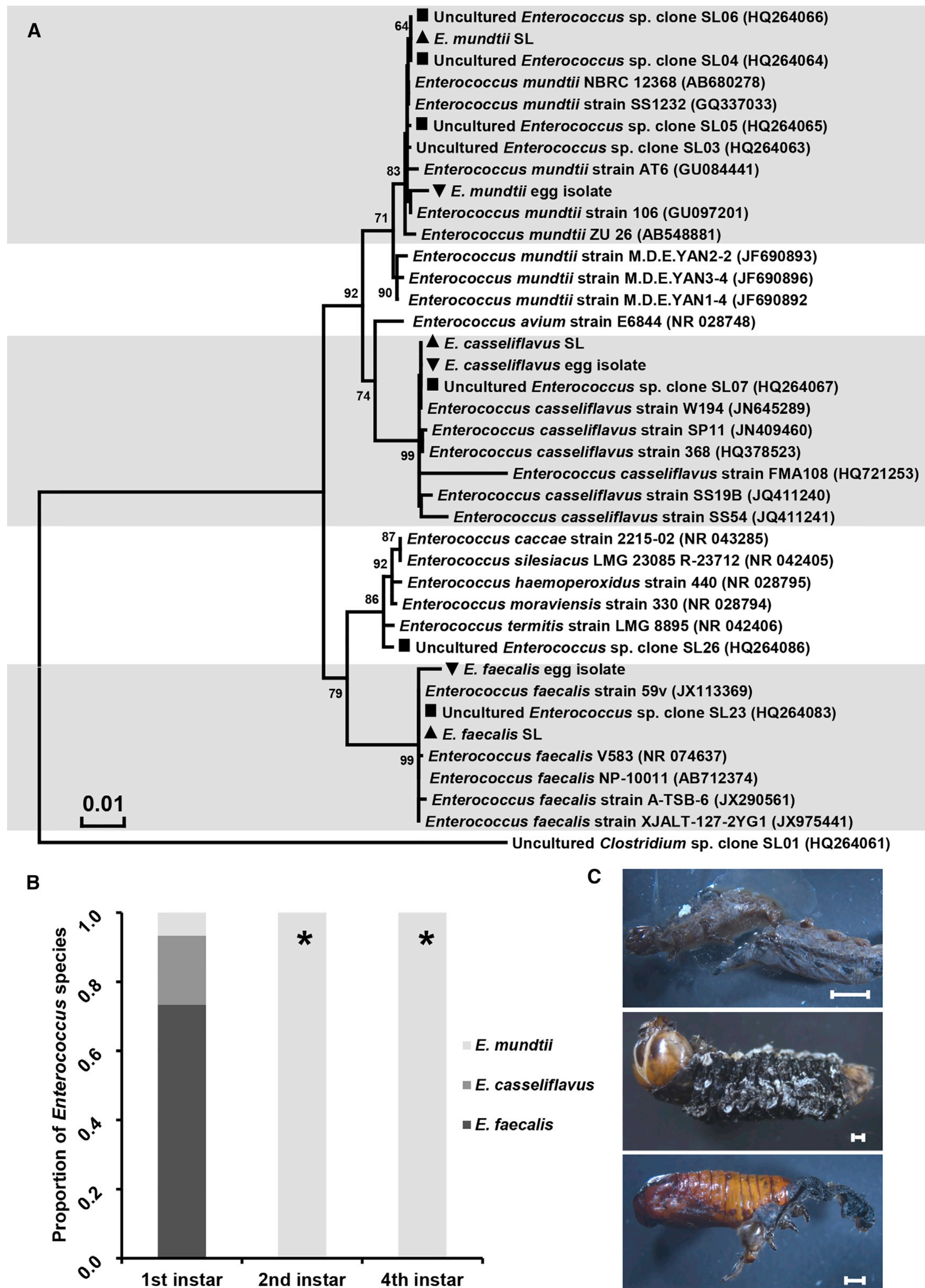
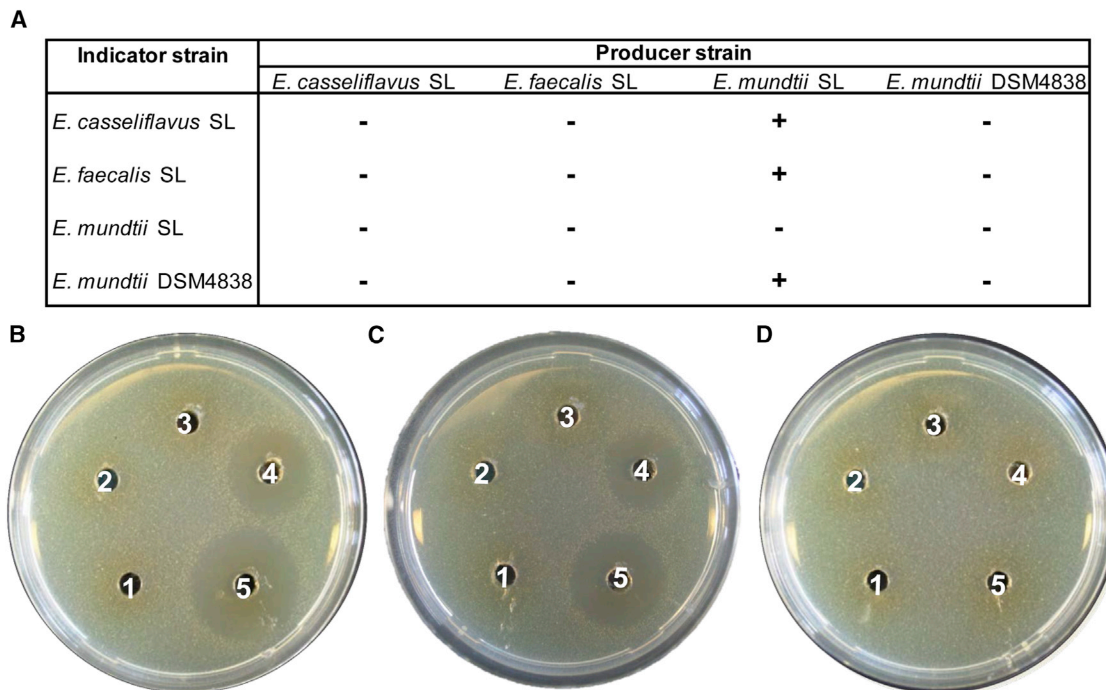


Figure 1. Survey and Characteristics of *Enterococcus* Bacteria from *S. littoralis*

(A) Phylogenetic position of enterococci identified from *S. littoralis*. Phylogeny was estimated by using the maximum likelihood method based on the Kimura two-parameter model. The scale 0.01 is the genetic distance. Bootstrap values (in percent) are based on 1,000 replications. The near full-length 16S rDNA sequences retrieved from previous clone-library-based studies of gut microbiota of *S. littoralis* are indicated by squares (■); the 16S rDNA sequences of *Enterococcus*

(legend continued on next page)



A

Indicator strain	Producer strain			
	E. casseliflavus SL	*E. faecalis* SL	*E. mundtii* SL	*E. mundtii* DSM4838
E. casseliflavus SL	-	-	+	-
E. faecalis SL	-	-	+	-
E. mundtii SL	-	-	-	-
E. mundtii DSM4838	-	-	+	-

Figure 2. The Native Gut Symbiont *E. mundtii* SL Efficiently Antagonizes Invading Enterococci

(A) Bacteriocin activity and immunity. +, growth inhibition of indicators; −, no inhibition of indicators; SL, enterococcal strains isolated from *S. littoralis*. *E. mundtii* DSM4838 is a type strain.

(B–D) (B) Typical results of agar diffusion assays using *E. faecalis* SL, (C) *E. casseliflavus* SL, and (D) *E. mundtii* SL as the indicator. 1, cell-free culture supernatant of *E. casseliflavus* SL; 2, cell-free culture supernatant of *E. faecalis* SL; 3, cell-free culture supernatant of *E. mundtii* DSM4838; 4, cell-free culture supernatant of *E. mundtii* SL; and 5, purified mundticin KS (10 ng/μL). Shown are representative results of at least ten independent experiments.

Symbiotic *E. mundtii* Directly Antagonizes Other Invading Enterococci through Its Antimicrobial Properties

The antagonistic effects of the identified enterococci were investigated by using inhibition assays on agar plates. The three representative strains showed different inhibition properties relative to each other (Figure 2A). *E. mundtii* SL exhibited specific antimicrobial activity against *E. faecalis* and *E. casseliflavus*. The cell-free *E. mundtii* SL culture supernatant inhibited the growth of indicator bacteria and resulted in a clear inhibition zone (Figures 2B and 2C), suggesting bactericidal activity that resulted from extracellular and diffusible substances. The mode of action was also confirmed in the liquid culture. A rapid decrease in the optical density at 600 nm, due to the lysis of cells, was observed after adding a purified fraction into the broth culture of indicators. Notably, there was no inhibitory effect from *E. mundtii* type strain DSM4838 on *E. faecalis* or *E. casseliflavus*, which served as a negative control. The growth of *E. mundtii* SL itself was not affected (Figure 2D), indicating that it is immune (self-protective) to the antimicrobials it produces. In contrast, *E. faecalis* and *E. casseliflavus* isolates were inactive against *E. mundtii*. Next, we focused on characterizing the antimicrobials produced by *E. mundtii* SL both in vitro and in vivo.

Antimicrobial Production, Inhibitory Spectrum and Identification

In contrast to the *E. mundtii* type strain, in which the best growth rate was observed at pH 7.0, *E. mundtii* SL grows well in high pH media. The highest cell density of strain SL can be reached within several hours at pH 9.0. Under these conditions, more bacterial biomass is produced than is produced in bacteria growing at neutral pH (Figure S2A), indicating that this strain is well adapted to the alkaline gut environment of *S. littoralis* larvae. The growth curve and antimicrobial production in a broth culture are shown in Figure 3A. An agar diffusion test indicated that *E. mundtii* cells already began secreting antimicrobial substances during the early exponential growth phase, and production increased as the bacteria grew. Maximum production was recorded at the stationary phase and remained stable up to 20 hr. Thus, antimicrobial production is associated with growth. *Enterococci*, which

isolates are indicated by triangles (▲, enterococci isolated from eggs of *S. littoralis*; ▼, enterococci isolated from the larval gut of *S. littoralis*). The numbers in parentheses are accession numbers extracted from GenBank.

(B) Change of *Enterococcus* population in the gut microbiota of *S. littoralis* larvae across different instars. Proportional data are modeled using the mean number of CFUs counted from five to nine larvae. Statistically significant changes (*$p < 0.05$: second instar versus first instar, fourth instar versus first instar) were determined with ANOVA and a post hoc Tukey honestly significant difference (HSD) test.

(C) *S. littoralis* larvae and prepupae infected with the *E. faecalis* SL strain. Upper panel, dead young larva; middle panel, shrunken cadaver with a characteristic short, dry condition; lower panel, some of the infected larvae attempted to spin a pupa and died as prepupae. Scale bar represents 1 mm.

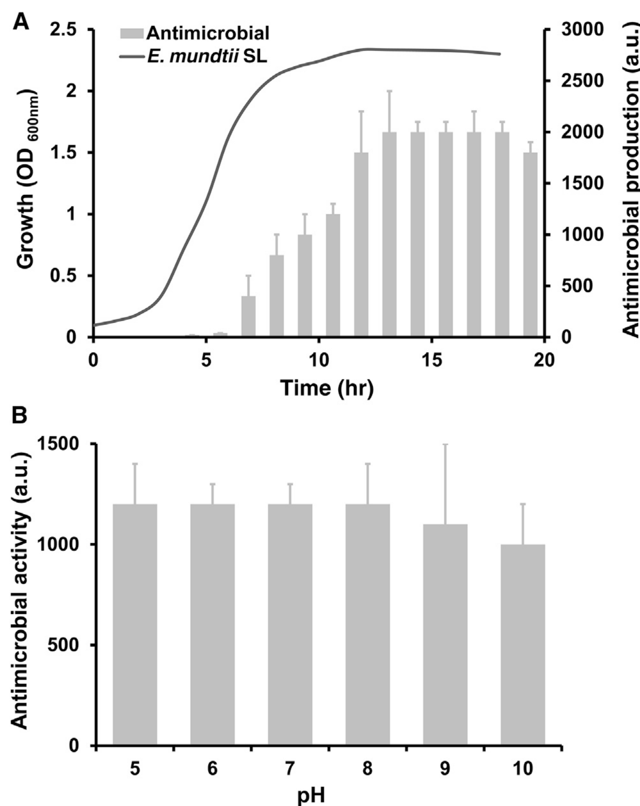


Figure 3. Characterizing Antimicrobial Production

(A) Growth curve and antimicrobial production of *E. mundtii* SL strain in liquid MRS medium at 30°C.

(B) Effect of pH on the activity of mundticin KS. Scale bars represent means from three independent experiments; error bars, SDs.

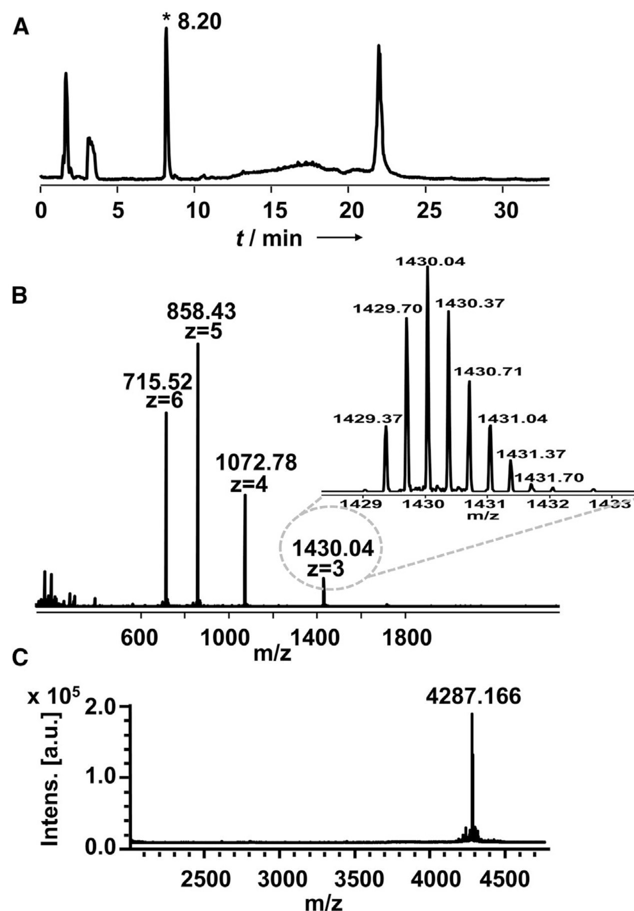


Figure 4. Identification of Inhibitory Molecule

(A) The reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram profile of the extract from the culture of *E. mundtii* SL. Elution of peptides was monitored by a UV detector at 280 nm. * Indicates the active fraction and retention time.

(B) Electrospray mass spectrum of the purified antimicrobial peptide with multiple charged molecular ions.

(C) MALDI-TOF/MS analysis of mundticin KS obtained from the second reversed-phase chromatography. Intens., intensity.

belong to lactic acid bacteria and produce organic acids during growth, decrease the medium pH (Figure S2B). However, we still observed a clear inhibition zone after the *E. mundtii* culture was neutralized; thus, the major bactericidal activity is not due to the production of acids. Notably, the bactericidal substance retains its activity after being incubated at a wide pH range (Figure 3B) and tolerates heat treatments, even at 100°C for 1 hr (Figure S3). These characteristics reveal the high stability of this antimicrobial compound, suggesting that it can function under the harsh gut conditions.

We further assessed the antimicrobial spectrum of *E. mundtii* SL (Table S1) and found it was particularly effective against some common Gram-positive pathogens, namely *Listeria monocytogenes* and *Listeria innocua*, which opportunistically infect a wide range of hosts including Lepidoptera and usually pose severe threats to larvae (Mukherjee et al., 2010). The test clostridia were insensitive to *E. mundtii* SL. *E. mundtii* SL displayed no significant activity against Gram-negative bacteria used in our assay, including *Escherichia* and *Pseudomonas* spp.

To elucidate the chemical composition of the antimicrobial substance, we performed a bioassay-guided isolation. After being purified by chromatography, a well-separated peak at a retention time of 8.20 min (the only active fraction against test enterococci) was finally collected from high-performance liquid chromatography (HPLC) and applied to mass spectrometry (MS) (Figure 4A). In liquid chromatography-electrospray ionization time-of-flight mass spectrometry (LC-ESI-TOF/MS), the bioactive compound displayed multiple charged molecular ions ranging from $[M+3H]^{3+}$ to $[M+6H]^{6+}$ (Figure 4B), which is very similar to the molecular mass of the class IIa bacteriocin mundticin KS by comparison with those of other known compounds held in protein databases. MALDI-TOF/MS of the purified substance further confirmed the molecular mass of 4,287.166 Da (Figure 4C). The fragmentation pattern obtained from the post-source decay (PSD) analysis revealed its amino acid sequence. The peptide is composed of 43 amino acid residues, and the sequence is similar to a peptide produced by *E. mundtii* strain NFRI 7393 (Kawamoto et al., 2002) (Figure S4). Taken together, these results suggest that *E. mundtii* SL, originated from *S. littoralis*, produces the bacteriocin mundticin KS as a weapon directly against other bacteria.

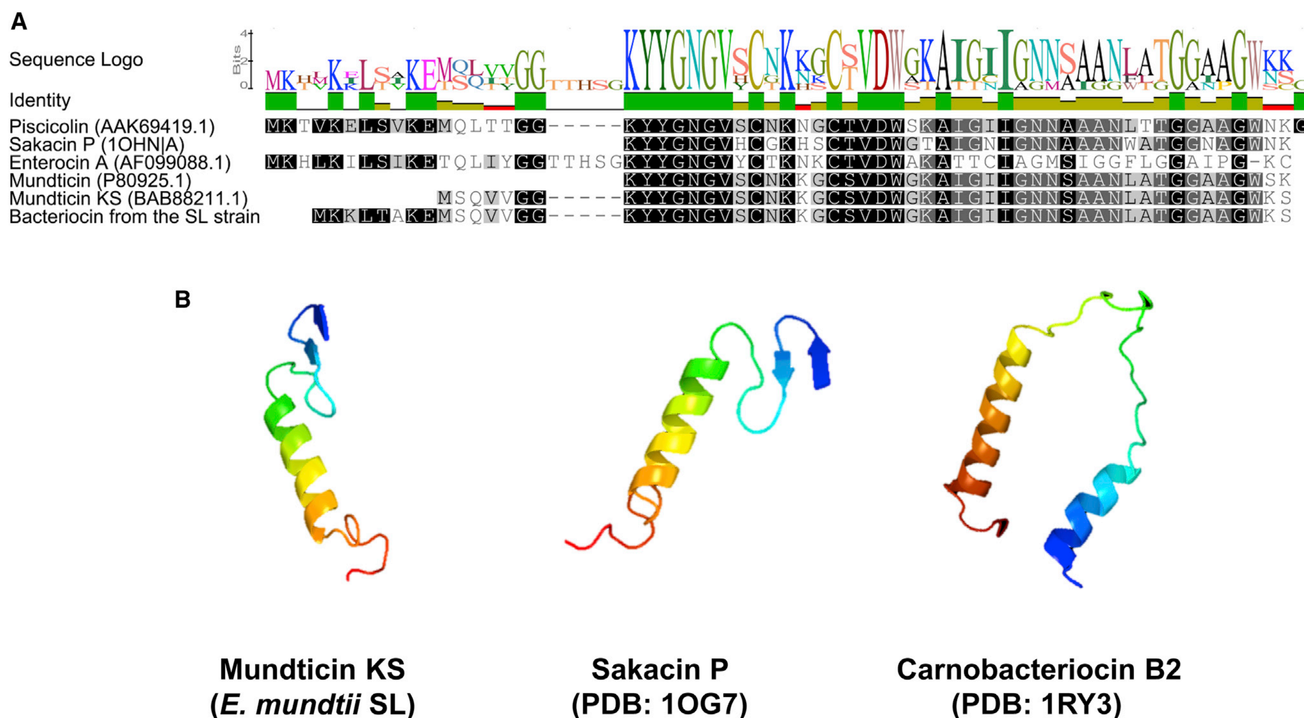


Figure 5. The Primary Sequence and Stereostructure of Mundticin KS

(A) Amino acid sequence alignment of the mundticin secreted by *E. mundtii* SL strain with other class IIa bacteriocins (access number extracted from NCBI is displayed). Color key for identity: green means that the residue at the position is the same across all sequences; yellow is for less than complete identity; red refers to a very low identity for the given position.

(B) Tertiary structure prediction for mundticin KS by Phyre2 in comparison with structures of Sakacin P (PDB: 1OG7) of *Lactobacillus sakei* and Carnobacteriocin B2 (PDB: 1RY3) of *Carnobacterium maltaromaticum* determined by nuclear magnetic resonance spectroscopy. Forty-three residues (100% coverage of mature mundticin KS) have been modeled with 99.9% confidence by the single highest-scoring template. Image colored by the rainbow N → C terminus.

Functional Gene Determination, Expression of Mundticin KS In Situ in the Gut, and Its Causal Role in the Clearance of Invading Enterococci

On the basis of the chemical identification, several primers (Table S2) were designed to amplify the responsible gene from the genomic DNA of *E. mundtii* SL. One positive amplicon with the expected size was sequenced, and the nucleic acid sequence revealed that *E. mundtii* SL possesses the mundticin KS biosynthesis gene, which matches perfectly with that in the strain NERI 7393 (Kawamoto et al., 2002). The sequence involves a region of *munA* and *munB*, which encodes the mundticin KS precursor peptide and a transport protein. In contrast, *E. mundtii* DSM4838 lacks the mundticin gene (*munA*) in agreement with its phenotypic characteristic (no antimicrobial activity, Figure 2). The deduced amino acid sequence confirms that the mature antimicrobial peptide consists of 43 amino acids with an estimated molecular mass of 4,289.73 Da, and the sequence is identical with the prediction made from PSD data (Figure 5A). A comparison of the primary structure of mundticin KS with that of other published class IIa bacteriocins provides insight into some important features of this kind of peptide antimicrobial. These bacteriocins all contain a highly conserved NH_2-terminal YGNGV motif and are largely unmodified except for a disulfide bridge formed by two cysteine residues between positions 32 and 37, which explained the difference between the observed mass (4,287 Da) and that calculated from the deduced amino acid sequence (4,289 Da). The mode of killing action of mundticin KS (the bactericidal mechanism) is likely to induce permeabilization of the cell membrane of sensitive bacteria (Cotter et al., 2013). After binding to target bacteria, these peptide molecules perturb the cytoplasmic membrane and induce pore formation and rapid dissipation of intracellular components, which eventually leads to cell death (Diep et al., 2007). Neither the detailed stereostructure of mundticin KS nor the exact nature of the pores formed on cell membrane has yet been described. By using computer modeling, we illustrated a three-dimensional structure of mundticin KS (Figure 5B). The N-terminal residues were predicted to form β strands to result in a β sheet domain, which was structurally stabilized by the disulfide bridge, while the C-terminal region harbored a α helices domain that ended with a structurally extended tail. The presence of helical peptides, confirmed by circular dichroism (CD) measurement, facilitates its penetration into the phospholipid bilayer of the cytoplasmic membrane of target bacteria (Figure S5).

During genome sequencing of *E. mundtii* SL (Chen et al., 2016a), we found the mundticin gene was plasmid encoded. After treatment with a plasmid-curing procedure, some *E. mundtii* SL colonies (*E. mundtii* SL *Mun*$^{-}$) lost their plasmids and the bacteriocin biosynthesis gene, which consequently failed to induce clear zones of inhibition of lawns of *E. faecalis* SL (Figure S6).

To test our hypothesis that mundticin KS contributes to the demise of *E. faecalis* and *E. casseliflavus* in the gut flora, we further assessed bacteriocin gene expression in vivo. We detected mundticin KS at the mRNA level and could thus infer that it is produced in the gut. We found that, although mundticin KS was not detected in the first instar, probably due to the low titer of *E. mundtii* cells at this very early time point, mundticin KS was highly expressed from the second instar in parallel with the increase of *E. mundtii* population inside the gut (Figure 6A). More importantly, mundticin KS was continually synthesized by indigenous symbionts across different instars, which reduces the risk of infection. We attempted to infect larvae with a high dose of *E. faecalis*; larvae reassociated with the *E. mundtii* SL strain effectively cleared *E. faecalis* from the gut. However, larvae reassociated with an *E. mundtii* strain lacking the mundticin biosynthesis gene could no longer inhibit *E. faecalis* (Figure 6B). Since other differences probably occur beyond bacteriocin production between these strains, the purified mundticin was further orally administrated to *E. faecalis*-infected larvae to assess its effect directly. The mean *E. faecalis* SL count recovered from the control larvae (PBS used as placebo) was 4.26×10^4 (mg larva gut)$^{-1}$, whereas in the mundticin-treated larvae there was a >60% reduction of *E. faecalis* SL 24 hr following the administration of the bacteriocin (Figure 6C). The curing effect of mundticin is significant ($p < 0.05$, ANOVA, a post hoc Tukey honestly significant difference test, df = 5) compared with the untreated control at each concentration (Figure 6C). By measuring the expression of mundticin KS and the direct curing experiment, we confirmed that mundticin KS exerts an antagonistic effect in situ against invading bacteria.

DISCUSSION

Most insects harbor abundant symbiotic bacteria in the gut. It is broadly accepted that the components of the native microbiota can serve beneficial functions, which range from enhancing host energy metabolism to shaping the immune system (Schneider and Chambers, 2008). A simple but specific bacterial community, composed of approximately 40% *E. mundtii*, was found within the larval gut of a generalist herbivore, *S. littoralis* (Tang et al., 2012). The association of *E. mundtii* with lepidopterans has been widely recorded in both laboratory-reared and wild-captured hosts (Chen et al., 2016b; Johnston and Rolff, 2015; Pilon et al., 2013; Tang et al., 2012; Teh et al., 2016; van Frankenhuyzen et al., 2010). For example, *E. mundtii* was found to be the major species in the gut microflora of spruce budworm, *Choristoneura fumiferana* (van Frankenhuyzen et al., 2010). However, to date the ecological role of these *Enterococcus* relatives to the herbivores remains obscure.

In this study, a representative of the predominant gut *E. mundtii* bacteria isolated from *S. littoralis* shows an antimicrobial activity that directly inhibits competitors and potent pathogens from developing in the same ecological niche. Even though the gut niche is blocked early, *E. mundtii* efficiently overcomes invading bacteria and proliferates rapidly to colonize the digestive tract. Subsequently, a mature population of *E. mundtii* is stably maintained throughout larval life. The level of *E. mundtii* is as high as 10^8 CFU in a normal healthy larva. A recent study using fluorescent in situ hybridization revealed that the high density of *E. mundtii* cells assemble on the gut epithelium and form a biofilm-like structure contributing to its persistence and colonization (Shao et al., 2014). Unlike native *E. mundtii*, other detected enterococcal bacteria are toxic, especially the *E. faecalis* SL strain, which carries a highly virulent factor, enterococcal gelatinase, which decomposes the host's extracellular matrix (Gaspar et al., 2009). Recent studies showed even commensal *E. faecalis* induces rapid death in Lepidoptera following translocation from the gut (Holt et al., 2015). Our cultivation-based observation indicated that *E. faecalis* bacteria are alive inside the host's gut. Once these bacteria successfully establish in large numbers, they damage host tissues and increase the risk of infection (Figure 1C and Table 1) (Doane and Redys, 1970). Not only actively against related *Enterococcus* species, *E. mundtii* SL is also able to inhibit a range of Gram-positive bacteria, in particular some well-known food-borne pathogens and entomopathogens (Table S1). Therefore, the dominant *E. mundtii* is most likely a defensive symbiont that facilitates the survival of its host in a changing environment during development. This phenomenon was also observed in other lepidopterans and insects, such as locusts, ants, and beetles (Dillon et al., 2000; Oh et al., 2009; Scott et al., 2008), a fact that may be common in insects. It has been demonstrated that removing the dominant *E. mundtii* from the larval gut can cause increased susceptibility of the spruce budworm to pathogens, suggesting that *E. mundtii* plays a protective role (van Frankenhuyzen et al., 2010). Similarly, certain *Lactobacillus* strains isolated from the honeybee intestine were able to inhibit growth of the honeybee pathogen *Paenibacillus larvae*, and to some extent reduced the development of disease symptoms in bee colonies (Forsgren et al., 2010).

Although this increasing evidence suggests that native gut bacteria participate in host defense, until now the molecular mechanism has not been clearly demonstrated. We found that *E. mundtii* SL actively secretes a stable class IIa bacteriocin, mundticin KS, which kills particular kinds of microbial invaders. Bacteriocins, which are ribosomally synthesized antimicrobial peptides, are evolutionarily conserved weapons directly against others (Riley and Wertz, 2002). They have attracted great interest because of their potential use as alternatives to conventional antibiotics and food preservatives (Cotter et al., 2013). For example, nisin, a lantibiotic bacteriocin from *Lactococcus lactis*, has been shown to be more effective than vancomycin against *Streptococcus pneumoniae* in a mouse infection model (Goldstein et al., 1998). *Ruminococcus flavefaciens*, an important member of the rumen microbiota, produces nine different lanthipeptides (Zhao and van der Donk, 2016). However, functional studies of these compounds in their ecological context are limited. We further showed that the symbiotic antibiotic-producing *E. mundtii* efficiently expresses this antimicrobial in situ in the gut, permitting direct control over the gut microbiota. Mundticin KS was constitutively synthesized across larval stages. Considering the demonstrated strong inhibitory effect, it is not surprising that sensitive *E. faecalis* and *E. casseliflavus* were quickly cleared from the host. Therefore, symbiont-derived bacteriocin participates in the selection process stabilizing the host gut microbiota. Mundticin KS could also inhibit other bacteria from outside, thus limiting pathogenic microbes in the gut lumen. And its constitutive expression, as an additional chemical barrier, further increases the threshold for disease establishment.

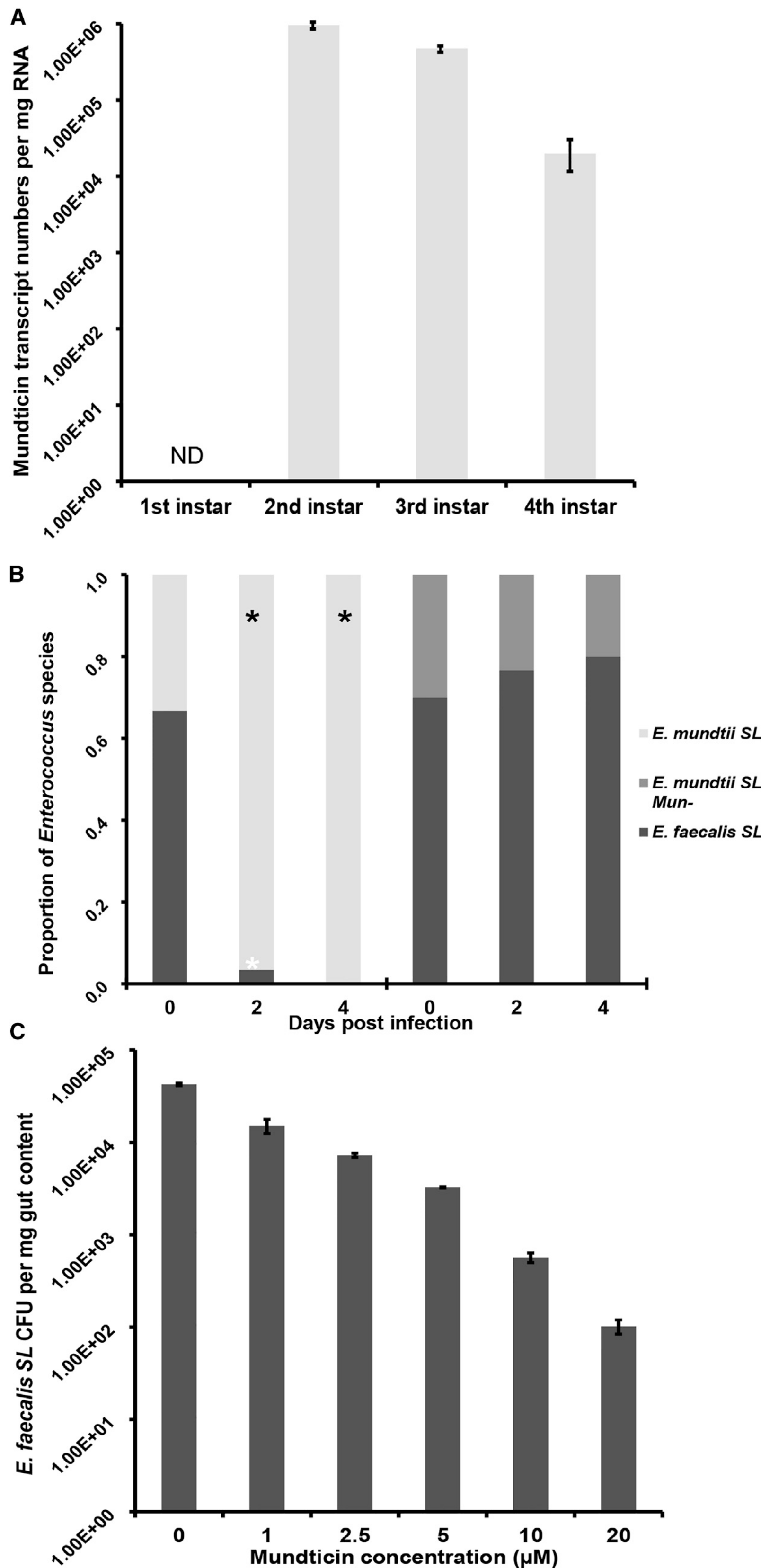


Figure 6. Production of Mundticin KS In Situ in the Gut and Suppression of Ingested Bacteria

(A) *E. mundtii munA* transcript numbers in the gut of *S. littoralis* larvae across different instars. Results are average values calculated from biological triplicates. ND, not detected. Error bars, SDs.

(B) Infection assays with *E. faecalis* SL. The same batch of larvae was firstly cured of their native *E. mundtii*, then was randomly divided into two groups and re-introduced of different *E. mundtii* strains. The larvae in both groups were intensively fed with *E. faecalis* SL once and the gut enterococcal population was analyzed. The larval gut re-associated with the *E. mundtii* SL strain could not be infected by ingested *E. faecalis* (left panel), whereas the larval gut reassociated with an *E. mundtii* strain lacking the mundticin biosynthesis gene does not inhibit *E. faecalis* (right panel). Proportional data are modeled using the mean number of CFUs counted from five to nine larvae. Statistically significant changes (*$p < 0.05$: 2 days post-infection [DPI] versus 0 DPI, 4 DPI versus 0 DPI) were determined with ANOVA and a post hoc Tukey HSD test.

(C) Purified mundticin cures *E. faecalis* SL-infected larvae. The curing effect of mundticin is significant ($p < 0.05$, ANOVA, a post hoc Tukey HSD test, df = 5) compared with the untreated control at each concentration. Bars represent the mean number of *E. faecalis* CFUs from three to five larvae and the experiment was repeated three times. Error bars, SDs.

Despite the energy costs associated with protein synthesis, the production of peptide antimicrobials by *E. mundtii* symbionts also benefits themselves by providing a competitive advantage in the gut for a safe living space and rich nutrient sources supplied by the host, thus contributing to their dominance within complex microbial settings and promoting long-term cooperative symbiosis between both parties. It is also noteworthy that not all *E. mundtii* bacteria exhibit antimicrobial activity, as the reference strain did not show any inhibition on the indicators. To our knowledge, the strain SL is the first bacteriocinogenic *E. mundtii* isolated from an insect herbivore. These data suggest that this unique feature of *E. mundtii* SL, namely, its ability to effectively produce a peptide antimicrobial, facilitates its prevalence and establishment into the gut niche, and contributes to the relatively simple microbiota inside the herbivore gut.

Because mundticin KS displays a selective antimicrobial spectrum, mainly toxic to the phylogenetically closely related bacteria including *E. faecalis*, *Streptococcus*, and *Lactobacillus*, as well as to the common food-borne pathogen *L. monocytogenes*, it does not influence other indigenous gut residents, resulting in the normal development of gut microbiota. This targeted approach to eliminating undesired bacteria while the normal flora remains relatively unaffected is safe to retain regular functions of the microbiota in energy metabolism and others. Recent reports have revealed that the normal gut microbiota not only confer "colonization resistance" (pathogen blockage by the intestinal microbiota) to protect the host, but also mediate novel pathogen clearance from the highly complex gut environment, which sufficiently complements host immune defenses (Endt et al., 2010). Similarly, Koch and Schmid-Hempel (2011) suggested that the host microbiota be considered an "extended immune phenotype". Our discovery, that the dominant gut symbiont effectively produces a narrow-spectrum bacteriocin in vivo, represents a molecular mechanism for suppressing invaders primarily mediated by the host native gut microbiota. Understanding the role of indigenous gut residents will contribute to the development of novel biocontrol strategies against herbivorous insect pests. Our observation that insects successfully rely on peptide antibiotic against enterococcal infections also has implications for the struggle against rapidly emerging multi-drug-resistant enterococcal pathogens in humans.

SIGNIFICANCE

Symbiotic bacteria as prevalent as *E. mundtii*, which are found worldwide in Lepidoptera, must have played important roles in the holobiont. Here we reveal that the extracellular symbiont *E. mundtii* of the cotton leafworm *Spodoptera littoralis* selectively clears pathobionts from the host gut lumen by secreting a stable bacteriocin, which facilitates the normal development of gut microbiota. The constitutively produced antimicrobials create an additional chemical barrier inside limiting pathobiont expansion, thus reducing risks of infection via the gut and enhancing host health. Our study together with other investigations into the microbial symbionts of leaf-cutting ants and locusts suggests that protective associations with antibiotic-producing bacteria is a common strategy of insects against microbial invaders; however, their antimicrobials are chemically diverse.

EXPERIMENTAL PROCEDURES

Bacteria were isolated from the normal *S. littoralis* insects and were identified based on both phenotypic characteristics and molecular techniques. For bacteria enumeration, freshly prepared gut bacterial suspensions (five to nine replicates for each sample) were serially diluted in brain heart infusion (BHI) broth (CM1135, Oxoid) and plated on *Enterococcus* Selective Agar (45183, Fluka) plates. Plates were grown at 30°C overnight and the resulting *Enterococcus* colonies were counted and identified. Virulence genes were screened by diagnostic PCR. The infection assay was performed by feeding larvae with each *Enterococcus* strain as described by the original publication (Doane and Redys, 1970). The second- and third-instar larvae were fed with a low amount of antibiotic (10 μg erythromycin/g artificial diet) to eliminate native *E. mundtii* and starved 1 or 2 days depending on age before the tests. Bacterial broth culture was adjusted to 10^8 CFU/mL with PBS and 100 μL was sprinkled directly on the artificial diet to provoke infection. The artificial diet was changed every day and the mortality was recorded until adult stage. The whole infection assay was performed in triplicate under the same rearing conditions. For further antimicrobial property study, the representative *Enterococcus* strains, including the isolated *E. faecalis* SL, *E. casseliflavus* SL, *E. mundtii* SL strain, and *E. mundtii* type strain (DSM4838), were cultivated 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 a 0.22 μm polyvinylidene fluoride membrane for testing. The related inhibitory spectrum was determined by using a well diffusion assay. The indicator strains were grown in the media (BHI or MRS) recommended by culture collections. The growth of gut symbiont *E. mundtii* was monitored by measuring biomass, and antimicrobial production was calculated from the result of an agar diffusion test. The antimicrobial substance produced by *E. mundtii* SL was purified to homogeneity by a three-step procedure consisting of cation-exchange chromatography, absorbent interaction, and reverse-phase HPLC equipped with a C18 column. MS techniques were used to determine the molecular mass of the purified antimicrobial peptide, followed by PSD analysis of its amino acid sequence. The corresponding bacteriocin-coding gene was amplified with a degenerated PCR approach from the *E. mundtii* SL genomic DNA template and confirmed by cloning and sequencing. The peptide secondary and tertiary structures were predicted by Phyre2 using default parameters and structure templates extracted from the PDB. The α helix domain was determined by performing the CD spectroscopy. The abundance of mundticin KS transcripts in the larval gut was calculated by quantitative real-time PCR using the absolute quantification method. 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. The infection assay with *E. mundtii Mun*$^-$ was performed according to the same procedure described above. For details on reagents, insect rearing, dissection, bacteria characterization, antimicrobial activity screening, peptide purification/sequencing, protein structure analyses, qPCR, infection assay, and other methods used in this study, see Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.11.015.

AUTHOR CONTRIBUTIONS

Y.S. and W.B. conceived the experiments. Y.S., K.I., C.S., and B.C. carried out the experiments, interpreted data, and assisted in the preparation of the manuscript. Y.S. and W.B. wrote the manuscript with contributions from C.H.

ACKNOWLEDGMENTS

We thank Andrea Perner and Maria Poetsch for MS measurements; Angelika Berg and Yitao He for technical assistance; and Emily Wheeler for editorial assistance. This work was supported by the National Natural Science Foundation of China (31601906), the "Hundred Talents Program" from Zhejiang University, the DFG (ChemBioSys, CRC1127), and the Max Planck Society.

Received: September 18, 2016
Revised: October 24, 2016
Accepted: November 21, 2016
Published: January 19, 2017

REFERENCES

Chen, B., Sun, C., Liang, X., Lu, X., Gao, Q., Alonso-Pernas, P., Teh, B.-S., Novoselov, A., Boland, W., and Shao, Y. (2016a). Draft genome sequence of *Enterococcus mundtii* SL 16, an indigenous gut bacterium of the polyphagous pest *Spodoptera littoralis*. Front Microbiol. *7*, 1676.

Chen, B., Teh, B.S., Sun, C., Hu, S., Lu, X., Boland, W., and Shao, Y. (2016b). Biodiversity and activity of the gut microbiota across the life history of the insect herbivore *Spodoptera littoralis*. Sci. Rep. *6*, 29505.

Cotter, P.D., Ross, R.P., and Hill, C. (2013). Bacteriocins – a viable alternative to antibiotics? Nat. Rev. Microbiol. *11*, 95–105.

Diep, D.B., Skaugen, M., Salehian, Z., Holo, H., and Nes, I.F. (2007). Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc. Natl. Acad. Sci. USA *104*, 2384–2389.

Dillon, R.J., Vennard, C.T., and Charnley, A.K. (2000). Exploitation of gut bacteria in the locust. Nature *403*, 851.

Doane, C.C., and Redys, J.J. (1970). Characteristics of motile strains of *Streptococcus-Faecalis* pathogenic to larvae of gypsy-moth. J. Invertebr. Pathol. *15*, 420–430.

Donia, M.S., Cimermancic, P., Schulze, C.J., Wieland Brown, L.C., Martin, J., Mitreva, M., Clardy, J., Linington, R.G., and Fischbach, M.A. (2014). A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. Cell *158*, 1402–1414.

Endt, K., Stecher, B., Chaffron, S., Slack, E., Tchitchek, N., Benecke, A., Van Maele, L., Sirard, J.C., Mueller, A.J., Heikenwalder, M., et al. (2010). The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal *Salmonella diarrhea*. PLoS Pathog. *6*, e1001097.

Forsgren, E., Olofsson, T.C., Vasquez, A., and Fries, I. (2010). Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. Apidologie *41*, 99–108.

Funke, M., Buechler, R., Mahobia, V., Schneeberg, A., Ramm, M., and Boland, W. (2008). Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. Chembiochem *9*, 1953–1959.

Gaspar, F., Teixeira, N., Rigottier-Gois, L., Marujo, P., Nielsen-LeRoux, C., Crespo, M.T.B., Lopes, M.D.S., and Serror, P. (2009). Virulence of *Enterococcus faecalis* dairy strains in an insect model: the role of fsrB and gelE. Microbiology *155*, 3564–3571.

Ghosh, A., Akhtar, M., Holderman, C., and Zurek, L. (2014). Significance and survival of *Enterococci* during the house fly development. J. Med. Entomol. *51*, 63–67.

Goldstein, B.P., Wei, J., Greenberg, K., and Novick, R. (1998). Activity of nisin against *Streptococcus pneumoniae*, in vitro, and in a mouse infection model. J. Antimicrob. Chemother. *42*, 277–278.

Herzner, G., Schlecht, A., Dollhofer, V., Parzefall, C., Harrar, K., Kreuzer, A., Pilsl, L., and Ruther, J. (2013). Larvae of the parasitoid wasp *Ampulex compressa* sanitize their host, the American cockroach, with a blend of antimicrobials. Proc. Natl. Acad. Sci. USA *110*, 1369–1374.

Holt, J.F., Kiedrowski, M.R., Frank, K.L., Du, J., Guan, C., Broderick, N.A., Dunny, G.M., and Handelsman, J. (2015). *Enterococcus faecalis* 6-phosphogluconolactonase is required for both commensal and pathogenic interactions with *Manduca sexta*. Infect. Immun. *83*, 396–404.

Hudault, S., Guignot, J., and Servin, A.L. (2001). *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. Gut *49*, 47–55.

Iatsenko, I., Yim, J.J., Schroeder, F.C., and Sommer, R.J. (2014). B. subtilis GS67 protects *C. elegans* from Gram-positive pathogens via fengycin-mediated microbial antagonism. Curr. Biol. *24*, 2720–2727.

Johnston, P.R., and Rolff, J. (2015). Host and symbiont jointly control gut microbiota during complete metamorphosis. PLoS Pathog. *11*, e1005246.

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.

Koch, H., and Schmid-Hempel, P. (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc. Natl. Acad. Sci. USA *108*, 19288–19292.

Lindow, S.E., and Brandl, M.T. (2003). Microbiology of the phyllosphere. Appl. Environ. Microbiol. *69*, 1875–1883.

Manero, A., and Blanch, A.R. (1999). Identification of *Enterococcus* spp. with a biochemical key. Appl. Environ. Microbiol. *65*, 4425–4430.

Mithoefer, A., and Boland, W. (2012). Plant defense against herbivores: chemical aspects. Annu. Rev. Plant Biol. *63*, 431–450.

Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A., and Chakraborty, T. (2010). *Galleria mellonella* as a model system for studying *Listeria pathogenesis*. Appl. Environ. Microbiol. *76*, 310–317.

Oh, D.C., Poulsen, M., Currie, C.R., and Clardy, J. (2009). Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nat. Chem. Biol. *5*, 391–393.

Palmer, K.L., Godfrey, P., Griggs, A., Kos, V.N., Zucker, J., Desjardins, C., Cerqueira, G., Gevers, D., Walker, S., Wortman, J., et al. (2012). Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. MBio *3*, e00318-11.

Pilon, F.M., Visotto, L.E., Guedes, R.N., and Oliveira, M.G. (2013). Proteolytic activity of gut bacteria isolated from the velvet bean caterpillar *Anticarsia gemmatalis*. J. Comp. Physiol. B *183*, 735–747.

Riley, M.A., and Wertz, J.E. (2002). Bacteriocins: evolution, ecology, and application. Annu. Rev. Microbiol. *56*, 117–137.

Ruffner, B., Pechy-Tarr, M., Ryffel, F., Hoegger, P., Obrist, C., Rindlisbacher, A., Keel, C., and Maurhofer, M. (2013). Oral insecticidal activity of plant-associated pseudomonads. Environ. Microbiol. *15*, 751–763.

Schneider, D.S., and Chambers, M.C. (2008). MICROBIOLOGY rogue insect immunity. Science *322*, 1199–1200.

Scott, J.J., Oh, D.C., Yuceer, M.C., Klepzig, K.D., Clardy, J., and Currie, C.R. (2008). Bacterial protection of beetle-fungus mutualism. Science *322*, 63.

Servin, A.L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. *28*, 405–440.

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.

Stecher, B., Berry, D., and Loy, A. (2013). Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. FEMS Microbiol. Rev. *37*, 793–829.

Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., Cordero, E.A., Andersen, G., Westermann, M., Heckel, D.G., and Boland, W. (2012). Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PLoS One *7*, e36978.

Teh, B.S., Apel, J., Shao, Y., and Boland, W. (2016). Colonization of the intestinal tract of the polyphagous pest *Spodoptera littoralis* with the GFP-tagged indigenous gut bacterium *Enterococcus mundtii*. Front Microbiol. *7*, 928.

Vallet-Gely, I., Lemaitre, B., and Boccard, F. (2008). Bacterial strategies to overcome insect defences. Nat. Rev. Microbiol. *6*, 302–313.

van Frankenhuyzen, K., Liu, Y., and Tonon, A. (2010). Interactions between *Bacillus thuringiensis* subsp. kurstaki HD-1 and midgut bacteria in larvae of gypsy moth and spruce budworm. J. Invertebr. Pathol. *103*, 124–131.

Vasquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L., and Olofsson, T.C. (2012). Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS One *7*, e33188.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature *415*, 389–395.

Zhao, X., and van der Donk, W.A. (2016). Structural characterization and bioactivity analysis of the two-component lantibiotic Flv system from a ruminant bacterium. Cell Chem. Biol. *23*, 246–256.

Cell Chemical Biology, Volume *24*

Supplemental Information

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

Yongqi Shao, Bosheng Chen, Chao Sun, Keishi Ishida, Christian Hertweck, and Wilhelm Boland

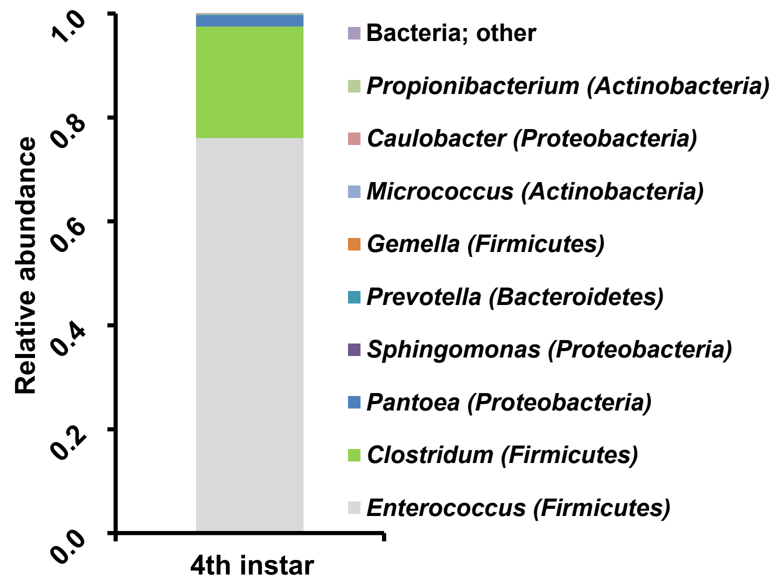
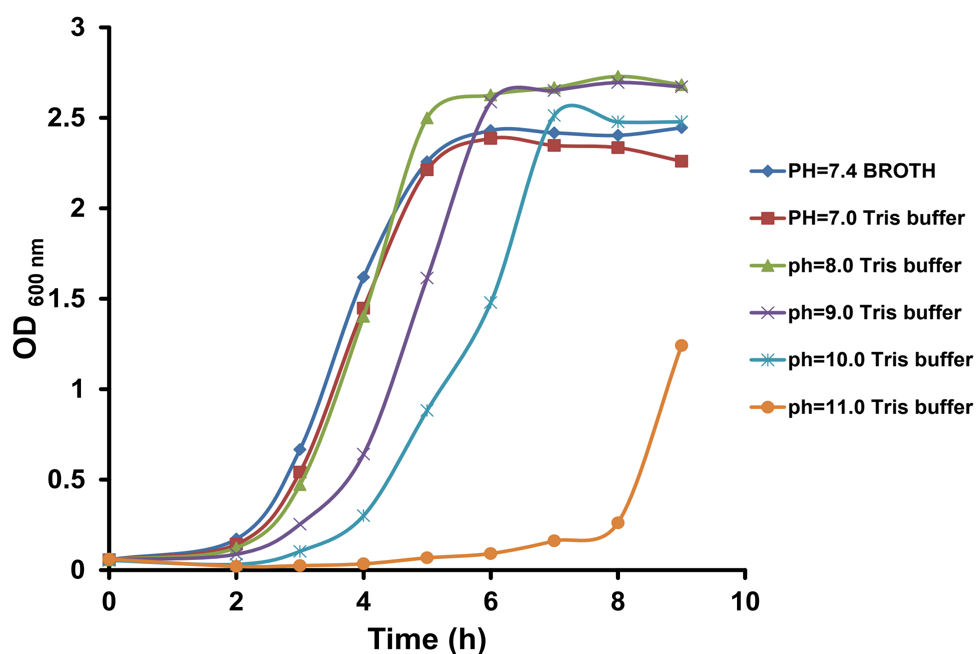


Figure S1, related to Figure 1. Sequencing analysis of the gut microbiota of fourth-instar *S. littoralis* larvae.

A



B

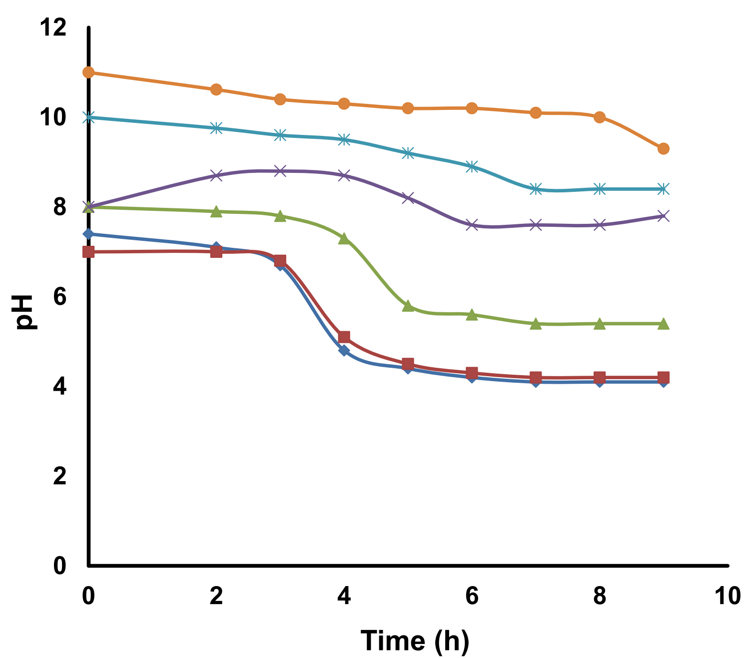


Figure S2, related to Figure 3. ***E. mundtii*** **SL grows well in alkaline pH condition. (*A*) Growth curves of *E. mundtii* SL strain inoculated under pH-buffered conditions. (*B*) Change of pH during growing.**

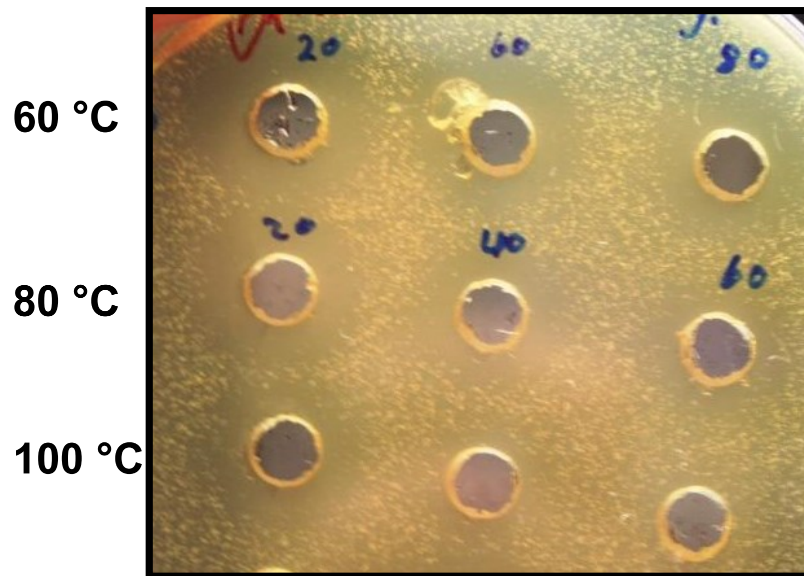


Figure S3, related to Figure 3. The effect of temperature on the inhibitory substance was tested by heating the supernatant at 60, 80 and 100°C, respectively.

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

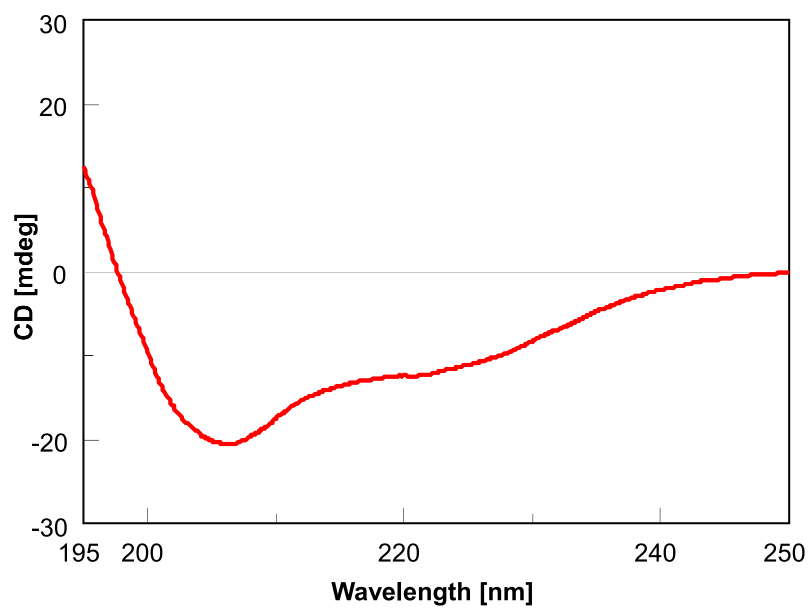


Figure S5, related to Figure 5. CD spectrum of mundticin KS in 90% 2, 2, 2-trifluoroethanol (0.1% trifluoroacetic acid final concentration, pH 2.5) at 20°C.

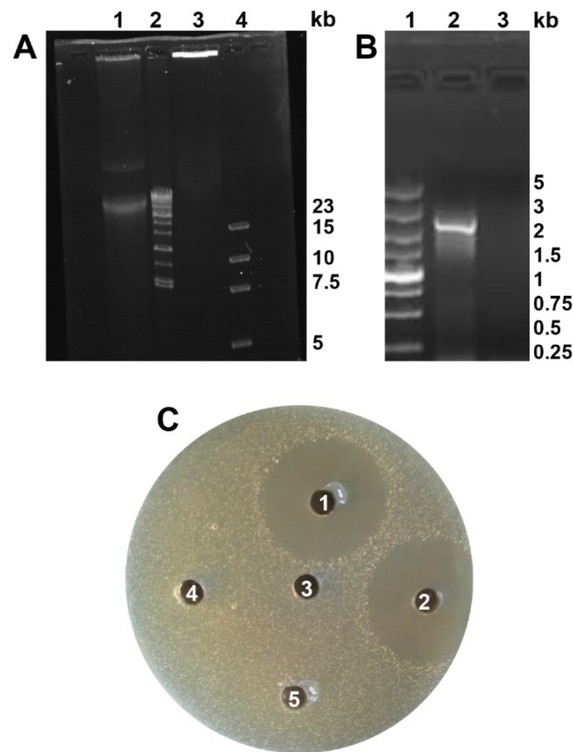


Figure S6, related to Figure 6. Curing of plasmids from *E. mundtii* SL. (*A*) Plasmid profiles. 1, plasmid DNA isolated from the native *E. mundtii* SL strain; 2, Lambda Mix marker; 3, no plasmid DNA was isolated from the novobiocin-treated *E. mundtii* SL colonies (*E. mundtii* SL *Mun*$^-$ strain); 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.