

INFLUENCE OF *Myriophyllum spicatum*-DERIVED TANNINS
ON GUT MICROBIOTA OF ITS HERBIVORE
Acentria ephemerella

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Abstract—The submerged living larvae of *Acentria ephemerella* were fed in the laboratory with either *M. spicatum* or *Potamogeton perfoliatus*, two of their host plants. Larvae exhibited a reduced growth when fed *M. spicatum*, a freshwater angiosperm that contains high concentrations of tannins, secondary metabolites known for their herbivore-deterrent and antimicrobial properties. In this study, we investigated the influence of food-derived tannins on gut microbiota. Bacterial densities in the guts did not differ between the food regimes, ranging from 2.8 to 13.3×10^6 cells per gut. Gut bacteria were characterized with cultivation techniques and subsequent identification of the strains by molecular methods. We isolated 17 bacterial strains belonging to all subdivisions, i.e., we identified α -, β -, and γ -proteobacteria, *Cytophyaga/Flavobacteria* (CF) and several Gram-positive bacteria. All except one Gram-positive strain were found in the guts of larvae fed with *P. perfoliatus*. Gram-positive bacteria and bacteria of the CF cluster were more sensitive to polyphenol-containing extracts of *M. spicatum* in an agar diffusion assay than strains of the α - or γ -proteobacteria subdivision. Our results suggest an influence of food-derived tannins on gut microbiota in *A. ephemerella*.

Key Words—Aquatic angiosperm, gut symbionts, hydrolyzable tannin, polyphenol, freshwater macrophytes.

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INTRODUCTION

Tannins (polyphenols) are secondary metabolites known for their ability to bind and precipitate proteins or enzymes (Spencer et al., 1988). However, some herbivores are well adapted to tannin-containing plants (Manuwoto and Scriber, 1986; Barbehenn and Martin, 1992). Despite numerous investigations, the precise mechanisms of tannin–herbivore interactions are still unknown.

An important function of tannins in plants is to provide protection against a wide range of potential phytopathogenic microorganisms (Field and Lettinga, 1992). Tannins possess diverse antimicrobial activities (Scalbert, 1991); thus, tannins could also be influencing gut microbiota of herbivores. Consequently, Schultz et al. (1992) hypothesized “that any negative impacts of plant polyphenols on insects or vertebrates may be no more than a fortuitous side effect of plant interactions with microbes.”

Larvae of the aquatic moth *Acentria ephemerella* (Lepidoptera: Pyralidae) feed on both tannin-containing and tannin-free freshwater macrophytes, causing substantial damage (Gross et al., 2001, 2002). *Acentria* grows significantly more slowly when fed *Myriophyllum spicatum* than when fed tannin-free pondweeds (Choi et al., 2002). *M. spicatum* contains approximately 10% tannins based on dry weight (Gross et al., 1996).

The impact of food-derived tannins on gut microbiota of lepidopteran larvae has been investigated only in the economically important species *Lymantria dispar* and its pathogen *Bacillus thuringiensis* (Appel and Schultz, 1994). The present study was conducted to characterize naturally occurring gut bacteria of the freshwater moth *Acentria ephemerella*. We compared the gut microbiota of larvae fed tannin-free *P. perfoliatus* and larvae fed tannin-containing *M. spicatum* to each other and to those epiphytic bacteria found on the respective host plants. Finally, we investigated the susceptibility of gut microbiota to extracts from both macrophytes and pure phenolic compounds.

METHODS AND MATERIALS

Sampling. *M. spicatum* and *Potamogeton perfoliatus*, both food plants of *Acentria* larvae (Gross et al., 2002), were sampled either by snorkeling or by SCUBA diving in Untersee, a shallow part of Lake Constance, Germany, in August 2001. Samples were stored in coolers and transferred to a 4°C cooling chamber at the institute. The fresh plant material was divided into three groups the next day. Apical shoot sections (upper 2–5 cm of shoots) and green leaves from the upper 25 cm were separated from the first group of plants. These were ground with a mortar and pestle in liquid nitrogen and freeze-dried separately for further

extraction. Individual leaves were picked for harvesting epiphytic bacteria from the second group. Leaves were chosen randomly from the upper 25 cm; no apical shoot sections were taken. Larvae were picked from the remaining plant material and kept in 1.5-liter glass jars in lake water. Larvae were only collected from *P. perfoliatus* since no larvae were present on *M. spicatum* at the sampling date. Larvae were divided into two groups and, for two days, fed constantly with apical shoot sections of either *M. spicatum* or *P. perfoliatus*.

Harvesting Bacteria. To isolate epiphytic bacteria, about 10 mg fresh weight (FW) of leaf material was suspended in 1 ml sterile sodium tetrapyrophosphate (PPi) solution (0.1 M) in an Eppendorf vial (1.5 ml). PPi had been shown to facilitate a good recovery of attached bacteria in arthropod guts (Cazemier et al., 1997). Preliminary experiments revealed PPi also as an appropriate detergent for harvesting epiphytic bacteria, since it did not show any influence on growth of isolated bacteria. The leaves were sonicated in an ultrasonic bath for 30 sec, shaken for 15 min at 1100 rpm in an Eppendorf thermomixer, and then sonicated for another 30 sec. One aliquot of this bacterial suspension was taken for direct counts of bacteria; the remaining suspension was used for cultivation.

In order to retrieve gut bacteria, larvae were first sterilized with 70% ethanol to avoid contamination with microorganisms attached to their surface. Larvae were dissected under a stereomicroscope; the complete intestinal tract was removed and separated into fore-, mid-, and hindgut. Each gut segment was separately immersed in 250 μ l sterile PPi, squeezed with a sterile preparation needle, and treated by sonication as described above to yield gut bacterial suspensions.

Bacterial Counts. One hundred microliters of bacterial suspensions, either from leaves or *Acentria* guts, were fixed with 100 μ l formaldehyde (10%, v/v) and diluted with water to a final volume of 1 ml. We used only ultrapure and sterilized water for microbiological and biochemical analysis. Staining was performed with 100 μ l of 4',6-diamidino-2-phenylindole (DAPI; 10 μ g/ml in 1% formaldehyde, v/v), and the mixture was incubated for 30 min in the dark. The stained bacteria were filtered on 0.2 μ m black Nucleopore filters and counted under a Nikon epifluorescence microscope (Labophot 2). Of each microscopic preparation, 10 microscopic fields (0.08 \times 0.08 mm) were counted.

The surface area of the freshwater macrophytes used was calculated from the dry weight of the plant samples according to Sher-Kaul et al. (1995). Bacterial densities in the gut of *Acentria* were quantified per gut segment (fore-, mid-, or hindgut) and per volume of gut fluid. For the latter we had to make some approximations. Although we used third to fourth instar larvae (for size classes see Gross et al., 2001, 2002), they were still too small to determine their gut volumes accurately. Consequently, we measured the head capsule widths to determine larval size. For an estimate of the gut volume, we assumed that the length of the larvae was correlated with the head capsule width and converted the latter to length

sizes (100 μm head capsule width set equivalent to 1.6 mm length). We further estimated a mean gut diameter of 1 mm and calculated gut volumes using these measures.

Isolation of Bacteria. Bacterial suspension in appropriate dilutions was spread on modified CPS agar (Collins and Willoughby, 1962) containing 0.5 g tryptone (Oxoid), 0.5 g peptone (Difco), 0.5 g soluble starch (Merck), 1 ml glycerol, 0.2 g K_2HPO_4 , and 0.05 g MgSO_4 . The plates were incubated aerobically for 3 days at 24°C. This method yielded distinct colonies. Representative colonies were selected on the basis of morphological criteria, such as colony size, pigmentation or surface structure, and recultured. Several isolates were obtained from guts and leaves, some from different sources looked similar. Therefore, all isolates were compared by means of denaturing gradient gel-electrophoresis (DGGE) for further characterization.

PCR and DGGE. Bacterial DNA of cultured cells was isolated in water at 100°C for 15 min. After centrifugation for 5 min at 13,600 g, 1 μl of supernatant containing bacterial DNA was taken as template for PCR. Small subunit rRNA genes were amplified by PCR using the primers 5'-GC-clamp-CCTACGGGAGGC AGCAG-3' (forward) and 5'-CCGTCAATTCMTTGTGAGTTT-3' (reverse) (Muyzer et al., 1998). PCR was performed in 50- μl assays with a GENIUS thermocycler (Techne, Cambridge, UK). One unit of Taq DNA polymerase (Eppendorf Master Taq) was used. PCR conditions were an initial denaturation step of 3 min at 94°C, followed by a touchdown protocol of 20 cycles in which the DNA was denatured for 1 min at 94°C, annealed for 1 min starting at 65°C, and decreased 0.5°C/cycle to 55°C and extended for 2 min at 72°C. This was followed by nine cycles in which the annealing temperature was 55°C. PCR products were examined using agarose gel electrophoresis and ethidium bromide staining.

DGGE of PCR products was performed by the method described by Muyzer et al. (1996) with the use of a DCode System (Bio-Rad Laboratories). Products were analyzed on a 6% (w/v) polyacrylamide gel with a linear gradient from 20 to 60% denaturant [100% denaturant contained 7 M urea and 40% (v/v) formamide]. Electrophoresis was performed for 16 hr at 95 V and a temperature of 60°C. After electrophoresis, gels were stained for 15 min with SYBR Gold (0.1 $\mu\text{l}/\text{ml}$; Molecular Probes), rinsed in water, and photographed on a UV transilluminator with a black and white Polaroid camera. Isolates with different banding patterns were taken as different bacterial species.

Sequencing. To further characterize isolates, DNA obtained as described above was taken as a template for another PCR. The annealing temperature started at 60°C; thus, the touchdown protocol consisted of only 10 cycles followed by 19 cycles in which the annealing temperature was 55°C. No GC clamp was added in this step. The PCR product was purified with a GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech) following the instruction manuals

of the manufacturer. Sequencing reactions were performed at GATC Biotech AG (Konstanz, Germany). The resulting sequences were analyzed with the online database BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Extraction of Plant Material. Freeze dried plant powder was extracted with 70% aqueous acetone (v/v; 10 ml/pg DW) for 2 hr at 15°C. Suspensions were continuously stirred in an Eppendorf thermomixer (interval mode, 1 min at 1100 rpm, 1 min pause). After centrifugation (10 min, 13,600g, 4°C), the supernatant was stored at 4°C, and the pellet was resuspended in acetone and extracted as above. Supernatants were combined and dried in a vacuum evaporator. The residue was redissolved in 50% methanol to a final concentration of 100 µg (DW)/ml.

Bacterial Assays. Bacterial assays were performed to analyze the inhibitory effects of crude plant extracts. We used CPS agar, adjusted with NaOH to pH 8.4 before autoclaving. Crude plant extracts were applied in 5-µl aliquots to agar plates and allowed to dry. Stepwise application of droplets yielded final extract concentrations equivalent to 0.5, 1, and 2 mg of extracted plant material. Controls used 20 µl of 50% methanol. Each extract and each concentration was tested in duplicate. As indicator organisms, isolated bacterial strains from agar plates were suspended in water and their optical density (OD₆₀₀) was adjusted to 0.1. These suspensions were diluted 1:50 with water in order to get distinct colonies on the plates. Then 0.1 ml of these suspensions was spread on the agar plates and incubated at 24°C for 48 hr. The presence of inhibition zones was recorded and their diameters measured. Bacterial assays were performed with upper leaf extracts from *M. spicatum* and *P. perfoliatus*. We also performed one series with extracts of apical shoot sections of *M. spicatum*, and one series with upper leaves of *M. spicatum*, both using CPS agar with a pH of 7.0 and small Petri dishes (32 mm diam.). In this case, only the highest extract concentration equivalent to 2 mg DW of plant material was used.

A third experiment was performed to verify that polyphenolic compounds in *M. spicatum* were responsible for antimicrobial effects in crude extracts. We first treated extract with polyvinyl polypyrrolidone (PVPP) to remove phenolic compounds. Secondly, we compared the inhibitory strength of extract with pure phenolic compounds applied in equivalent concentrations. PVPP-treatment of the crude extract to remove phenolic compounds was performed as described recently (Gross et al., 1996). The content of phenolic compounds was measured with the Folin-Ciocalteu assay and expressed as tannic acid equivalents; the concentration of tellimagrandin II was quantified by HPLC (Gross et al., 1996). Extract used in experiment III contained 160 µg tannic acid equivalents and 27 µg tellimagrandin II per milligram of plant dry weight. Accordingly, we used 27 and 160 µg of (+)-catechin, gallic acid, and tannic acid (Sigma Aldrich) in the assays. Purified tellimagrandin II was available in only a limited amount, and we used 10 µg per assay. All tests were performed in triplicate.

RESULTS

Bacterial Counts. Bacteria in the gut of *Acentria* may, at least in part, be derived from epiphytic bacteria of their host plants. Therefore, we measured bacterial density on both *M. spicatum* and *P. perfoliatus*. The former had $1.43 \pm 0.83 \times 10^6$ cells/cm² and the latter $2.51 \pm 0.89 \times 10^6$ cells/cm² surface area ($N = 5$ each; mean \pm SD; Student's *t* test; $t = 2.17$, $P = 0.055$; Table 1).

Instar stages of *Acentria* larvae can be derived from the size of their head capsule (Gross et al., 2002). Head capsule width ranged from 462 to 897 μ m ($641 \pm 212 \mu$ m, $N = 5$) in *P. perfoliatus* and from 550 to 1482 μ m ($871 \pm 337 \mu$ m, $N = 6$) in *M. spicatum* fed larvae. Table 1 summarizes densities of bacteria in the fore-, mid-, and hindgut of larvae fed with *P. perfoliatus* or *M. spicatum*. Bacterial counts varied from 0.39 to 10.01×10^6 ($1.9 \pm 1.8 \times 10^6$) cells per gut segment. There was no significant difference between cell numbers in the different gut segments nor was there a difference between cell numbers in larvae raised on different food sources. Bacterial densities did not correlate with head capsule width.

Characterization of Bacterial Isolates. We selected bacterial strains from agar plates based on colony size, morphology, and color. Dominant morphotypes of epiphytic bacteria from both plants were selected for further characterization. This yielded 17 strains. Furthermore, 29 isolates were obtained from guts of larvae fed with *M. spicatum* and 18 from those fed with *P. perfoliatus*. Of the 64 isolates selected based on morphological differences, only 17 were genetically different according to DGGE. These strains were further characterized by sequence analyses (Table 2).

Small subunit rRNA gene sequences of these 17 isolates were compared with the online database BLAST. Sequence similarities ranged between 97 and 100%.

TABLE 1. BACTERIAL COUNTS IN *Acentria* GUTS AND ON MACROPHYTE LEAVES

| Treatment | Bacterial counts ($\times 10^6$) ^a | N |
|--|---|---|
| <i>Acentria</i> fed with <i>P. perfoliatus</i> | | |
| Foregut | 1.8 ± 1.2 | 5 |
| Midgut | 2.1 ± 0.9 | 5 |
| Hindgut | 1.3 ± 1.4 | 4 |
| <i>Acentria</i> fed with <i>M. spicatum</i> | | |
| Foregut | 3.6 ± 3.5 | 6 |
| Midgut | 1.1 ± 0.8 | 6 |
| Hindgut | 1.7 ± 1.0 | 6 |
| <i>P. perfoliatus</i> leaves | 2.5 ± 0.9 | 6 |
| <i>M. spicatum</i> leaves | 1.4 ± 0.8 | 6 |

^a Values are the mean \pm SD. Counts for gut segments are cells/segment; for leaves, cells/cm² leaf area.

TABLE 2. INHIBITION OF BACTERIAL ISOLATES FROM MACROPHYTES AND GUTS BY *M. spicatum* EXTRACTS

| Subgroup | Bacterial isolates ^a closest relative | 16S rDNA fit (%) | Source ^b | | | | | | Inhibition zone diameter (mm) ^c | | | | | |
|---------------|---|------------------|---------------------|-----|-----|--------------------------|--------------|------|--|--------|------|---------------------|--------|----|
| | | | PFL | | | MSG | | | pH 7.0 ^d | | | pH 8.4 ^d | | |
| | | | MFL | PFG | MSG | Shoot, ^e 2 mg | Leaves, 2 mg | 2 mg | 1 mg | 0.5 mg | 2 mg | 1 mg | 0.5 mg | |
| α | <i>Brevundimonas</i> | 100 | | | x | | | ND | ND | ND | ND | ND | ND | ND |
| α | <i>Afpia</i> | 100 | | x | | | >32 | >32 | >32 | 20 | 12 | 0 | | |
| α | <i>Sphingomonas</i> | 99 | | | x | | ND | ND | ND | 15 | 8 | 0 | | |
| β | <i>Comamonas</i> | 97 | | x | | | 20 | 16 | 16 | 24 | 20 | 18 | | |
| γ | <i>Aeromonas</i> | 100 | x | | | | 14 | 10 | 6 | 0 | 0 | 0 | | |
| γ | <i>Enterobacter</i> | 100 | | x | | | 0 | 0 | 0 | 0 | 0 | 0 | | |
| γ | <i>Acinetobacter</i> (sp. 1) | 100 | | | x | | 18 | 15 | 15 | ND | ND | ND | | |
| γ | <i>Acinetobacter</i> (sp. 2) | 100 | | | x | | 18 | 15 | 15 | 23 | 16 | 7,5 | | |
| γ | <i>Pseudomonas</i> | 99 | | | x | | ND | ND | ND | 20 | 14 | 10 | | |
| Gram+ low GC | <i>Bacillus</i> | 100 | | | | x | ND | ND | ND | 20 | 17 | 10 | | |
| Gram+ high GC | <i>Gordonia</i> (sp. 1) | 99 | | | | x | 22 | 17 | 17 | 25 | 22 | 18 | | |
| Gram+ high GC | <i>Gordonia</i> (sp. 2) | 100 | | | | x | 24 | 18 | 18 | 31 | 25 | 20 | | |
| Gram+ high GC | <i>Leucobacter</i> | 97 | | | x | | >32 | >32 | >32 | 20 | 15 | 10 | | |
| Gram+ high GC | <i>Rhodococcus</i> | 100 | | | x | | ND | ND | ND | ND | ND | ND | | |
| Gram+ high GC | <i>Tsakumurella</i> | 100 | | | x | | 22 | 19 | 19 | 25 | 20 | 17 | | |
| CF cluster | previously uncultured | 97 | | | x | | 23 | 19 | 19 | 38 | 30 | 24 | | |
| CF cluster | <i>Chryseobacterium</i> | 97 | x | x | | x | 20 | 16 | 16 | >40 | >40 | 40 | | |

^a As revealed by BLAST.
^b Source of the respective strains: PFL = *P. perfoliatus* leaves; MSL = *M. spicatum* leaves; PFG = gut of *P. perfoliatus* fed larva; MSG = gut of *M. spicatum* fed larvae.
^c 1 SD of replicates was always below 1.
^d pH of agar plates.
^e Apical shoot sections.

Ten strains showed 100% identity with known bacteria. The four isolates with only 97% homology probably represent previously unknown bacteria. Members of α -, β -, or γ -proteobacteria, the CF cluster (*Cytophaga/Flavobacteria*), and Gram-positive bacteria were identified.

Sequence analyses revealed that only three bacterial strains dominated the cultivable fraction of epiphytic bacterial population. Two of them belonged to the CF cluster. One showed the closest relation to an as yet uncultivated epilithic bacterium, and the other to *Chryseobacterium defluvium*. Both cases exhibited only 97% conformity with described species. The third strain showed 100% similarity with the common freshwater bacterium *Aeromonas veronii*. All three epiphytic strains were also detected in guts of *Acentria*. One of them and two other proteobacteria were found in *Acentria* guts of both treatments (Table 2). A surprisingly high proportion of Gram-positive strains were found in larvae fed with *P. perfoliatus* (5 of 11) compared with larvae fed with *M. spicatum* (1 of 9).

Bacterial Assays. The antimicrobial activity of plant extracts was measured with a modified agar disc diffusion assay. None of the bacterial isolates was inhibited by extracts of *P. perfoliatus*, even when applied in the highest concentration equivalent to 2 mg plant biomass (DW) extracted. Tannin-containing extracts of *M. spicatum* inhibited most strains, even at the lowest concentration used (Table 2). The size of the inhibition zone increased with higher extract concentrations used. Extracts made with apical shoot sections of *M. spicatum* caused larger inhibition zones than those made with upper leaves using the same concentration. HPLC analysis showed that in our extracts apical shoot sections contained 30% more tellimagrandin II based on dry weight compared to upper leaf extracts. Tellimagrandin II is the major hydrolyzable tannin in *M. spicatum* (Gross et al., 1996). The inhibition of bacterial isolates also differed with the pH of the agar plates used and the *M. spicatum* parts extracted. Using extract equivalent to 2 mg plant dry weight, we observed smaller inhibition zones of bacteria on neutral agar plates (pH 7.0) compared to alkaline conditions (pH 8.4) with 9 of 11 strains.

The susceptibility of bacterial isolates towards *M. spicatum* extract varied on alkaline media. Isolates belonging to the α - or γ -proteobacteria exhibited only a weak inhibition. Two of the α -proteobacteria were not inhibited with the lowest extract concentration. One strain, *Enterobacter* sp. (γ -proteobacteria) was not affected at all. Another member of this subgroup, *Aeromonas* sp., was only slightly inhibited with the highest concentration used. Two other γ -proteobacteria (*Acinetobacter* and *Pseudomonas*) exhibited inhibition zones almost as large as those observed for Gram-positive bacteria. The inhibition zone of Gram-positives at the lowest concentration ranged from 10 to 20 mm. Inhibition of the only β -proteobacterium isolated was comparable to that of Gram-positive bacteria. Isolates belonging to the CF cluster exhibited the highest susceptibility towards *M. spicatum* extract.

TABLE 3. INHIBITION^a OF SELECTED BACTERIAL STRAINS BY *M. spicatum* EXTRACTS AND PHENOLIC COMPOUNDS

| | <i>Comamonas</i> | <i>Pseudomonas</i> | <i>Chryseobacterium</i> |
|-----------------------------------|------------------|--------------------|-------------------------|
| <i>M. spicatum</i> extract (1 mg) | 17 ± 0 | 23 ± 1 | 23 ± 2 |
| PVPP treated (1 mg) | 0 | 0 | 14 ± 1 |
| Tannic acid | | | |
| 160 µg | 20 ± 1 | 22 ± 0 | 30 ± 1 |
| 27 µg | 13 ± 2 | 14 ± 1 | 19 ± 2 |
| Gallic acid | | | |
| 160 µg | >40.0 | >40.0 | >40.0 |
| 27 µg | 13 ± 0 | 26 ± 1 | 23 ± 1 |
| (+)-Catechin (160 µg) | 0 | 0 | 0 |
| Tellimagrandin II (10 µg) | 4 ± 0 | 9 ± 2 | 11 ± 0 |

^a Mean ± SD (mm).

The susceptibility of three selected strains were tested with *M. spicatum* extract, extract treated with PVPP, and pure phenolic compounds on alkaline media (Table 3). PVPP treatment caused a disappearance of inhibition in two of three strains and greatly reduced the inhibition in the CF strain. Gallic and tannic acid as well as tellimagrandin II inhibited all selected strains, whereas catechin was inactive. This indicates a high susceptibility of bacterial isolates towards tannins in *M. spicatum*.

DISCUSSION

Our study investigated the allelochemical interactions between freshwater macrophytes, the herbivorous aquatic moth *Acentria ephemerella* and their gut symbionts. We fed larvae of *Acentria* with a tannin-free (*Potamogeton perfoliatus*) or a tannin-rich (*Myriophyllum spicatum*) macrophyte. Tannins are well-known herbivore deterrents (Bernays, 1981), but they also have antimicrobial effects (Scalbert, 1991; Field and Lettinga, 1992). Our hypothesis is that food-derived tannins influence the naturally occurring gut microbiota of lepidopteran larvae, which may lead to an altered performance of the larvae. Larvae exhibited reduced growth when fed with *M. spicatum* (Choi et al., 2002). Results of this study show that gut bacteria of *Acentria* are affected by secondary metabolites of the freshwater macrophytes on which larvae were feeding.

We found evidence for the presence of gut symbionts in larvae of *Acentria*. Direct DAPI counts of bacteria revealed densities of 2.8–13.3 × 10⁶ cells/gut. Converting this to gut volume (see Methods and Materials section), we determined bacterial densities of 0.3–2.2 × 10⁶ cells/µl. For other arthropods, 0.07–190 × 10⁶ cells/µl gut fluid have been reported (Cazemier et al., 1997). To our knowledge,

our study is the first to present quantitative data on bacterial densities in guts of Lepidoptera.

Bacterial numbers did not vary between fore-, mid-, or hindgut, and no correlation between the bacterial counts and the size of the larvae was found. So far, it has not been possible to evaluate which factors influence the density of gut bacteria in Lepidoptera, since so little is known in this field. The large variability observed in bacterial densities of different gut segments or larvae may have several explanations. First, bacterial densities in Lepidoptera may naturally show large fluctuations. A few days before metamorphosis, lysozyme is secreted in the midgut in some Lepidoptera larvae (Russell and Dunn, 1991), which would account for lower densities at that time. Second, the rearing procedure can influence bacterial densities. We observed higher densities of bacteria in larvae kept for a prolonged time in the laboratory compared to larvae taken fresh from the field (data not shown). This indicates that laboratory conditions can alter densities of gut bacteria.

We assume that the gut microbiota in *Acentria* is mainly derived from inoculates through the respective food plants. The bacterial densities on the two plants used in our study differed only marginally (Table 1). We did not observe any difference between the mean abundance of bacteria in larvae fed with *P. perfoliatus* or *M. spicatum*. At least two possibilities could account for this: either, tannins from *M. spicatum* may not affect gut bacteria or, the gut bacterial community may have been shifted to more tannin-tolerant strains.

To investigate the inhibitory effect of plant extracts on *Acentria* gut bacteria, we isolated several strains from larvae on different food treatments and performed agar diffusion assays. Sequence analysis revealed only a little overlap in the bacterial isolates from larvae fed with either *P. perfoliatus* or *M. spicatum* (Table 2). None of the strains were inhibited by extracts of leaves from *P. perfoliatus*, even when high concentrations were used. In contrast, extracts of upper leaves from *M. spicatum* inhibited the growth of all but one strain (Table 2).

The antimicrobial effect of *M. spicatum* extracts is, for the most part, due to polyphenols, as was shown by the PVPP assay and comparison with pure phenolic compounds. At present, we cannot fully exclude that some nonphenolic compounds have additional effects, since one strain (*Chryseobacterium* sp.) still exhibited an inhibition after PVPP treatment of the extract.

These experiments were performed using CPS agar with alkaline conditions (pH 8.4 before autoclaving) to mimic Lepidoptera midgut conditions (Appel and Martin, 1990; Clark, 1999). Alkaline conditions promote the autoxidation of tannins and phenolic compounds of lower molecular weight (Appel, 1993). This enhances their toxicity towards microorganisms (Field and Lettinga, 1992). Accordingly, the inhibition zones were always smaller with all but two strains tested when using neutral CPS agar (pH 7.0, Table 2). The inhibition zones in the assay increased when the same amount of extract from apical shoot sections of

M. spicatum were used. Apical shoot sections contain much higher concentrations of polyphenols, among them tellimagrandin II, as was shown with our extracts and in previous studies (Gross, 2000). This indicates a dose-dependent growth inhibition of bacteria by milfoil polyphenols.

We observed considerable differences in the susceptibility of the strains towards these extracts. In our study, both α - and γ -proteobacteria displayed the weakest inhibition of all isolates. The two strains belonging to the CF cluster (*Cytophaga/Flavobacteria*) exhibited the largest inhibition. We found these strains in high densities on the plants, indicating that they are transient, and that the larvae obtained them with their food. To our knowledge, inhibition of flavobacteria by tannins has not been investigated before.

The high susceptibility of Gram-positive bacteria towards polyphenols is in accordance with previous findings (Henis et al., 1964; Puupponen-Pimia et al., 2001). Gram-negative bacteria are thought to be better protected against tannins because of the structure of their outer membrane (Field and Lettinga, 1992). Gram-positive bacteria with a high GC content were found exclusively in guts from larvae fed with *P. perfoliatus*. We assume that the absence of Gram-positive bacteria with high GC content in guts of larvae fed with *M. spicatum* may be a result of the higher sensitivity towards polyphenols in this freshwater macrophyte.

Our results provide the first evidence for the allelochemical effect of *M. spicatum* tannins on gut symbionts in *Acentria*. Due to the small proportion of culturable bacteria, culture studies like ours do not provide sufficient information to observe bacterial community shifts. Consequently, culture-independent studies are needed to verify these findings for the whole bacterial community.

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REFERENCES

- APPEL, H. M. 1993. Phenolics in ecological interactions: the importance of oxidation. *J. Chem. Ecol.* 19:1521–1552.
- APPEL, H. M. and MARTIN, M. M. 1990. Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.* 16:3277–3290.
- APPEL, H. M. and SCHULTZ, J. C. 1994. Oak tannins effectiveness of thuricide (*Bacillus thuringiensis*) in the gypsi moth (Lepidoptera, Lymantriidae). *J. Econ. Entomol.* 87:1736–1741.
- BARBEHENN, R. V. and MARTIN, M. M. 1992. The protective role of the peritrophic membrane in the tannin-tolerant larvae of *Orgyia leucostigma* (Lepidoptera). *J. Insect Physiol.* 38:973–980.
- BERNAYS, E. A. 1981. Plant tannins and insect herbivores: an appraisal. *Ecol. Entomol.* 6:353–360.
- CAZEMIER, A. E., HACKSTEIN, J. H. P., OP DEN CAMP, H. J. M., ROSENBERG, J., and VAN DER DRIFT, C. 1997. Bacteria in the intestinal tract of different species of arthropods. *Microbiol. Ecol.* 33:189–197.

- CHOI, C., BAREISS, C., WALENCIAK, O., and GROSS, E. M. 2002. Impact of polyphenols on the growth of the aquatic herbivore *Acentria ephemerella* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 28.
- CLARK, T. M. 1999. Evolution and adaptive significance of larval midgut alkalization in the insect superorder Mecoptera. *J. Chem. Ecol.* 25:1945–1960.
- COLLINS, V. G. and WILLOUGHBY, L. G. 1962. The disruption of bacterial and fungal spores in Blelham Tarn with particular reference to an experimental overturn. *Arch. Microbiol.* 43:294–307.
- FIELD, J. A. and LETTINGA, G. 1992. Toxicity of tannic compounds to microorganisms, pp. 673–692 in R. W. Hemingway and P. E. Laks (eds.). *Plant Polyphenols*. Plenum Press, New York.
- GROSS, E. M. 2000. Seasonal and spatial dynamics of allelochemicals in the submersed macrophyte *Myriophyllum spicatum* L. *Verh. Int. Verein. Limnol.* 27:2116–2119.
- GROSS, E. M., MEYER, H., and SCHILLING, G. 1996. Release and ecological impact of algicidal hydrolyzable polyphenols in *Myriophyllum spicatum*. *Phytochemistry* 41:133–138.
- GROSS, E. M., JOHNSON, R. L., and HAIRSTON N. G., JR. 2001. Experimental evidence for changes in submersed macrophyte species composition caused by the herbivore *Acentria ephemerella* (Lepidoptera). *Oecologia* 127:105–114.
- GROSS, E. M., FELDBAUM, C., and CHOI, C. 2002. High abundance of herbivorous Lepidoptera larvae (*Acentria ephemerella* Denis & Schiffmüller) on submersed macrophytes in Lake Constance (Germany). *Arch. Hydrobiol.* 155:1–21.
- HENIS, Y., TAGARI, H., and VOLCANI, R. 1964. Effect of water extract of carob pods, tannic acid, and their derivatives on the morphology and growth of microorganisms. *Appl. Microbiol.* 12:204–210.
- MANUWOTO, S. and SCRIBER, J. M. 1986. Effects of hydrolyzable and condensed tannin on growth and development of two species of polyphagous lepidoptera: *Spodoptera eridania* and *Callosamia promethea*. *Oecologia* 69:225–230.
- MUYZER, G. T., HOTTENTRAEGER, S., TESKE, A., and WAWER, C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities, pp. 3.4.4:1–23 in A. D. L. Akkermans, J. D. van Elsas and F. J. de Bruijn (eds.). *Molecular Microbial Ecology Manual*, 2nd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- MUYZER, G. T., BRINKHOFF, T., NÜBEL, U., SANTEGOEDS, C., SCHÄFER, H., and WAWER, C. 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, pp. 3.4.4:1–27 in A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (eds.). *Molecular Microbial Ecology Manual*, 3rd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- PUUPPONEN-PIMIA, R., NOHYNEK, L., MEIER, C., KAHKONEN, M., HEINONEN, M., HOPIA, A., and OKSMAN-CALDENTY, K.-M. 2001. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.* 90:494–507.
- RUSSELL, V. W. and DUNN, P. E. 1991. Lysozyme in the midgut of *Manduca sexta* during metamorphosis. *Arch. Insect Biochem. Physiol.* 17:67–80.
- SCALBERT, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30:3875–3883.
- SCHULTZ, J. C., HUNTER, M. D., and APPEL, H. M. 1992. Antimicrobial activity of polyphenols mediates plant-herbivore interactions, pp. 621–692 in R. W. Hemingway and P. E. Laks (eds.). *Plant Polyphenols*. Plenum Press, New York.
- SHER-KAUL, S., OERTLI, B., CASTELLA, E., and LACHAVANNE, J.-B. 1995. Relationship between biomass and surface area of six submersed aquatic plants species. *Aquat. Bot.* 51:147–154.
- SPENCER, C. M., CAI, Y., MARTIN, R., GAFFNEY, S. H., GOULDING, P. N., MAGNOLATO, D., LILLEY, T. H., and HASLAM, E. 1988. Polyphenol complexation—Some thoughts and observations. *Phytochemistry* 27:2397–2409.