Supplementary Information

Faeces of *Rhinolophus euryale* (Chiroptera) from the winter season contain inorganic matter

Maxinová E., Arrizabalaga-Escudero A., Arriolabengoa M., Aloria K., Zaldibar B., Boldogh S., Uhrin M., Goiti U., Aihartza J., Garin I.

DNA analysis

The polymerase chain reaction (PCR) of the partial sequence of the 16S rRNA gene was performed in a 20 µl PCR reaction using the HotStarTaq Plus Master Mix Kit (Qiagen Ltd., Crawley, West Sussex, UK). Each PCR reaction contained: 13 µl dd H_2O, 2 µl 10× Buffer, 0.4 µl $MgCl_2$, 0.4 µl dNTP at 200 µM (final concentration), 1 µl of each forward and reverse primers at 0.5 µM (final concentration), 0.12 µl HotStarTaq plus DNA polymerase and 2 µl from each extracted faecal DNA. PCR was carried out on a Mastercycler pro S thermocycler (Eppendorf) with the following conditions: initial denaturation at 95 °C for 15 minutes followed by 30 cycles of 94 °C for 30 seconds, 58 °C for 1 minute and 72 °C for 1 minute, followed by a final extension of 6 minutes at 72 °C. We included one negative and one positive (DNA extracted from a tissue sample of *R. euryale*) control for each PCR.

A Multiplex PCR Kit (Qiagen Ltd., Crawley, West Sussex, UK) was used for the amplification of a 157 bp length insects' COI fragment. The PCR mix for each sample (20 µl) consisted of: 10 µl 2× Multiplex Buffer, 0.4 µl each of forward and reverse primers at 0.2 µM (final concentration), 8.2 µl dd H_2O and 1 µl from each extracted faecal DNA. PCR conditions were: initial denaturation at 95 °C for 15 minutes followed by 50 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 30 seconds, followed by a final extension of 6 minutes at 72 °C (Bohman et al., 2011). One negative and one positive (insect DNA from typical consumptive summer faeces of *R. euryale*) control were included for each PCR. The PCR products were visualised on 1.5% agarose gel stained with RealSafe running at 100 V for 20 minutes.

Protein analysis

Faecal pellets were placed in homogenization tubes (Micro tube 2 ml, Sarstedt AG & Co., Nümbrecht, Germany) containing 1.0 mm Ø zirconia/silica beads (Biospec) and 400 µl of 0.15% (v/v) Trifluoroacetic acid. The tubes were placed in a Precellys©24 homogenizer, and homogenization was performed (5000 rpm – 3 × 15 sec. – 005). Samples were centrifuged at 13000 rpm at 4 °C in a Hettich Mikro 200R (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), and the soluble supernatant was recovered for subsequent analysis. The total protein content of the faecal extracts was determined using the Bradford Reagent (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. Sample protein concentration was 0.38 mg/ml.

20 µg of protein were precipitated using a 2-D Clean-Up Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) according to the manufacturer's instructions. Once precipitated, the proteins were digested using an in-solution digestion protocol. Briefly, the protein pellet was suspended in 20 µL of 0.2% RapiGest SF solution (Waters Corporation, Milford, MA, USA), vortexed and incubated at 85 °C for 15 minutes with agitation (1150 rpm). Once suspended, the proteins were reduced with DTT (5 mM final concentration) and heating for 30 minutes at 60 °C with agitation. After cooling to room temperature, the sample was alkylated by incubation with Iodoacetamide (IAA, 15 mM final concentration) for 30 minutes in the dark. Proteins were digested with Trypsin (F. Hoffmann-La Roche Ltd., Basel, Switzerland) at a ratio of 1:20 (w/w) and incubated at 37 °C for 16 h. Digestion was terminated by acidification with 35% HCl (incubation for 30 minutes at 37 °C, followed by centrifugation), which hydrolized RapiGest and induced the precipitation of the insoluble material. Peptides were desalted using a C18 Micro SpinColumn (Harvard Apparatus, Holliston, MA, USA), evaporated to dryness and re-suspended in 20 µl of 0.1% formic acid in water.

LC-MS/MS analysis was performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) interfaced with an Easy-nLC 1000 nanoUPLC System (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Digested peptides were loaded onto an Acclaim PepMap100 precolumn (75 µm × 2 cm, Thermo Fisher Scientific Inc., Waltham, MA, USA) connected to an Acclaim PepMap RSLC (50 µm × 15 cm, Thermo Fisher Scientific Inc., Waltham, MA, USA) analytical column. Peptides were eluted at a flow rate of 300 nl/min directly onto the nanoES Emitter (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a 180 min linear gradient from 5% to 30% of acetonitrile in 0.1% of formic acid.

The Q Exactive was operated in a Top 15 data dependent mode. Survey scans were acquired at a resolution of 70000 (m/z 200) and a fragmentation spectra of 17500 (m/z 200). Peptide selection was done with an isolation window of 2.0 Th, and normalized collision energy of 28 was applied for peptide fragmentation. The maximum injection time was 120 ms for survey and MS/MS scans, and AGC target values of 3×10^6 for survey scans and 5×10^5 for MS/MS scans were used. Raw files were processed and searched with Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Precursor and fragment mass tolerances were set to 10 ppm and 0.05 Da, respectively, and up to 1 missed cleavage was allowed. Carbamidomethylation of Cys was set as the fixed modification, and oxidation of Met as the variable modification. False Discovery Rate (FDR) was set to 1% and when a spectrum indentified more than one different peptide, the peptide with the highest score has only been counted.