Supplementary Material

The excreted microbiota of bats: evidence of niche specialization
based on multiple body habitats

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Text S1. Bat sampling protocol.

To minimize potential bias due to variability in bacterial communities over time and space, samples were collected in the same geographic location and during a short period of time. Bat sampling was thus conducted in February 2015, during a 4-night period, at the Gatkop cave (S 24.61806; E 027.65223) and its surrounding environment (< 3kms; S 24.61737; E 027.68773) in the Limpopo province, South Africa. This cave hosts different insectivorous bat species, including *Miniopterus natalensis*, *Rhinolophus simulator* and *Nycteris thebaica* (Kearney, Keith and Seamark 2016). *Neoromicia capensis* is also present in this area and roosts in trees as well as man-made structures. In order to make our sampling as heterogeneous as possible, we have included these different species in our sampling scheme. Bats were captured using harp-traps placed at the entrance of a cave and using mist nets in the surrounding area. Trapped bats were kept separately in numbered cotton cloth bags and immediately processed on site. Bats were morphologically identified using taxonomic keys (Monadjem *et al.* 2010; ACR 2015) and we also recorded the sex, age (adult/sub-adult, based on the complete fusion of phalangeal epiphyses; Anthony 1988), weight and forearm length. All individuals were in a non-reproductive state (not pregnant for females, non-scrotal for males). All bats were released after sampling, with the exception of the *N. capensis*, which was euthanatized by an overdose inhalation of isoflurane, and deposited as a voucher specimen in the Ditsong National Museum of Natural History.

Text S2. DNA extraction protocol.

The protocol includes the addition of the peptidoglycan-degrading enzymes mutanolysin and lysozyme, which ensure robust lysis of Gram-positive and Gram-negative species. Two hundred microliters of filtered buffer (consisting of 20 mM Tris-Cl (pH 8), 2 mM EDTA, 1.2% Triton X-100, and 20 µg/ml lysozyme), supplemented with 30 µl of filtered mutanolysin (5,000 U/ml; Sigma-Aldrich, St. Louis, MO), was added to samples. The mixture was incubated for 1 h at 37°C and the lysates were processed through the QIAmp DNA Micro kit (Qiagen, Valencia, CA), which is adapted for small sample volumes. We used the protocol for isolation for genomic DNA from tissues according to the manufacturer's instructions with slight modifications. We used a 3 hours incubation time at 56°C, the addition of carrier RNA, and faecal samples were centrifuged (2 min. at $604g$) and the supernatant collected before the transfer to the Qiagen column. DNA was eluted into 20 µl of buffer AE and stored at -20°C.

Text S3. Illumina sequencing and bioinformatics.

Negative and positive PCR controls, in the form of an ultrapure water sample and a mock community (DNA of eleven known bacterial species) respectively, were provided by Genoscreen and included in the preparation of the libraries, which were all prepared at the same time. The indexed DNA libraries were equimolar pooled and diluted at a final concentration of 4 nM. Paired-end (2 x 250) sequencing was performed in a single run using a MiSeq Reagent Nano v2 kit on an Illumina MiSeq instrument, and PhiX DNA (15%) as a spike-in control for the estimation of the error rate during sequencing.

Libraries were de-multiplexed using Casava version 1.8 and raw reads were recovered as FASTQ files. Forward and reverse primers were removed at 100% nucleotide identity by Genoscreen and sequences were quality trimmed when Q score < 30. Alignment of PhiX reads to the reference genome yielded an error rate of 1.56% for read 1 and 1.64% for read 2 during sequencing. The correct taxonomic assignation of the 11 bacterial species present in the positive control was validated by Genoscreen, using the Greengenes reference database (DeSantis *et al.* 2006). Assembled reads were quality trimmed based on their length prior to alignment against the MOTHUR-formatted SILVA database. Preclustering of the data was performed using a 4-bp difference, following by the detection and removal of chimeras using the UCHIME algorithm(Edgar *et al.* 2011). We then classified sequences using the MOTHUR-formatted version of the RDP training set (v.9), and any unknown, chloroplast, mitochondrial, archaeal, or eukaryotic sequences were removed. Remaining sequences were clustered into phylotypes using a 97% identity threshold.

Text S4. Removal of potential background contaminants.

Removal of potential contaminant bacteria during DNA extraction and PCR steps was performed at the phylotype level with R (R Core Team 2013) and the scripts provided by Meadow *et al.* (2014). The relative abundance of each phylotype in samples and the DNA extraction and PCR controls was compared, and when the distribution was positively correlated, we considered that it is suggestive of a spurious signal generated in the absence of a preferentially amplified template (Meadow, Altrichter and Green 2014). To assess the influence of potential contaminant phylotypes and their removal on bacterial community diversity and structure, we produced four datasets from null to full removal of contaminant phylotypes:

- the first dataset ('nocont') with no contaminant phylotypes removed
- the second dataset ('2cont') with a conservative approach, removing contaminant phylotypes with a relative abundance > 10% in the controls;
- the third dataset ('28cont') where contaminant phylotypes with a relative abundance > 0.001% in the controls were removed;
- the fourth dataset ('allcont') with all contaminant phylotypes removed.

Table S1. Potential contaminant phylotypes removed from sequencing data prior to alpha- and beta-diversity analyses. Bacterial phyla are indicated in bold and the rest of the name is composed of Class / Order / Family / Genus. Removed phylotypes in each dataset are indicated by a cross.

Phylotype ID	2cont	28cont	allcont
Acidobacteria			
Acidobacteria_Gp10 / Acidobacteria_Gp10_order_incertae_sedis / Acidobacteria_Gp10_family_incertae_sedis / Gp10			X
Acidobacteria_Gp4 / Acidobacteria_Gp4_order_incertae_sedis / Acidobacteria_Gp4_family_incertae_sedis / Gp4			X
Acidobacteria_Gp6 / Acidobacteria_Gp6_order_incertae_sedis / Acidobacteria_Gp6_family_incertae_sedis / Gp6			X
Acidobacteria_Gp7 / Acidobacteria_Gp7_order_incertae_sedis / Acidobacteria_Gp7_family_incertae_sedis / Gp7			X
Actinobacteria			
Actinobacteria / Acidimicrobiales / unclassified / unclassified			X
Actinobacteria / Actinomycetales / Bogoriellaceae / *Georgenia*			X
Actinobacteria / Actinomycetales / Brevibacteriaceae / *Brevibacterium*			X
Actinobacteria / Actinomycetales / Cellulomonadaceae / *Cellulomonas*			X
Actinobacteria / Actinomycetales / Corynebacteriaceae / *Corynebacterium*			X
Actinobacteria / Actinomycetales / Dietziaceae / *Dietzia*			X
Actinobacteria / Actinomycetales / Geodermatophilaceae / *Blastococcus*			X
Actinobacteria / Actinomycetales / Intrasporangiaceae / *Ornithinimicrobium*			X
Actinobacteria / Actinomycetales / Intrasporangiaceae / *Tetrasphaera*			X
Actinobacteria / Actinomycetales / Intrasporangiaceae / unclassified			X
Actinobacteria / Actinomycetales / Microbacteriaceae / *Agrococcus*			X
Actinobacteria / Actinomycetales / Microbacteriaceae / *Microbacterium*			X
Actinobacteria / Actinomycetales / Microbacteriaceae / unclassified			X
Actinobacteria / Actinomycetales / Micrococcaceae / *Arthrobacter*			X
Actinobacteria / Actinomycetales / Micrococcaceae / *Nesterenkonia*			X
Actinobacteria / Actinomycetales / Micrococcaceae / unclassified			X
Actinobacteria / Actinomycetales / Micrococcaceae / *Rothia*			X
Actinobacteria / Actinomycetales / Micromonosporaceae / *Actinoplanes*			X
Actinobacteria / Actinomycetales / Micromonosporaceae / unclassified			X
Actinobacteria / Actinomycetales / Mycobacteriaceae / *Mycobacterium*			X
Actinobacteria / Actinomycetales / Nocardiaceae / *Rhodococcus*			X
Actinobacteria / Actinomycetales / Nocardiaceae / *Smaragdicoccus*			X
Actinobacteria / Actinomycetales / Nocardiaceae / *Williamsia*			X
Actinobacteria / Actinomycetales / Nocardioidaceae / *Nocardioides*			X
Actinobacteria / Actinomycetales / Promicromonosporaceae / *Cellulosimicrobium*	X	X	X
Actinobacteria / Actinomycetales / Promicromonosporaceae / *Isoptericola*			X
Actinobacteria / Actinomycetales / Promicromonosporaceae / unclassified		X	X
Actinobacteria / Actinomycetales / Propionibacteriaceae / *Propionibacterium*			X
Actinobacteria / Actinomycetales / Pseudonocardiaceae / *Pseudonocardia*			X
Actinobacteria / Actinomycetales / Pseudonocardiaceae / unclassified			X
Actinobacteria / Actinomycetales / Streptomycetaceae / *Streptomyces*			X
Actinobacteria / Actinomycetales / unclassified / unclassified		X	X
Actinobacteria / Coriobacteriales / Coriobacteriaceae / unclassified			X
Actinobacteria / Rubrobacterales / Rubrobacteraceae / *Rubrobacter*			X
Actinobacteria / Solirubrobacterales / Conexibacteraceae / *Conexibacter*			X
Actinobacteria / Solirubrobacterales / Solirubrobacteraceae / *Solirubrobacter*			X
Actinobacteria / Solirubrobacterales / unclassified / unclassified			X
Actinobacteria / unclassified / unclassified / unclassified		X	X
Bacteroidetes			
Bacteroidia / Bacteroidales / Porphyromonadaceae / *Odoribacter*			X
Bacteroidia / Bacteroidales / Porphyromonadaceae / *Parabacteroides*			X
Bacteroidia / Bacteroidales / Porphyromonadaceae / unclassified			X

Taxonomy		
Flavobacteria / Flavobacteriales / Flavobacteriaceae / *Chryseobacterium*		X
Flavobacteria / Flavobacteriales / Flavobacteriaceae / *Cloacibacterium*		X
Flavobacteria / Flavobacteriales / Flavobacteriaceae / *Salinimicrobium*		X
Flavobacteria / Flavobacteriales / Flavobacteriaceae / unclassified		X
Sphingobacteria / Sphingobacteriales / Cytophagaceae / *Dyadobacter*		X
Sphingobacteria / Sphingobacteriales / Sphingobacteriaceae / *Pedobacter*	X	X
Sphingobacteria / Sphingobacteriales / Sphingobacteriaceae / *Sphingobacterium*		X
Sphingobacteria / Sphingobacteriales / Sphingobacteriaceae / unclassified		X
unclassified / unclassified / unclassified / unclassified	X	X

Fibrobacteres

Fibrobacteria / Fibrobacterales / Fibrobacteraceae / *Fibrobacter*		X

Firmicutes

Bacilli / Bacillales / Bacillaceae_1 / *Anoxybacillus*	X	X
Bacilli / Bacillales / Bacillaceae_1 / *Bacillus*	X	X
Bacilli / Bacillales / Bacillaceae_1 / unclassified		X
Bacilli / Bacillales / Bacillaceae_2 / unclassified		X
Bacilli / Bacillales / Bacillales_Incertae_Sedis_X / *Thermicanus*		X
Bacilli / Bacillales / Bacillales_Incertae_Sedis_XI / *Gemella*		X
Bacilli / Bacillales / Paenibacillaceae_1 / *Paenibacillus*		X
Bacilli / Bacillales / Paenibacillaceae_1 / *Saccharibacillus*		X
Bacilli / Bacillales / Planococcaceae / *Planomicrobium*	X	X
Bacilli / Bacillales / Planococcaceae / *Sporosarcina*		X
Bacilli / Bacillales / Staphylococcaceae / *Staphylococcus*	X	X
Bacilli / Bacillales / unclassified / unclassified		X
Bacilli / Lactobacillales / Enterococcaceae / *Enterococcus*		X
Bacilli / Lactobacillales / Lactobacillaceae / *Lactobacillus*	X	X
Bacilli / Lactobacillales / Streptococcaceae / *Lactococcus*		X
Bacilli / Lactobacillales / Streptococcaceae / *Streptococcus*		X
Bacilli / unclassified / unclassified / unclassified		X
Clostridia / Clostridiales / Clostridiaceae_1 / *Clostridium*_sensu_stricto		X
Clostridia / Clostridiales / Clostridiaceae_1 / unclassified		X
Clostridia / Clostridiales / Clostridiales_Incertae_Sedis_XIII / *Mogibacterium*		X
Clostridia / Clostridiales / Lachnospiraceae / *Cellulosilyticum*	X	X
Clostridia / Clostridiales / Lachnospiraceae / *Clostridium*_XlVa		X
Clostridia / Clostridiales / Lachnospiraceae / Lachnospiracea_incertae_sedis	X	X
Clostridia / Clostridiales / Lachnospiraceae / unclassified	X	X
Clostridia / Clostridiales / Ruminococcaceae / *Pseudoflavonifractor*		X
Clostridia / Clostridiales / Ruminococcaceae / unclassified	X	X
Clostridia / Clostridiales / unclassified / unclassified		X
Negativicutes / Selenomonadales / unclassified / unclassified		X
Negativicutes / Selenomonadales / Veillonellaceae / unclassified		X
unclassified / unclassified / unclassified / unclassified		X

Fusobacteria

Fusobacteria / Fusobacteriales / Leptotrichiaceae / *Sebaldella*		X

Gemmatimonadetes

Gemmatimonadetes / Gemmatimonadales / Gemmatimonadaceae / *Gemmatimonas*		X

Proteobacteria

Alphaproteobacteria / Caulobacterales / Caulobacteraceae / *Brevundimonas*		X
Alphaproteobacteria / Caulobacterales / Caulobacteraceae / *Caulobacter*		X
Alphaproteobacteria / Rhizobiales / Bradyrhizobiaceae / unclassified		X
Alphaproteobacteria / Rhizobiales / Methylobacteriaceae / *Methylobacterium*	X	X
Alphaproteobacteria / Rhizobiales / Methylobacteriaceae / *Microvirga*		X
Alphaproteobacteria / Rhizobiales / Methylocystaceae / *Pleomorphomonas*	X	X
Alphaproteobacteria / Rhizobiales / Phyllobacteriaceae / *Mesorhizobium*		X
Alphaproteobacteria / Rhizobiales / Rhizobiaceae / *Rhizobium*		X
Alphaproteobacteria / Rhizobiales / unclassified / unclassified		X
Alphaproteobacteria / Rhodobacterales / Rhodobacteraceae / *Loktanella*		X
Alphaproteobacteria / Rhodospirillales / Acetobacteraceae / unclassified		X
Alphaproteobacteria / Sphingomonadales / Sphingomonadaceae / *Novosphingobium*		X
Alphaproteobacteria / Sphingomonadales / Sphingomonadaceae / *Sphingomonas*	X	X
Alphaproteobacteria / unclassified / unclassified / unclassified		X
Betaproteobacteria / Burkholderiales / Alcaligenaceae / *Pigmentiphaga*		X

Taxonomy			
Betaproteobacteria / Burkholderiales / Burkholderiaceae / *Pandoraea*		X	X
Betaproteobacteria / Burkholderiales / Burkholderiales_incertae_sedis / *Tepidimonas*			X
Betaproteobacteria / Burkholderiales / Comamonadaceae / *Acidovorax*			X
Betaproteobacteria / Burkholderiales / Comamonadaceae / *Delftia*			X
Betaproteobacteria / Burkholderiales / Comamonadaceae / *Hydrogenophaga*			X
Betaproteobacteria / Burkholderiales / Comamonadaceae / *Pelomonas*			X
Betaproteobacteria / Burkholderiales / Comamonadaceae / *Variovorax*			X
Betaproteobacteria / Burkholderiales / Oxalobacteraceae / *Janthinobacterium*		X	X
Betaproteobacteria / Burkholderiales / Oxalobacteraceae / unclassified			X
Betaproteobacteria / Burkholderiales / Oxalobacteraceae / *Undibacterium*			X
Betaproteobacteria / Burkholderiales / unclassified / unclassified			X
Betaproteobacteria / Neisseriales / Neisseriaceae / *Aquitalea*			X
Betaproteobacteria / Rhodocyclales / Rhodocyclaceae / *Azospira*			X
Betaproteobacteria / Rhodocyclales / Rhodocyclaceae / unclassified			X
Deltaproteobacteria / Desulfobacterales / Desulfobacteraceae / unclassified			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Buttiauxella*			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Citrobacter*			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Escherichia_Shigella*	X	X	X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Klebsiella*			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Pantoea*			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Serratia*			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Trabulsiella*			X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / *Yersinia*		X	X
Gammaproteobacteria / Enterobacteriales / Enterobacteriaceae / unclassified		X	X
Gammaproteobacteria / Legionellales / Legionellaceae / *Legionella*			X
Gammaproteobacteria / Pasteurellales / Pasteurellaceae / *Actinobacillus*			X
Gammaproteobacteria / Pasteurellales / Pasteurellaceae / unclassified			X
Gammaproteobacteria / Pseudomonadales / Moraxellaceae / *Acinetobacter*		X	X
Gammaproteobacteria / Pseudomonadales / Moraxellaceae / *Enhydrobacter*			X
Gammaproteobacteria / Pseudomonadales / Pseudomonadaceae / *Pseudomonas*		X	X
Gammaproteobacteria / Pseudomonadales / Pseudomonadaceae / unclassified			X
Gammaproteobacteria / Xanthomonadales / Xanthomonadaceae / *Pseudoxanthomonas*			X
Gammaproteobacteria / Xanthomonadales / Xanthomonadaceae / *Stenotrophomonas*		X	X
Gammaproteobacteria / Xanthomonadales / Xanthomonadaceae / unclassified			X
Gammaproteobacteria / Xanthomonadales / unclassified / unclassified			X
Gammaproteobacteria / unclassified / unclassified / unclassified		X	X
unclassified / unclassified / unclassified / unclassified			X

Synergistetes

Synergistia / Synergistales / Synergistaceae / unclassified			X

Tenericutes

Mollicutes / Mycoplasmatales / Mycoplasmataceae / *Mycoplasma*			X

Verrucomicrobia

Subdivision3 / Subdivision3_order_incertae_sedis / Subdivision3_family_incertae_sedis / 3_genus_incertae_sedis			X
Subdivision5 / Subdivision5_order_incertae_sedis / Subdivision5_family_incertae_sedis / 5_genus_incertae_sedis			X

TM7

TM7_class_incertae_sedis / TM7_order_incertae_sedis / TM7_family_incertae_sedis / TM7_genus_incertae_sedis			X

Unclassified

unclassified / unclassified / unclassified / unclassified		X	X

Figure S1. Relative abundance of phylotypes in the 18 bat samples and the two negative controls (DNA extraction and PCR) according to the different body habitats: (a) urine, (b) faeces, (c) saliva, and (d) for all samples after the removal of the two main contaminant phylotypes. The ratio 1:1 is shown by the grey dash line. Names of the main contaminant phylotypes are indicated.
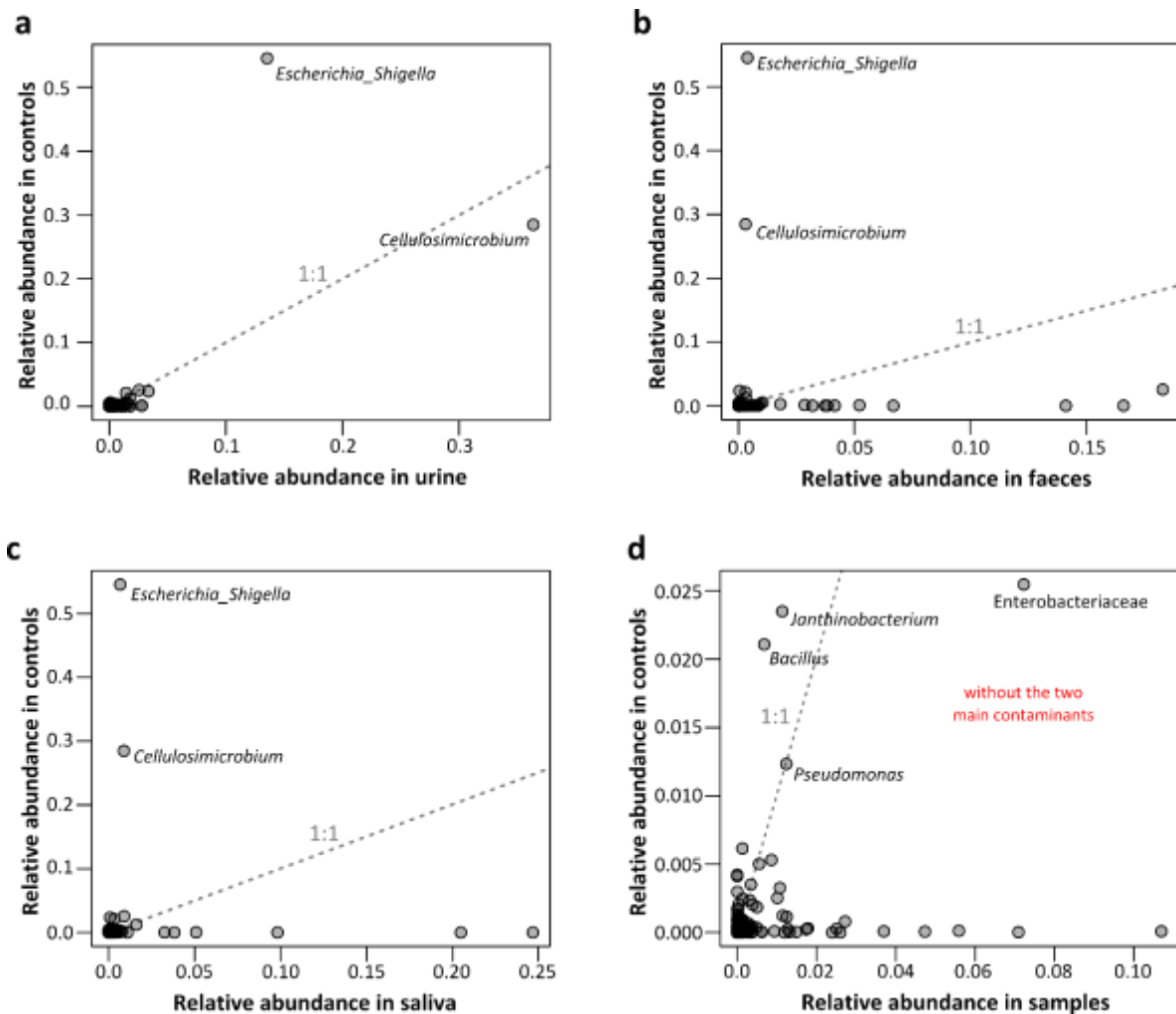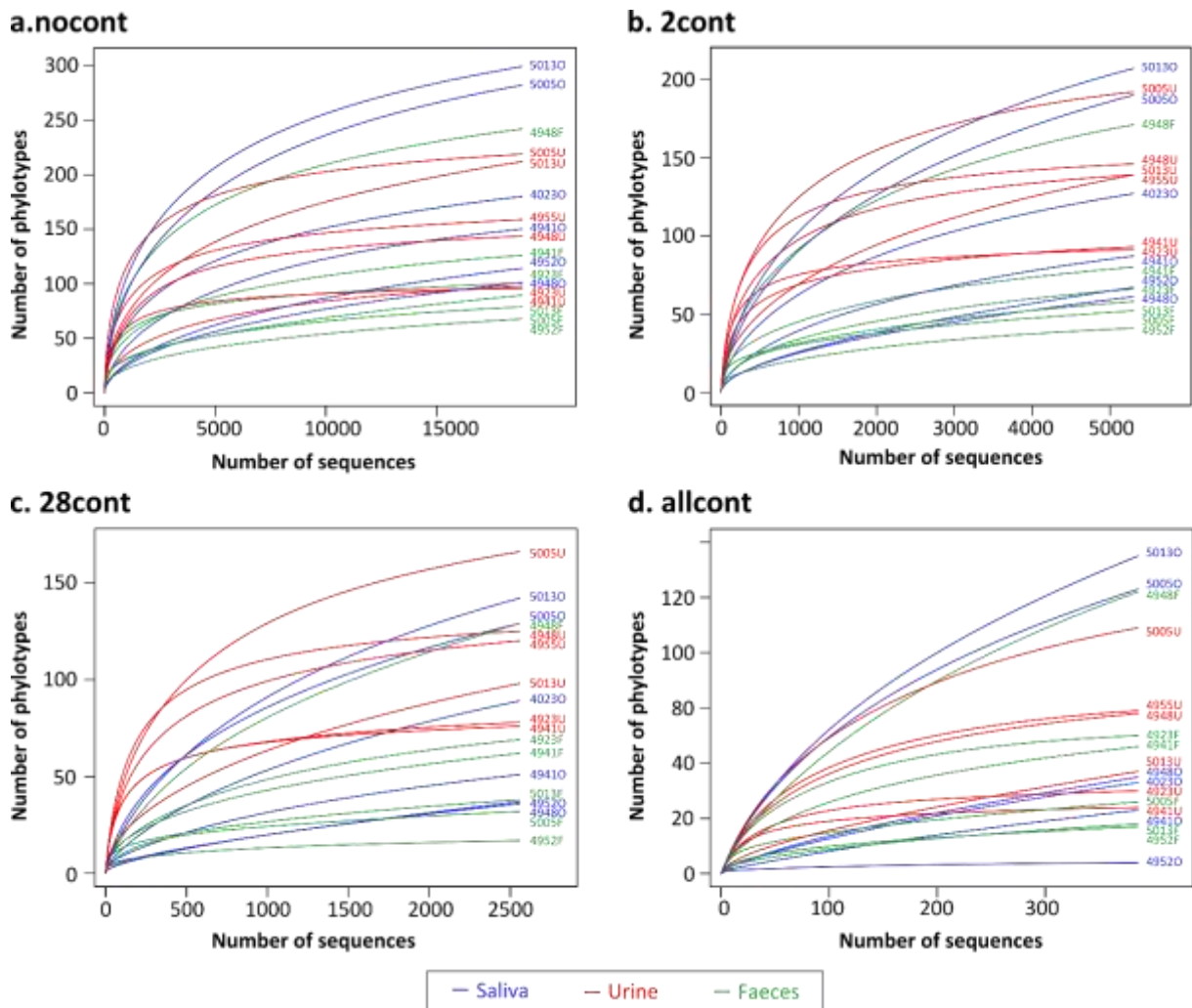
Figure S2. Phylotype rarefaction curves for the 18 bat samples based on the four different datasets. (a) nocont: no contaminant phylotypes removed, (b) 2cont: removal of the two most offensive contaminant phylotypes, (c) 28cont: removal of the first 28 contaminant phylotypes, and (d) allcont: all contaminant phylotypes removed.

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