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

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One sentence summary: Body habitat is a main driver of bacterial community composition in bats with the majority of phylotypes being body habitat-specific and major zoonotic bacteria excreted in all body habitats.

ABSTRACT

Animal-associated microbiotas form complex communities, which play crucial functions for their host, including susceptibility to infections. Despite increasing attention to bats as reservoirs of zoonotic pathogens, their microbiota is poorly documented, especially for samples potentially implicated in pathogen transmission such as urine and saliva. Here, using low-biomass individual samples, we examined the composition and structure of bacterial communities excreted by insectivorous bats, focusing on three body habitats (saliva, urine and faeces). We show that niche specialization occurs as bacterial community composition was distinct across body habitats with the majority of phylotypes being body habitat-specific. Our results suggest that urine harbors more diverse bacterial communities than saliva and faeces and reveal potentially zoonotic bacteria such as *Leptospira*, *Rickettsia*, *Bartonella* and *Coxiella* in all body habitats. Our study emphasized that, in addition to the traditional use of gut-associated samples such as faeces, both urine and saliva are also of interest because of their diverse microbiota and the potential transmission of pathogenic bacteria. Our results represent a critical baseline for future studies investigating the interactions between microbiota and infection dynamics in bats.

Keywords: microbiota, bats, Africa, saliva, urine, faeces

INTRODUCTION

Bats are receiving increasing attention as potential reservoirs for a wide range of zoonotic pathogens, including bacteria (Calisher *et al.* 2006; Mühldorfer 2013). Most of our understanding of bat-borne pathogen diversity has focused on a single pathogen at a time. Recent technical advances have made it possible to scrutinize the microbial community (or microbiota) in bats. However, to date, bacterial metagenomic studies in bats have focused mainly on digestive tract derived samples, such as intestine (Banskar, Mourya and Shouche 2014; Carrillo-Araujo *et al.* 2015), colon (Phillips *et al.* 2012), faeces and guano (Veikkolainen *et al.* 2014; De Mandal *et al.* 2015). Thus, our knowledge of bacterial community composition in other body habitats remains completely unknown, especially for potentially pathogen transmission routes such as urine and saliva.

Technically, the collection of biological samples from bats using non-lethal capture provides only small amounts of material, especially with small body-sized insectivorous bat species. In this context, metagenomic sequencing of microbial communities from individual samples (and not pooling samples) can be challenging. In part, this is due to limited biological content of low-biomass samples, which may provide little template DNA to compete for amplification with background contaminant bacterial DNA that are ubiquitous in extraction kits and other laboratory reagents (Biesbroek *et al.* 2012; Salter *et al.* 2014). The inclusion of controls and accurate post-processing of sequences is thus highly recommended (Jervis-Bardy *et al.* 2015) in order to identify and filter these background bacterial contaminants from datasets, but this has been rarely applied to date (but see Meadow *et al.* 2014; Rynkiewicz *et al.* 2015).

In this study, we report findings of the excreted microbiota of bats by investigating the bacterial community composition and structure in saliva, urine and faecal samples at the individual level. We focused on insectivorous bat species found in Africa, a continent for

which no bacterial metagenomic data were previously available. Our objectives were (i) to assess the reliability of low-biomass individual samples to infer bacterial communities in bats, (ii) to analyze how the diversity and composition of bacterial communities varied between body habitats, and (iii) to assess the excretion of potential zoonotic bacteria from different body habitats.

MATERIALS AND METHODS

Field sampling

Bat sampling was conducted in February 2015 at the Gatkop cave (S 24.61806; E 027.65223) in the Limpopo province, South Africa (Table 1 and Text S1, Supporting Information). We collected saliva samples and, as far as possible, urine and faeces from the same bat individual. Saliva was collected by carefully swabbing the tongue, the palate and the inside of the lips using sterile cotton swabs. A urine droplet, when available, was collected using a pipette at the urethral opening. One faecal pellet, when available, was collected straight from the bats during handling. Each sample was placed in a sterile vial and stored in liquid nitrogen prior to transfer to a -80°C freezer. The sampling protocol was approved by the University of Pretoria Animal Ethics committee (EC054-14) and carried out in strict accordance with the terms of the research permit 0089-CPM401-00015 issued by the Department of Economic Development, Environment & Tourism (Limpopo province).

Sample preparation and sequencing

DNA extraction was performed using a modified QIAamp DNA Micro kit (Qiagen, Valencia, CA) protocol (see details in Text S2, Supporting Information). Care was taken to avoid bacterial DNA contamination by utilizing DNA-free reagents when applicable, filter all solutions through a 0.2 µM filter, and working in a PCR-clean hood. Before DNA extraction,

faeces samples were weighted and the volume of urine samples was measured. All samples were processed in the same extraction batch, including a negative control (reagents only) to control for the introduction of contaminating DNA. DNA purity and yield were evaluated with a NanoDrop spectrophotometer and a Qubit fluorometer respectively. We then subjected DNA extracts and the negative extraction control to V3-V4 region 16S rRNA PCR and barcoded Illumina MiSeq paired-end sequencing, following the Metabiote® protocol developed by Genoscreen (Lille, France), which includes the addition of a negative PCR control (see details in Text S3, Supporting Information).

Bioinformatics and statistical analyses

DNA sequences were analyzed using MOTHUR v.1.33.3 following the MiSeq SOP Pipeline (Schloss *et al.* 2009; Kozich *et al.* 2013) (see details in Text S3, Supporting Information). Using both the extraction and PCR controls, we identified potential background contaminants from laboratory reagents, and produced four datasets with different levels of contaminant removal: none ('nocont'), two ('2cont'), twenty-eight ('28cont') and all ('allcont') contaminants removed (Text S4, Supporting Information). Details of phylotypes removed as presumed bacterial contaminants to produce the four datasets are provided in Table S1 (Supporting Information). We calculated alpha- and beta-diversity metrics along with rarefaction plots from the rarefied phylotype tables. We calculated microbial richness using the inverse Simpson diversity index and performed comparison between body habitats using ANOVA. Nonmetric multidimensional scaling (NMDS) ordination were conducted on Bray-Curtis dissimilarities, calculated from rarefied sequence counts, after square root transformation and Wisconsin standardization. Permutational MANOVA (PERMANOVA) tests with 999 permutations were performed to test the structure of bacterial communities among body habitats.

The taxonomic composition of bacterial communities were explored based on the non-rarefied 'allcont' dataset, to avoid including any potential contaminant phylotypes as well as eliminating rare phylotypes during the rarefaction step. We analyzed the more abundant phylotypes, as well as the specific microbiota of each body habitat. Finally, we focused on those bacterial genera that included species known, or suspected, to be pathogenic for humans. The 16S rRNA sequences and phylotype table are archived at Dryad: http://dx.doi.org/10.5061/dryad.q0h60

RESULTS

Bat samples

Samples were obtained from four insectivorous bat species (*Nycteris thebaica*, *Miniopterus natalensis*, *Rhinolophus simulator* and *Neoromicia capensis*), the first three were roosting in the same cave, and *N. capensis* was trapped in the surrounding area. Seven bats were sampled, and faeces, saliva and urine samples were obtained from each individual, with the exception of the female *N. thebaica*, as samples were from two different individuals (saliva and faeces from UP4952 and urine from UP4955). In total we analyzed 18 samples (six for each body habitat, Table 1). As expected when using non-lethal capture on small body-sized insectivorous bats, samples were characterized by small amounts of initial material and low DNA yields. However, DNA purity and quality was good for all samples and although the variance between samples was high (Table 1), faecal samples generated greater DNA yields compared to saliva and urine ($P = 0.0432$).

Phylotype classification and removal of background contamination

Bioinformatic analysis yielded 292,565 sequences that were classified in 576 unique phylotypes. The genera *Cellulosimicrobium* and *Escherichia_Shigella* both showed a strong

positive correlation in their relative abundance between controls and samples (Fig. S1, Supporting Information). *Cellulosimicrobium* represented the major phylotype (56.6%) in the DNA extraction control, indicating that its presence was due to contamination of the kit or reagents used for extraction. *Escherichia_Shigella* represented 97.6% of phylotypes in the PCR control and may be associated with contamination of the Taq polymerase during its production. Rarefaction analysis indicated sequencing effort started to saturate the diversity of the 16S rRNA gene fragment for all the body habitats, but after various levels of contamination removal, some of the rarefaction curves were not approaching a horizontal asymptote, indicating that current sequencing effort had not saturated diversity (Fig. S2, Supporting Information).

Bacterial community diversity and structure

Bacterial community diversity and structure were analyzed in parallel with the four rarefied datasets. Based on the Inverse Simpson diversity index, we found a significant difference of the bacterial community diversity among body habitats after the removal of 2 or 28 contaminant phylotypes (2cont and 28cont: both $P < 0.001$), with the highest level of alpha-diversity found in urine compared to saliva and faeces samples (Fig. 1). However, this difference was no longer significant when all contaminant phylotypes were removed (allcont: $P = 0.233$).

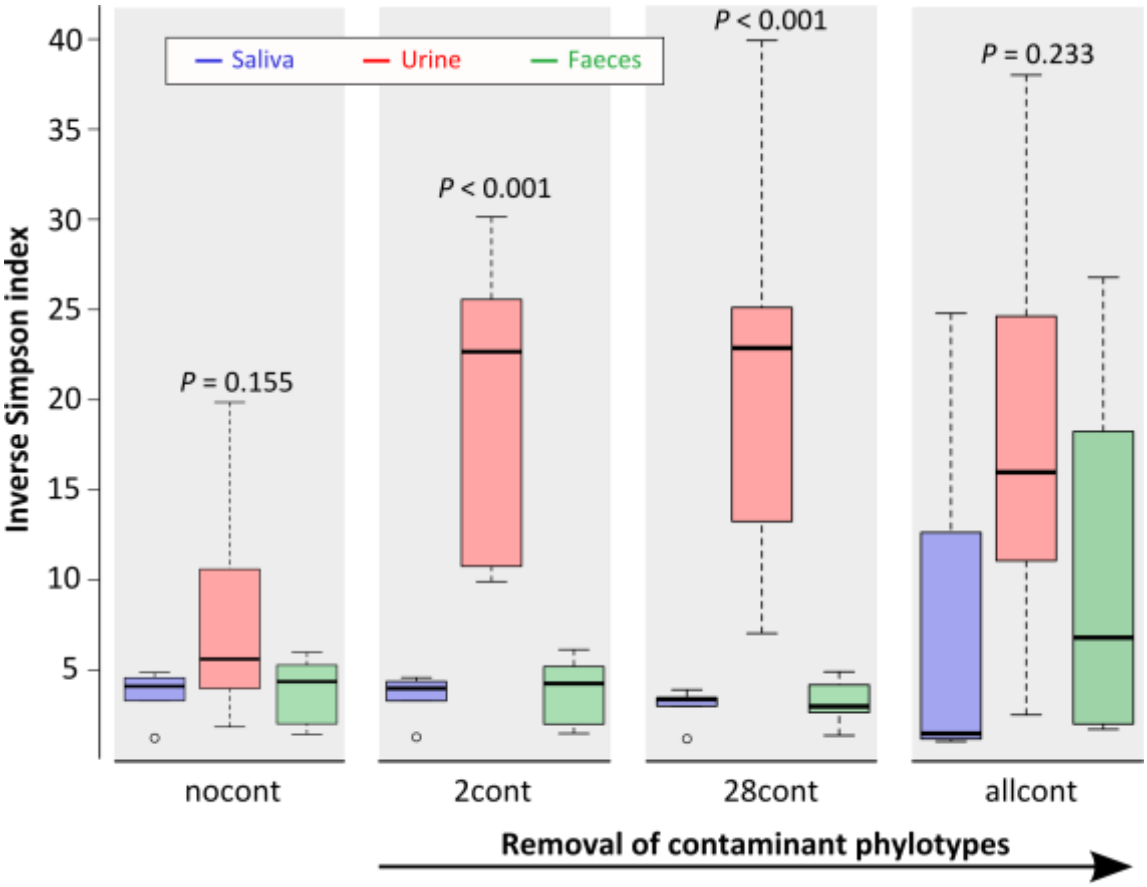

Figure 1. Comparison of bacterial diversity in saliva, urine and faecal samples from insectivorous bats. The inverse Simpson index is shown for the rarefied phylotype table of the four datasets. Median values, and the lower and upper quartiles, are shown. The P-value of the ANOVA is indicated for each dataset.

Permutational MANOVA showed that the bacterial community composition was significantly different among body habitats (nocont, 2cont and 28cont: all $P = 0.001$); however this difference was no longer significant when all contaminant phylotypes were removed (allcont: $P = 0.101$). For all datasets, NMDS plots showed no or little overlap in microbial community structure across all three body habitats (Fig. 2).

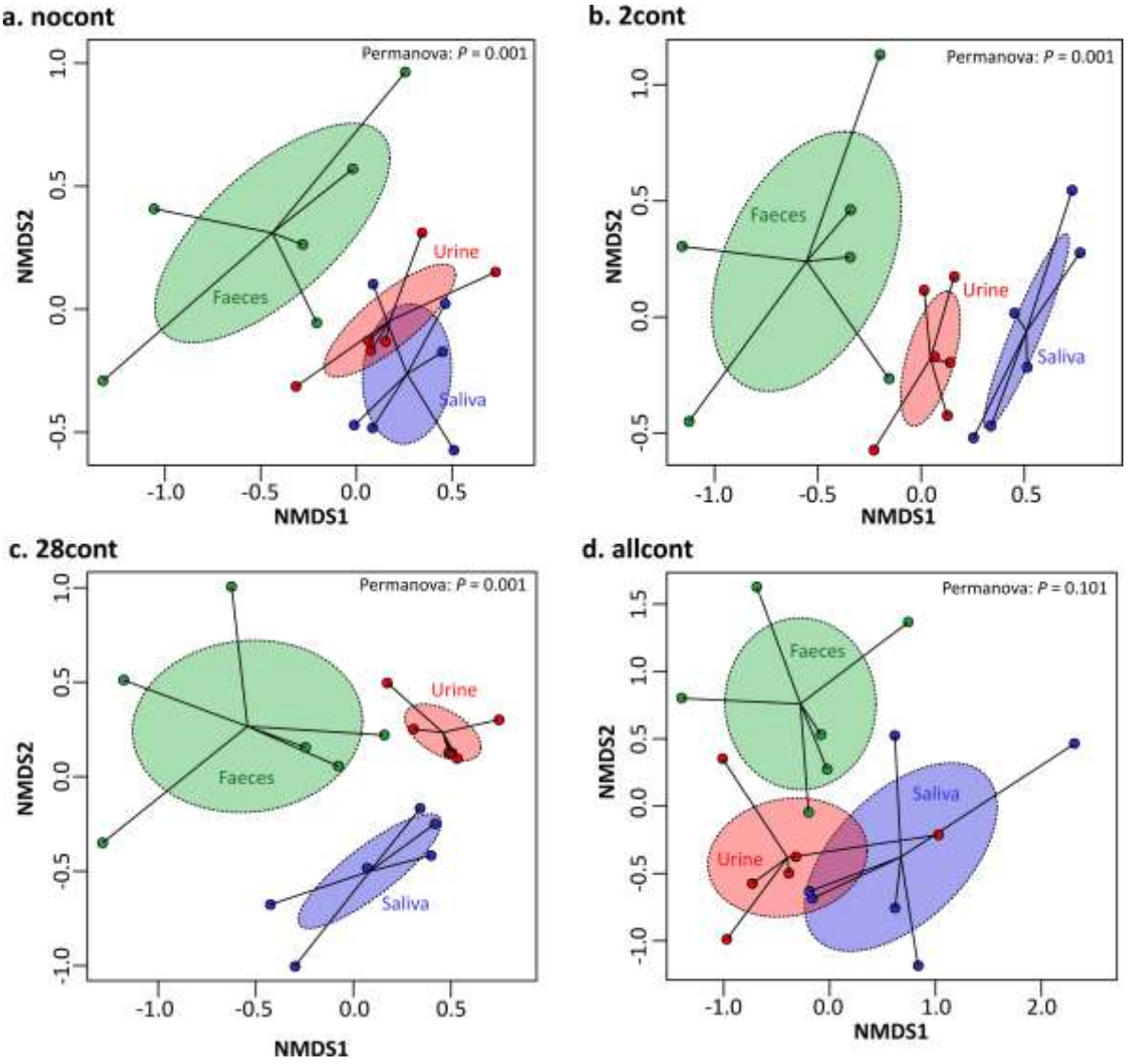

Figure 2. Structure of bacterial communities in insectivorous bats in relation to the body habitat and after different levels of background contamination removal. NMDS plots were generated using Bray–Curtis distance matrices from (a) the 'nocont', (b) '2cont' (c) '28cont' and (d) 'allcont' datasets. The P-value of the PERMANOVA is indicated in the upper right corner.

Microbiota composition

At the phylum level, saliva samples were dominated by Proteobacteria (> 90% of the sequences, Fig. 3), with noticeable differences between bat species (Fig. 4a). In contrast, most abundant phyla in faecal and urine samples were Firmicutes (41.5% and 39.3% of the sequences, respectively), followed by Proteobacteria (32.1% and 32.6%) and Actinobacteria

(17.6% and 13.1%, Fig. 3) and high inter-individual variability was observed for both faeces and urine (Fig. 4b-c).

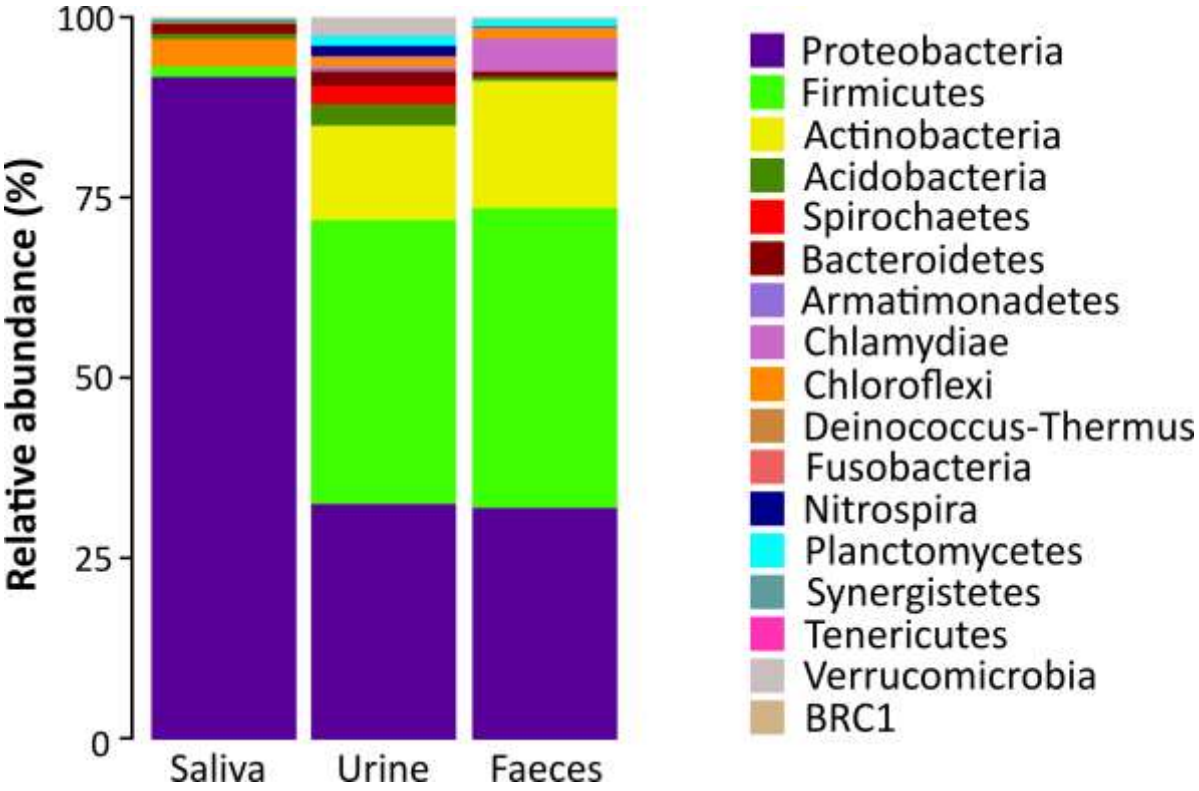

Figure 3. Relative bacterial abundance at the phylum level in saliva, urine and faeces of insectivorous bats.

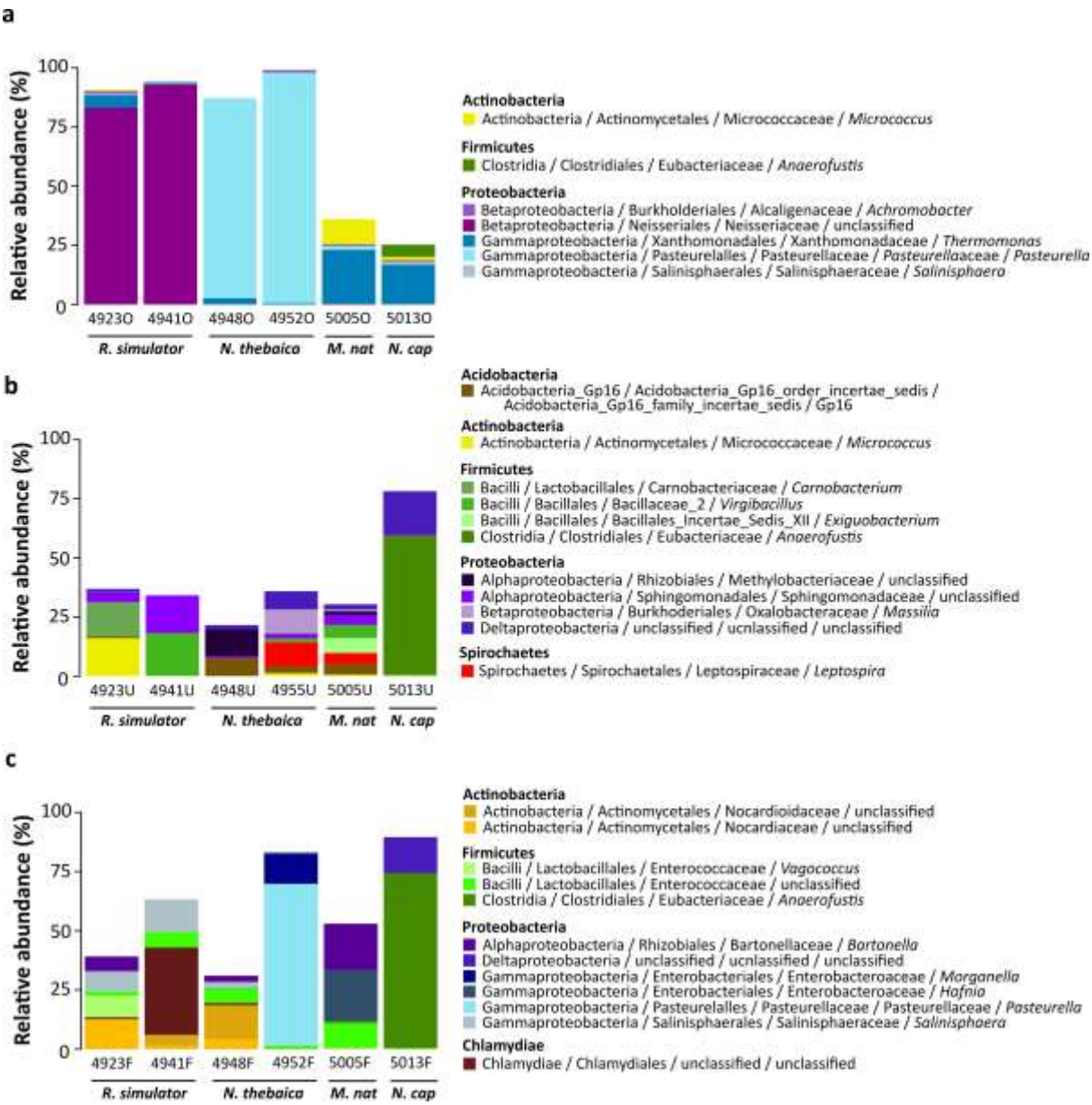

Figure 4. Most abundant phylotypes in (a) saliva, (b) urine and (c) faeces samples from different insectivorous bat species. Shown phylotypes include the two most abundant for each individual sample. Bacterial phyla are indicated in bold and the rest of the name is composed of Class / Order / Family / Genus. Bat species names are indicated below the sample ID: '*M. nat*' and '*N. cap*' refer to *M. natalensis* and *Neoromicia capensis*, respectively. Percentages represent the relative abundance of phylotypes in each individual sample.

Among all phylotypes, 46.6% were unique to a single body habitat. The highest number of specific phylotypes was observed in saliva (26.2% of all phylotypes in saliva), compared to faeces (11.6%) and urine (8.8%). The most abundant phylotype in the saliva-

specific microbiota was the Betaproteobacteria *Schlegelella*, and was found in 100% of the saliva samples. The faecal-specific microbiota included the Enterobacteria *Hafnia* (found in the *M. natalensis* sample: UP5005) and *Nitrococcus* (found in 50% of the faecal samples). Among the specific-urine phylotypes shared by at least two bat individuals, we identified the spirochaete *Leptospira* (2.4% of all phylotypes in urine), the Proteobacteria *Phyllobacterium*, *Bauldia* and *Microvirgula*, as well as the Actinobacteria *Euzebya* (all < 1%).

Most well-known zoonotic bacteria for which bats are reservoirs were detected, notably *Leptospira* and the arthropod-borne bacteria *Bartonella*, *Rickettsia* and *Coxiella*. *Leptospira* was excreted in urine by two bat individuals: *M. natalensis* (UP5005) and *N. thebaica* (UP4955), and was among the three most abundant phylotypes of the urinary microbiota for these bats. *Rickettsia* was only observed in faeces of two different bat species: *R. simulator* and *N. capensis*, and *Coxiella* in the faeces of one species: *N. thebaica*. In contrast, *Bartonella* was present in all three body habitats, but mainly in faeces (67% of the samples) compared to urine (33%) and saliva (17%). *Bartonella* was the second most abundant phylotype in the faeces (19% of faecal phylotypes) and was also excreted in urine and saliva of one bat in particular (*M. natalensis* – UP5005). Finally, we detected a number of genera that include opportunistic pathogens in humans under certain conditions, such as *Pasteurella*, *Haemophilus*, *Aeromonas*, *Neisseria*, *Nocardia*, *Burkholderia*, *Helicobacter* and *Treponema*. Only *Bartonella*, *Burkholderia* and *Helicobacter* were found in the all three body habitats.

DISCUSSION

We studied bacterial communities excreted by insectivorous bats, focusing on multiple body habitats: saliva, urine and faeces. Our results argue that bacterial communities can be inferred from low-biomass individual samples, but that negative controls should be systematically

included at different experimental stages. Indeed, we showed that background contamination occurred at least during DNA extraction and library preparation. Our result support moreover that a stringent removal of contaminant phylotypes (100% of contaminant phylotypes removed) has a major effect on the alpha- and beta-diversity (Salter *et al.* 2014; Jervis-Bardy *et al.* 2015). Indeed, by removing all phylotypes identified in controls, we might have also removed true phylotypes that were present in samples and thus have loss information. This may explain why differences in bacterial community composition and diversity were no longer significant when all contaminants were removed. Our results thus underline the importance of using negative controls and applying accurate post-processing of sequences for the identification and filtering of potential contaminant bacteria present in individual bat samples.

We found that body habitat was a major determinant of bacterial community composition within the bat community studied, with almost half of total phylotypes specific to a single body habitat. This is consistent with human and other animal microbiome studies (Costello *et al.* 2009; Alfano *et al.* 2015; Cheng *et al.* 2015; Chiarello *et al.* 2015) and suggests niche specialization within individuals. The composition of the saliva bacterial community in these bats shared several common features with other animal species, given the dominance of Proteobacteria (Li *et al.* 2013; Sturgeon *et al.* 2014; Alfano *et al.* 2015) and in particular the presence of truly endogenous (or highly host-associated) genera from the Pasteurellaceae and Neisseriaceae families. Potential zoonotic bacteria such as *Bartonella* were also detected in saliva, but only in conjunction with a high abundance in faeces and presence in urine. The concomitant detection of *Bartonella* in multiple body habitats of the same individual might correspond to an acute infection. Although transmission of *Bartonella* in bats is usually associated with arthropod vectors and faecal droppings (Billeter *et al.* 2008; Veikkolainen *et al.* 2014; Wilkinson *et al.* 2016), these bacteria have also been reported in

dog and cat saliva (Duncan, Maggi and Breitschwerdt 2007; Oskouizadeh, Zahraei-Salehi and Aledavood 2010), suggesting *Bartonella* may also be transmitted within bat populations through behaviours involving saliva transmission, such as biting and grooming.

The faecal microbiota of bats was distinct from bacterial communities colonizing their oral cavity, and was characterized by high inter-individual variability. It was dominated by Firmicutes, which are a common component of the animal gastrointestinal tract (Ley *et al.* 2008). Proteobacteria, which are a most predominant phyla retrieved from intestine samples in different bat species (Banskar, Mourya and Shouche 2014; Carrillo-Araujo *et al.* 2015), were also well represented in our faecal samples, but also with a high variability among individuals. We were able to detect *Coxiella* DNA in one faecal sample from *N. thebaica*. The only reports of *Coxiella* in bats are recent, and describe positive samples for *Coxiella burnetii*, the agent of Q fever, in urine of flying foxes (Tozer *et al.* 2014) and in a bat tick *(Ixodes verspertilionis*) (Leulmi *et al.* 2016). Our result thus supports that bats may be a source of *Coxiella* transmission (the species still remains to be determined), and warrants further research aimed at determining the exact mode of transmission (e.g. tick vectors as usually seen for other mammal species, or faecal pellets).

It is only recently that the bacterial microbiota in urine has been studied, and only in humans (Whiteside *et al.* 2015). Thus, to our knowledge, our study is the first to investigate bacterial communities in urine in wild animals. Previous metagenomic work has identified diverse viruses in bat urine, and especially high abundance of adenoviruses (Baker *et al.* 2013). Our result show that urine also harbored diverse bacterial communities, and suggest that this diversity is even higher than in saliva and faeces, which altogether support the fact that urine is not sterile and has its own microbiota (Siddiqui *et al.* 2011; Pearce *et al.* 2014). Our study revealed common features with the human urinary microbiome, such as the predominance of Firmicutes, Proteobacteria and Actinobacteria (Pearce *et al.* 2014). As in

faecal samples, urinary microbiota was also mostly characterized by high variability among bat individuals, suggesting that host-associated factors may be important in driving bacterial communities in bat urine and faeces. Additional sampling, including higher number of samples per bat species, will be necessary to verify this hypothesis. Interestingly, one of the urine-specific phylotypes was the pathogenic spirochete *Leptospira*, which is known to be specifically transmitted by the urine of its reservoir hosts (Bharti *et al.* 2003). Bats have been found to be infected with this bacteria, although their role in human leptospirosis remains unclear (Dietrich *et al.* 2015). Our study is the first detecting *Leptospira* in bats using metagenomic tools, but more importantly, based on the relative abundance of *Leptospira* compared to other phylotypes found in urine, our metagenomic data supports acute infection and/or high shedding of this bacteria in bat urine.

In conclusion, by investigating in parallel the main excretion routes (saliva, faeces and urine), our results provide a global view of the bacterial communities excreted within a bat community. We emphasized that, in addition to the traditional use of gut-associated samples such as faeces, both urine and saliva are also of interest because of their diverse microbiota and the potential transmission of pathogenic bacteria. Given the role of bats as potential carriers of zoonotic infectious agents, analyzing the interactions between the microbiota and infection dynamics in bats becomes an urgent challenge.

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Conflict of interest. None declared.

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