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Fecal bacterial communities in insectivorous bats from the Netherlands and their role as a possible vector for foodborne diseases

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Bats are commonly regarded as vectors for viruses, but little is known about bacterial communities in bats and the possible role of bats in the transmission cycle of foodborne diseases. To gain more insight, microbial communities in fecal samples from 37 insectivorous bats of different species from the Netherlands were investigated by polymerase chain reaction and denaturant gradient gel electrophoresis (PCR-DGGE). Subsequently, 10 samples from the following bat species: common pipistrelle (*Pipistrellus pipistrellus*; $n = 3$), Daubenton's bat (*Myotis daubentonii*; $n = 3$), serotine bat (*Eptesicus serotinus*; $n = 1$), whiskered bat (*Myotis mystacinus*; $n = 1$), Geoffroy's bat (*Myotis emarginatus*; $n = 1$) and Natterer's bat (*Myotis nattereri*; $n = 1$) were selected and used in bacterial 16S rDNA cloning and sequencing. The fecal microbiota in bats was found to be diverse with predominant bacterial genera *Carnobacterium*, *Serratia*, *Pseudomonas*, *Enterococcus* and *Yersinia*. The presence of opportunistic pathogens *Citrobacter freundii*, *Escherichia coli, Enterococcus faecalis*, *Serratia fonticola* and *Rahnella aquatilis* was also recorded. Based on cloning results, we found no proof that bats in the Netherlands are a major vector for the transmission of bacterial zoonotic diseases, although previous findings in literature reported isolation of foodborne pathogens from bats.

Key words: bats, microbial diversity, bacteria, DGGE

INTRODUCTION

Bats (Chiroptera) are widely distributed animals over the world except polar regions. Among mammals, bats are unique in their capacity to fly and some species cover long distances during their seasonal migration. Their ability to inhabit various ecological niches and environments, in combination with the habit of many bat species to form large colonies, make bats an interesting and successful clade. However, the same characteristics inherently make them highly potent vectors for diseases. When focusing on the transmission of diseases, so far bats are mostly known for their transmission of viruses like rabies, Hendra, SARS, Ebola, Marburg and Nipah (Calisher *et al*., 2006; Field *et al*., 2011; Schountz, 2014). The fact however, that they could be a reservoir host for (foodborne) pathogens cannot be neglected since they have been associated with *Salmonella*, *Shigella*, *Yersinia* and *Campylobacter* among many others (Rozalska *et al*., 1998; Mühldorfer *et al*., 2010; Reyes *et al*., 2011; Mühldorfer, 2013; Hazeleger *et al*., 2018; Vengust *et al*., 2018). Bats have the ability to fly and, as birds, might have the ability to contaminate food products (crops) or infect livestock. Wild and pet birds have been shown to carry *Campylobacter* sp., thereby facilitating the transmissions of *Campylobacter* to live stock and farm areas, and also humans (Hald *et al*., 2015). Also for *Salmonella* sp. this has been shown (Boseret *et al*., 2013), and transmission to humans was established for *Salmonella* (Smith *et al*., 2005).

Intestinal microbiota is important for digestion and breakdown of ingested food compounds and can therefore play a crucial role in health and illness of animals (Guarner and Malagelada, 2003; Skrodenytė-Arbačiauskienė *et al*., 2008). Knowledge on the microbiota of bats is also of importance since these bacteria can be dispersed into the environment, where they can contribute to possible infection of humans via e.g., interaction with wild animals, intake of raw food, or drinking of contaminated water (Bengtsson-Palme *et al*., 2018). Several studies exist on the intestinal microbiota in bats (Mühldorfer, 2013), but not much is known on the presence of bacterial pathogens in live bats while

this information could contribute to unravelling a possible role of bats in the transmission of bacterial zoonotic diseases. Many different factors might influence the microbiota. Host family, geographical origin and environment of the host, the host's diet, the developmental stage of the host and stress factors are known to affect the composition of the microbiota (Phillips *et al*., 2012; Ingala *et al*., 2018). Other factors may be roost size and roosting behaviour or certain foraging preferences (Catto *et al*., 1996; Zahn *et al*., 2010). Studies on the effect of diet on gut microbiota indicate that it influences the host's intestinal communities in different ways (Carrillo-Araujo *et al*., 2015). The current study was conducted to gain insight in the fecal bat microbiota from live animals, using a Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)-molecular cloning approach, allowing random identification of bacterial species present in the bat droppings.

Materials and Methods

Bat Fecal Samples

Bat droppings of 37 bats (Appendix) were collected in limestone mines, in close vicinity to villages, in the Southern part (province of Limburg) of the Netherlands during the autumn of 2008. Bat captures were carried out under Flora and Fauna Act license FF/75A/2003/150 and with permission of all site owners (Staatsbosbeheer; Limburgs Landschap) during a study after swarming behaviour (van Schaik *et al*., 2015). All procedures were in compliance with Dutch legislation on animal handling and welfare and wildlife conservation. All bats were released within one hour of the point of capture. No bats were caught specifically for the current study and droppings were only collected if deposited naturally by the bat (either from the cotton bag or directly from the animals if defecation occurred during handling of the bats). Droppings were stored in -20°C until further use (2010).

DNA Extraction and PCR-DGGE

Total genomic DNA was extracted from 37 bat fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol with elution in 70 µl of elution buffer. Microbial genomic DNA isolated from fecal samples of bats was used directly to amplify the V6-V8 region of the bacterial 16S rRNA gene, using the set of primers described by Nübel *et al*. (1996). The PCR mixtures (50 µl) included 2U of Taq DNA polymerase (Native; Fermentas), 5 µl of 10xTaq buffer, 1.5 mM $MgCl_2$ (Fermentas), 400 µM deoxynucleoside triphosphate mix (Fermentas), 0.4 µM each primer, 0.1 mg/ml BSA, 1 µl template DNA (10 to 50 ng/µl), and sterile Milli-Q water. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) using the following conditions: 7 min at 95°C; 40 cycles, each consisting of 1 min at 95°C, 30 s at 56°C, 1 min at 72°C; and a final extension of 10 min at 72°C. DGGE was performed using the DCode System (Bio-Rad Laboratories, Hercules, CA, USA) according to the initially described method by Muyzer *et al*. (1993) with the changes incorporated by Martín *et al*. (2007). In our study a denaturing gradient of 30–60% proved to be optimal. Gels were silver stained according to Sanguinetti *et al*. (1994) with minor modifications (~10 µg sodium borohydride was added to the developer solution. In addition, gels were, after staining and fixation, preserved in a demi water solution containing 25% v/v ethanol (96% v/v), 10% v/v glycerol and dried overnight at 60°C). Gel images were digitized on a GS800 calibrated densitometer (Bio-Rad) and BioNumerics software v. 4.0 (Applied Maths) was used to align the gels and perform clustering (Dice correlation coefficient) and band matching analysis.

DNA Clone Library Construction and Sequencing

Ten fecal samples were selected for clone libraries construction. This selection was based on the incorporation of different bat species and the diversity of these species as shown on the DGGE gels. To cover both intra- and interspecies differences the following samples were selected: *P. pipistrellus* (three samples, B2, B7, B12), *M. daubentonii* (three samples, B8, B18, B33) and one sample each from *E. serotinus* (B9), *M. mystacinus* (B15), *M. emarginatus* (B27) and *M. nattereri* (B35). Amplified 16S rRNA gene, using 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') universal primers, was purified and cloned in *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) as described by Lima *et al*. (2012). PCR mixtures were set up as described for the PCR-DGGE (only with an increased primer concentration of 1µM each and 0.2 µM dNTP) and amplified under the following conditions: 7 min at 95°C; 30 cycles, each consisting of 1 min at 95°C, 30 s at 56°C, and 1 min at 72°C; and a final extension of 30 min at 72°C and cooling down to 4°C. The expected size (approximately 1,500 bp) was checked in recombinant colonies using T7 and Sp6 pGem-T-specific primers (Lima *et al*., 2012).

For each of ten fecal bat samples, randomly selected clones containing the plasmid with insert were sent for sequencing to GATC Biotech (Germany) with the bacterial universal primer 16S-27f (5- AGAGTTTGATCMTGGCTCAG-3). Obtained sequences were trimmed using Chromas v. 2.3.1 (Technelysium Pty Ltd.). DNA baser software (Heracle BioSoft, Piteşti, Romania) was used to create multifasta formats which were analyzed for the presence of chimeric sequences using Bellerophon (Huber *et al*., 2004). Chimeric sequences were discarded before further analysis using BLAST (NCBI).

Analysis of Clones on DGGE Fingerprints

Clones with the sequences of interest were analyzed alongside the original sample by DGGE with the V6-V8 primers as mentioned above. BioNumerics software v. 4.0 (Applied Maths) was used to align the gels and for data interpretation.

Shannon diversity index calculation

Shannon (diversity) index (Haegeman *et al*., 2013) was calculated for the ten cloned samples using the following equation:

$$-\sum (A_i \times Ln(A_i)), \text{ where}$$

$$A_i = \frac{\text{species abundance}}{\sum \text{total species abundance}}$$

Results

The composition of the fecal microbiota differed between bat samples (Fig. 1), ranging from only a few bands up to 15 bands per sample on the DGGE gel. When comparing all 37 samples, some bands were present in most of them, while others were only present in one sample, indicating a variability between the samples. Most fecal samples used in this study (B6, B8, B11, B16–B19, B21, B23–B26, B33) originated from *M. daubentonii*, which gave the opportunity to take a closer look at intraspecies similarities or differences. Clustering analysis of *M. daubentonii* samples with BioNumerics and Dice correlation showed some bands that were common in the majority of samples, although all samples differed in their number of bands and band intensities (Fig. 2).

Based on Fig 1, 10 samples (samples codes underlined in Fig 1) were selected and subjected to molecular cloning. This selection encompassed six different species of bats (*P. pipistrellus*, *M. daubentonii*, *E. serotinus*, *M. mystacinus*, *M. emarginatus* and *M. nattereri*) and for two species (*P. pipistrellus*

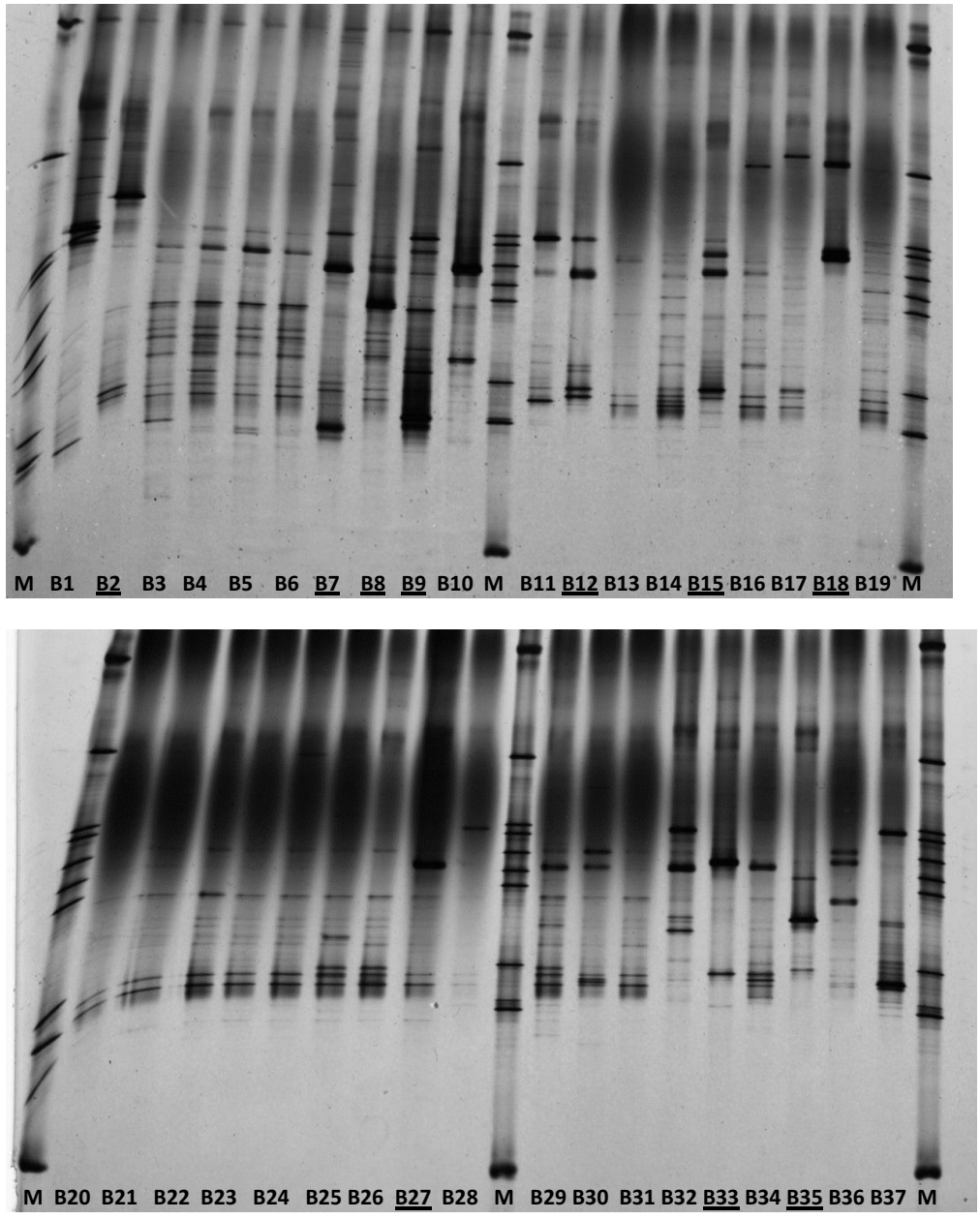

Fig. 1. DGGE profiles of bacterial communities of bat fecal samples (B1–B37). Sample codes correspond to the samples listed in the Appendix. M represent the Marker used as an external gel migration control. Underlined samples were subjected to cloning

and *M. daubentonii*) three samples each were taken along. Random molecular cloning and sequencing of the ten selected samples revealed in total 30 bacterial genera with *Serratia*, *Carnobacterium* and *Enterococcus* being the predominant ones; *Serratia* being present in seven out of ten samples and *Carnobacterium* and *Enterococcus* present in six out of 10 samples (Table 1).

At species level, *Carnobacterium maltaromaticum* was by far the most abundant species, being present in half of the samples (B2, B7, B12, B15 and B35) subjected to cloning. In four out of these five samples *C. maltaromaticum* represented more than 50% of the clones. *Pseudomonas* and *Yersinia* were present in respectively three (B7, B8 and B12) and two (B12 and B18) out of ten samples, both being predominant clones within these samples, except for B12. Although no obligate food pathogens were detected in the current study, interesting observations were the presences of opportunistic pathogens *Citrobacter freundii*, *E. coli*, *Enterococcus faecalis*, *Serratia fonticola* and *Rahnella aquatilis*. Aligning the DGGE bands of the clones with the bands of the related samples revealed that, for some samples, the majority of the clones was found back in the original sample's DGGE profile (between 50 and 100% for B7, B8, B12, B15, B27, B33 and B35) while for B2, B9 and B18 respectively 40, 20 and 25% could be recovered (data not shown). To get an overall view on the diversity of the microbiota present in the cloned samples the Shannon index was used. The Shannon index is an index that is commonly applied to characterize species diversity in a community. The Shannon index (Fig. 3) varied from 0.57 to 1.84, indicating that there is diversity (both inter- and intraspecific) between the cloned samples. The higher the Shannon index, the more diverse (based on number of different species and the abundance of each species present) the microbial community within each sample.

Discussion

In this study the focus was on unravelling the microbial community and its diversity in fecal samples of live bats and to investigate whether foodborne pathogens in particular were present. Variety, both inter- and intraspecific, was shown in both DGGE patterns (Fig. 1) and by Shannon index (Fig. 3) and clustering analysis gave a good impression of the degree of variation possible within one species (Fig. 2). The diversity found in DGGE profiles could not be completely recovered through cloning and sequencing. On the one hand, this could be due to the random cloning (instead of DGGE followed by gel band excision and sequencing), resulting in a selective overview of the species present. On the other hand, DGGE has been proven to be able to detect members of the bacterial community, which make up at least one percent of the total community (Muyzer and Smalla, 1998). Bacteria, representing less than one percent might therefore not be detected in the gel, but could however be picked up with random cloning. Another factor could be a reduced recovery from the prolongued frozen storage of the fecal samples. Although Metzler-

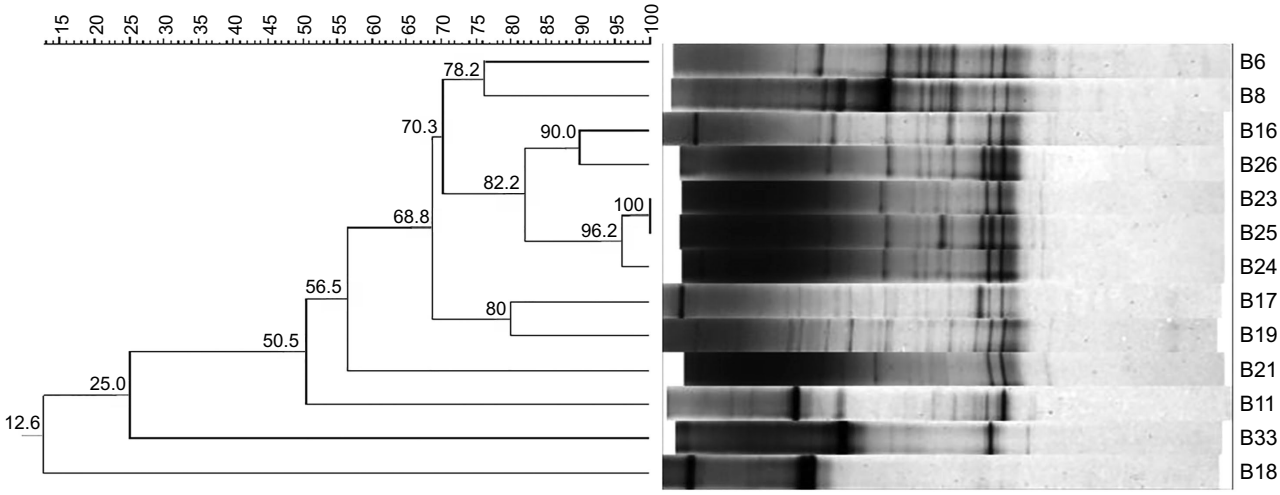

Fig. 2. Cluster analysis of *M. daubentonii* samples. The dendrogram includes similarity values based on Dice correlation coefficient

TABLE 1. Overview of the bacterial diversity in cloned samples. B2, B7, B12, *P. pipistrellus* (Ppip); B8, B18, B33, *M. daubentonii* (Mdau); B9, *E. serotinus* (Eser); B15, *M. mystacinus* (Mmys); B27, *M. emarginatus* (Mema); B35, *M. natterri* (Mnat). Numbers indicate the relative percentage of a species' presence within one sample based on sequencing results

% similarity/Bacteria	Bat sample									
	Ppip			Mdau			Eser	Mmys	Mema	Mnat
	B2	B7	B12	B8	B18	B33	B9	B15	B27	B35
99/Acetobacteraceae			4.0	16.2						
99/*Acinetobacter* sp.							75.0			
97/*Asaia lannensis*	4.1									
97/*Asaia* sp.	5.4									
99/*Carnobacterium divergens*						10.0				
100/*C. maltaromaticum*	86.5	16.9	70.7					53.2		52.8
99/*Cedecea neteri*						20.0				
98/*Citrobacter freundii*							20.8			
99/Enterobacteriaceae		15.7								
100/*Enterococcus faecalis*								6.3		
99/*E. rotai*						30.0				
99/*Enterococcus* sp.	2.7	1.2	4.0					19.0		15.7
99/*E. termitis*			2.7							
100/*Erwinia acidophila*						10.0				
99/*E. billingiae*		1.2								
100/*E. persicina*		3.6								
99/*Erwinia* sp.			6.7							
99/*Escherichia coli*							4.2			
100/*Kaistia* sp.									1.1	
100/*Lactococcus lactis* ssp. *lactis*	1.4							8.9		
99/*Leucobacter* sp.										2.2
99/*Leuconostoc citreum*		1.2								
99/*Methylobacterium*									1.1	
99/*Moellerella wiconensis*										1.1
99/*Ochrobactrum pseudogrigrionense*			1.3							
98/*Pectobacterium carotovorum*					1.1					
100/*Providencia rettgeri*								2.5		
99/*Pseudomonas fragi*				20.3						
100/*P. orientalis*		3.6								
99/*P. psychrophila*				35.1						
99/*Pseudomonas* sp.		24.1	1.3	23.0						
99/*Rahnella aquatilis*		2.4								
99/*Rahnella* sp.			2.7							
99/*Rhodobacter* sp.			1.3							
99/*Rickettsia* sp.			2.7							
99/*Schineria* sp.										6.7
100/*Serratia fonticola*		1.2				30.0		2.5		
100/*S. grimesii*					58.2				21.6	
99/*S. liquefaciens*									11.4	
100/*S. liquefaciens*/*proteamaculans*/ *grimesii*									38.6	
99/*Serratia* sp.		28.9		2.7				7.6	23.9	2.2
99/*Serratia*/*S. fonticola*				2.7						
100/*Staphylococcus capitis*									1.1	
100/*Streptococcus sanguinis*									1.1	
99/*Vagocuccus* sp.										19.1
99/*Yersinia intermedia*					37.4					
100/*Y. kristensenii*			1.3							
100/*Y. massiliensis*			1.3							
99/*Yersinia* sp.					3.3					

Zebeli *et al*. (2016) found that freezing storage could have an effect on sensitive microorganisms, with shorter storage time span, Lauber *et al*. (2010) found little influence of storage time and condition. Sequencing revealed that most of the clones belong to the family of the Enterobacteriaceae (ca. 37%)

and Leuconostocaceae (ca. 32%); both groups being common among the natural gut microbiota of different animals including bats (Mühldorfer 2013; Di Bella *et al*., 2014; Li *et al*., 2018).

To date, several studies exist that focussed on the microbiota of bats. There is quite some overlap between the genera reported in literature and found in this study. Genera reported include *Carnobacterium*, *Staphylococcus*, *Enterococcus*, *Pseudomonas*, *Yersinia*, *Providencia*, *Serratia*, *Vagococcus*, *Leuconostoc*, *Enterobacter*, *Lactococcus*, *Citrobacter*, *Acinetobacter*, *Pseudomonas*, and *Escherichia* (Di Bella *et al*., 2014; Veikkolainen *et al*., 2014; Vengust *et al*., 2018). Although several of the bat species investigated in these studies were also investigated here, identified genera differ partly, suggesting that the microbiota of bats can be influenced by a variety of factors, like feeding habits. Banskar *et al*. (2016) profiled the bacterial community in the intestines of both frugivorous and insectivorous bats and reported common and specific bacterial communities associated with their feeding habits. In addition, different isolation- and detection techniques could contribute to these different findings. In our study, no obligate food pathogens were detected in the bat microbiota, which is in contrast to previous studies where *Campylobacter jejuni*, *Salmonella* and *Clostridium* sp. were isolated from bat feces (Child, 1994; Hazeleger *et al*., 2018; Vengust *et al*., 2018) indicating that bats can be carriers of intestinal bacterial pathogens. Many of the bacterial species found however can be classified as opportunistic pathogens. These can cause disease in immunocompromised people, thereby being a factor to take into consideration. For example, *C. freundii*, *E. coli, S. fonticola* and *R. aquatilis* were found in our study. *Citrobacter freundii* was reported previously by Di Bella *et al*. (2014). Our single occurance of *E. coli* is in contrast to previous studies (Jarzembowski, 2002; Rozalska *et al*., 2008). *Serratia fonticola* and *R. aquatilis* (pathogenic to humans), have soil and water as primary habitat. Van Hoek *et al*. (2015) showed that both these *Serratia* and *Rahnella* species are natural carriers of ESBL (extended spectrum β-lactamases) genes that could contribute to antibiotic resistance.

Bat species included in this study exclusively feed on insects and water. Since prey depends a lot on availability there can be an overlap between different bat species, contributing to interspecific similiarities in gut microbiota (Banskar *et al*., 2016). Bacteria present in insects might play an important role assuming that the diet directly influences the microbiota and bacteria, taken up with the diet, can invade the gastrointestinal tract. It is therefore not surprising that many of the bacteria identified in the bat samples have previously been isolated from insects. Houseflies (*Musca domestica*, Diptera)) for example has been reported to carry *Streptococcus sanguinis* (Zurek *et al*., 2000), and *E. coli* O157:H7 (Alam and Zurek, 2004; Butler *et al*., 2010). *Enterococcus faecalis* and *Enterobacter* sp. were identified in the midgut of gypsy moth larvae (Lepidoptera) fed different diets. In Broderick's study, the diet was found to influence midgut microbial diversity significantly (Broderick *et al*., 2004). Members of the genera *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Rahnella*, *Serratia*, *Yersinia*, *Ochrobactrum* and *Enterococcus* were found in

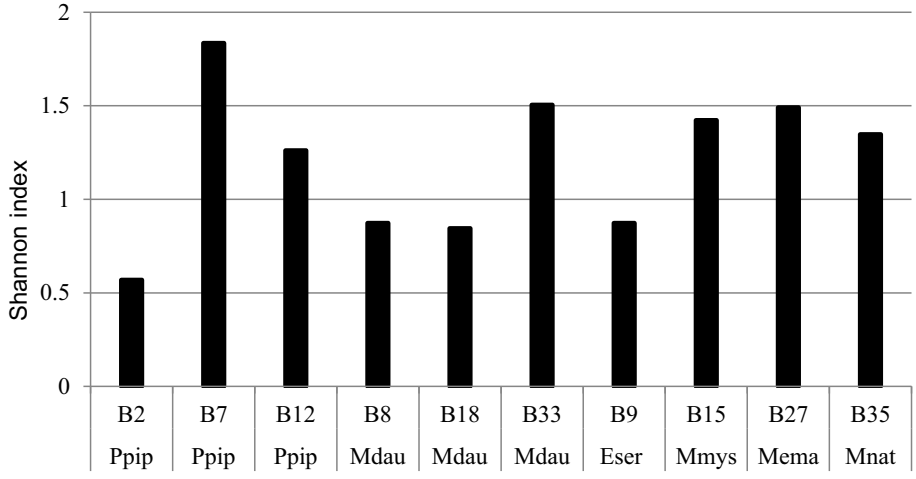

FIG. 3. Shannon (diversity) index of cloned samples: B2, B7, B12, *P. pipistrellus*; B8, B18, B33, *M. daubentonii*; B9, *E. serotinus*; B15, *M. mystacinus*; B27, *M. emarginatus*; B35, *M. nattereri*

the gut of larvae from wood- and bark-inhabiting longhorned beetles (Coleoptera) (Grünwald *et al*., 2010). *Citrobacter freundii*, *E. faecalis* and *Pseudomonas* sp. were identified in the intestinal tract of the Carabidae family (Coleoptera) (Lehman *et al*., 2009) showing a large overlap with the bacteria found in the bat droppings in our study. Also transmission of bacteria via water might be possible and is certainly of importance since pathogenic bacteria have been found in environmental water samples (Horman *et al*., 2004). Since bats drink water and can prey on aquatic insects from the water surface, this leads to ingestion of water and its associated bacteria, which could settle in the gut. In Europe, this might especially be relevant for *M. daubentonii* and pond bat (*Myotis dasycneme*) as they are highly dependent on aquatic insects (Vaughan, 1997). Ingested bacteria may be spread widely through fecal droppings, hence bats have the ability to contaminate surface water, crops and livestock feed. Therefore more insight in their fecal microbial composition will contribute to knowledge on possible transmission routes of bacteria and associated diseases.

Conclusions

The microbial composition of the bat feces investigated in this study showed diversity, both intra- and interspecific. Genera were found that have known pathogenic members but no pathogenic species were found, although some species might be opportunistic pathogens. Many of the bacterial genera found are commonly present in other animals and surface waters as well. Based on cloning results, no evidence was found that bats in the Netherlands are a vector in the transmission of bacterial zoonotic diseases, although previous findings in literature reported isolation of foodborne pathogens from bats. Since bats are so widely dispersed around the globe and come in close vicinity of human populated areas (live stock, surface water and crops) it is of importance to know what disease associated bacteria they might carry and could transfer. The current findings, which are based on data recovered from live animals, can be used to acquire an understanding of the microbiota in bats. The fact that opportunistic pathogens were found gives reason for further study, contributing to the knowledge whether bats can transmit bacterial pathogens.

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Appendix

Overview of bat samples used (B1–B37)

Code	Bat species	Geographical origin of sample
B1	*Myotis emarginatus*	Brabant
B2	*Pipistrellus pipistrellus*	South Limburg (Koelebos)
B3	*Myotis nattereri*	South Limburg (Schark)
B4	*Myotis mystacinus*	South Limburg (Koelebos)
B5	*Myotis dasycneme*	South Limburg (Koelebos)
B6	*Myotis daubentonii*	South Limburg
B7	*P. pipistrellus*	South Limburg (Schark)
B8	*M. daubentonii*	South Limburg (Koelebos)
B9	*Eptesicus serotinus*	South Limburg
B10	*E. serotinus/M. daubentonii*	South Limburg
B11	*M. daubentonii*	South Limburg
B12	*P. pipistrellus*	South Limburg (Schark)
B13	*M. mystacinus*/*M. daubentonii*	South Limburg (Koelebos)
B14	*P. pipistrellus*	South Limburg (Schark)
B15	*M. mystacinus*	South Limburg
B16	*M. daubentonii*	South Limburg
B17	*M. daubentonii*	South Limburg
B18	*M. daubentonii*	South Limburg
B19	*M. daubentonii*	South Limburg
B20	*M. dasycneme*	South Limburg (Koelebos)
B21	*M. daubentonii*	South Limburg
B22	*M. emarginatus*	South Limburg
B23	*M. daubentonii*	South Limburg
B24	*M. daubentonii*	South Limburg
B25	*M. daubentonii*	South Limburg
B26	*M. daubentonii*	South Limburg
B27	*M. emarginatus*	South Limburg
B28	no identification	South Limburg
B29	no identification	South Limburg (Schark)
B30	no identification	
B31	*E. serotinus*	South Limburg (Schark)
B32	no identification	
B33	*M. daubentonii*	South Limburg
B34	no identification	
B35	*M. nattereri*	
B36	no identification	
B37	no identification	