Biological cycle and persistence of zoonotic agents in a closely-monitored free-roaming house mouse (*Mus musculus domesticus*) population

Final report

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1. Brief description of the project.

1.a. Context and specific aims.

Recently, it was estimated that 60% of all emerging infectious diseases are zoonoses (Jones et al., 2008). Zoonoses are complex to investigate because of the involvement of several hosts and vectors linking the biological cycle of the causative agents to ecology and environmental factors (Altizer et al., 2006; Fisman, 2007; Jones et al., 2008). Additionally, data about the lifestyle of zoonotic microorganisms can be even more complicated to obtain because of the complexity of the host microbiota and the difficulty of monitoring secretive small mammal species, their ectoparasites and the surrounding environment. For these reasons, little knowledge is available about spread and persistence of some of these pathogens.

The project, co-financed by the Swiss Expert Committee for Biosafety (EFBS) and the Federal Office for the Environment (FOEN), represents a unique research situation. Sources, spread and persistence of zoonotic bacteria will can be investigated in a barn housing closely-monitored free-ranging mice that experienced an outbreak of tularemia in 2012 (Origgi et al., 2015). This mouse population is strictly monitored for behavioral studies and population genetics purposes. The project is separated in two parts: 1) Zoonotic diseases in rodents (financed by the EFBS) and 2) ectoparasites (fleas) of mice as source of zoonotic diseases (financed by the FOEN).

1.b. Introduction.

A large number of emerging diseases, like tularemia, bartonellosis, borreliosis, leptospirosis or coxiellosis, are emerging zoonoses and their causative agents display a complex biological cycle related to ecological and environmental parameters (Taylor et al., 2001; Altizer et al., 2006; Fisman, 2007; Jones et al., 2008). Some of them can alternate among the environment, vectors and several hosts (Mörner, 1992; Maurin and Raoult, 1999; Pilo and Frey, 2011). These characteristics complicate the understanding of these microorganisms at the level of their spread and persistence in the environment, possibly resulting in the sudden and unpredictable appearance of outbreaks or of sporadic cases. The lack of knowledge about the ecology of these microorganisms leads to the difficulty encountered to prevent and limit outbreaks in humans and animals.

We recently described an outbreak of tularemia among free-ranging house mice (*Mus musculus domesticus*) (Dobay et al., 2015; Origgi et al., 2015). This investigation was exceptional because a mouse population of circa 360 individuals, at the time of the epidemic, is closely-monitored for research purposes since more than 10 years. We could observe that the outbreak exhausted by itself after approximately three months without treating the animals with antibiotics. About 7% of the mouse population died during that time period (Origgi et al., 2015). Moreover, several researchers working with the mice were potentially exposed to *Francisella tularensis*, the causative bacterium of tularemia. However, no transmission to humans could be confirmed (Origgi et al., 2015). Interestingly, no ticks were observed, while mice harbored fleas and mites (Origgi et al., 2015). For these reasons, this mouse population is a unique opportunity to unravel the biological cycle of zoonotic pathogenic bacteria.

Rodents are hosts of many zoonotic agents and are a threat because of their close proximity to human households (Wobeser et al., 2009; Firth et al., 2014). However, little is known about the prevalence of zoonotic agents in these animals and their ectoparasites. Complete and accurate investigations about the carriage of zoonotic agents by free-ranging rodents, their ectoparasites and the close environment is challenging because of the lack of comprehensive information about animal populations and because of cost restrictions. Normally, only selected microbial species are targeted by specific PCRs, missing the broad spectrum of microorganisms present. Another crucial point is the paucity of information concerning shedding and persistence of those agents in the environment meaning that comprehensive data about the whole biological cycle of zoonotic agents is missing.

The recent advent of deep sequencing methods led to the possibility to acquire information about the full microbial community present in tested samples with a PCR reaction targeting variable regions of the *rrs* gene encoding for the 16S rRNA and subsequent sequencing (Miller et al., 2013). This technology is revolutionary since it theoretically leads to identify bacteria without restriction in the number of detectable species and the composition bacterial populations as a whole can be investigated.

The presented project aimed to identify bacterial communities, highlighting zoonotic agents, in free-ranging rodents (house mice) and their ectoparasites (fleas) and to follow a population of

mice that experienced a natural tularemia outbreak (Origgi et al., 2015). Two approaches were used direct PCR and microbiota analysis (PCR followed by deep sequencing).

2. Material and Methods

2.a. Sampling

The location of the field study is in Illnau in Canton Zürich. The permit for the field study with house mice was issued by the Veterinary Office of Zurich, Switzerland (Kantonales Veterinäramt Zürich, no. 056/13 and 091/16). This mouse population is strictly monitored for behavioral studies and population genetics purposes. The population is monitored with 2–3 visits per week depending on season (König and Lindholm, 2012). Checks of nest box contents are regularly made and the entire population is captured every two months. Fleas falling from their host during capture and carcasses of freshly dead mice were collected. Fleas were stored in 70% ethanol until further process. Mice were necropsied and spleen, urinary bladder, kidney and a portion of the large intestine were sampled to target organs important for bacterial shedding (urinary bladder, kidney, intestine) and an organ relevant in terms of infection (spleen) and frozen at -80°C until further process.

2.b. DNA extraction, PCR and Sanger sequencing

The total genomic DNA of individual fleas was extracted with the DNeasy Tissue Kit (Qiagen, Switzerland) according to the manufacturer's protocol. During the lysis incubation, fleas were squashed using individual sterile plastic pestles. Total genomic DNA was further used as template for PCR targeting the following genes of the host or of specific bacteria as previously described: *COII*: fleas genus identification (Zhu et al., 2015), *fopA*: *Francisella* spp. (Wicki et al., 2000), *ssrA*: *Bartonella* spp. (Diaz et al., 2012), *LipL32*: pathogenic *Leptospira* spp. (Stoddard et al., 2009), IS*1111*: *Coxiella burnetii* (Christensen et al., 2006), *fla*: *Borrelia burgdorferi* sensu lato (Schwaiger et al., 2001), *ftsZ*: *Wolbachia* spp. (Fischer et al., 2002). Sanger sequencing was carried out with the primer sets used for DNA amplification of the *COII* amplicons with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer’s instructions.

2.c. Standard curves and detection limit

In order to validate the realtime PCR assays, the targeted DNA fragments were amplified by conventional PCR and cloned in the pGEM-T easy vector (Promega). Plasmids were used to determine standard curves and limit of detection of the tested bacteria.

2.d. Microbiota analysis

To sequence the V4 regions of the bacterial 16S rRNA gene, two-step PCR libraries using the primer pair NGS_PCR1_515F (5′- 5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA-3' -3′) and NGS_PCR1_806R (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG** GGACTACHVGGGTWTCTAAT-3') were created. Subsequently the Illumina MiSeq platform and a v2 500 cycles kit were used to sequence the PCR libraries. The produced paired-end reads which passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina's real time analysis software (no further refinement or selection). The quality of the reads was checked with the software FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).The locus specific V4 adaptors were trimmed from the sequencing reads with the software cutadapt v1.9.2.dev0 (Martin, 2011). Paired-end reads were discarded if the adaptor could not be trimmed. Trimmed forward and reverse reads of the paired-end reads were merged considering a minimum overlap of 15 bases using the software USEARCH version 8.1.1861 (Edgar, 2010). Merged sequences were then quality filtered allowing a maximum of one expected error per merged read and also discarding those containing ambiguous bases. The remaining reads were clustered at a 97% similarity level using USEARCH to form operational taxonomic units (OTUs) discarding singletons and chimeras in the process (Edgar, 2013). OTUs were aligned against the reference sequences of the SILVA v128 (Quast et al., 2013) database and taxonomies were predicted considering a minimum confidence threshold of 0.6 using USEARCH. Libraries, sequencing and generation of the OTU table were performed at Microsynth AG (Balgach, Switzerland). The bacterial distribution at the phylum, class, order, family and genus level was summarized and plotted from the BIOM table to group the samples by organisms (fleas and mice) and by organs (large intestine, spleen, kidney and urinary bladder) using the script *summarize_taxa_through_plots.py* in QIIME 1.9.1 (Caporaso et al., 2010). Results were

analyzed similarly to Vidal et al. (Vidal et al., 2017). The sequencing depth was normalized by sub-sampling the dataset randomly to 1,000 reads per sample. The OTU dataset was normalized by log2-transformation. Paleontological Statistics (PAST; v3.12) software (Hammer et al., 2001) was used for alpha-diversity analyses including observed species richness, the mean number of OTUs; Shannon Diversity Index, a measure of species that combines species abundance and evenness; and Chao-1, an estimation of true species diversity. Data ordination by principal component analysis (PCA) and assessment of differences between microbial profiles of organisms and mouse organs by one-way PERMANOVA (Bray–Curtis similarity distance) was performed. The significant differences in alpha-diversity were calculated in all type of samples using the Mann–Whitney U test in PAST; v3.12. The *p* values were corrected using Bonferroni correction. $p < 0.01$ were considered statistically significant.

3. Results

3.a. Sampling

Five field samplings were performed (25.08.2015; 20.10.2015; 18.12.2015; 07.03.2016 and 26.04.2016) until October 2016. Three hundred and thirty fleas from 102 mice were collected. Additionally, 19 mouse carcasses were sampled.

3.b. DNA extraction, PCR and Sanger sequencing

A total of 144 individual fleas and 48 organ samples were subjected to DNA extraction. The PCR for the identification of the fleas was performed with all samples and the targeted fragment of the *COII* gene was successfully amplified and further sequenced. We obtained 144 highly similar sequences of 676 bp. Among them, 119 were identical while 25 harbored few single-nucleotide polymorphisms (SNPs). Briefly, five SNPs were identified and the maximal number of SNPs per sequence was 2. All sequences showed 98% identity with 93% coverage by BLAST with the genus *Ctenophthalmus*. Additionally, 4.2% (6/144) of the fleas were positive for *Bartonella* spp., 39.6% (57/144) were positive for *Wolbachia* spp. and all were negative for *Francisella* spp., pathogenic *Leptospira* spp., *C. burnetii* and *B. burgdorferi* sensu lato. The mouse organs were all negative for the above-mentioned bacteria.

3.c. Standard curves and limit of detection

The following values were measured as minimum detected copies of the targeted DNA fragment: one copy of *fopA* for *F. tularensis* per reaction at a Ct of 36.65, 10 copies of *ssrA* for *Bartonella* sp. per reaction at a of Ct 37.50, 100 copies of *lipl32* for pathogenic *Leptospira* sp. per reaction at a Ct of 30.58 and ten copies of *fla* for *B. burgdorferi* sensu lato per reaction at a Ct of 36.69. For *C. burnetii*, the detection limit was measures as one copy of IS*1111* per reaction at a Ct of 34.66. It has to be mentioned that in this last case, the PCR targets an IS element that is present in multiple copies in a single cell and thus the detection limit does not correspond to a genomic equivalent.

Concerning the results of the standard curves, the realtime PCR for the detection of *Francisella* spp. is very efficient (Slope at -3.324) and accurate (R^2 at 0.999). The realtime PCR for the detection of *Bartonella* spp. is efficient (Slope at -3.763) and accurate (R^2 at 0.991). The realtime PCR for the detection of pathogenic *Leptospira* spp. is less accurate (R^2 at 0.9422). The realtime PCR for the detection of *B. burgdorferi* sensu lato is moderately efficient (Slope at -3.991) but accurate (R^2 at 0.988). The realtime PCR for the detection of *C. burnetii* is very efficient (Slope at -3.438) and accurate (R^2 at 0.988). For reference: a Slope at -3.3 indicates 100% of PCR amplification efficiency and R^2, which indicates the correlation coefficient obtained for the standard curve, should be > 0.99.

3.d. Microbial profile analysis

All DNA extractions from fleas and organs were subjected to the *rrs* PCR using a primer pair specific for the V4 region. A fragment of the expected size of approximatively 350 bp was amplified in 137 flea samples out of 144 and in 33 organ samples out of 48. Briefly, 41.1% (7/17) of the spleens, 73.3% (11/15) of the kidneys, 100% (7/7) of the large intestines and 88.9% (8/9) of the urinary bladders were positive for bacterial DNA. Among the positive PCR products, we selected a total of 96 samples (69 from fleas, 27 from organs) that were subsequently purified and sent for microbiota analysis.

Sequencing overview

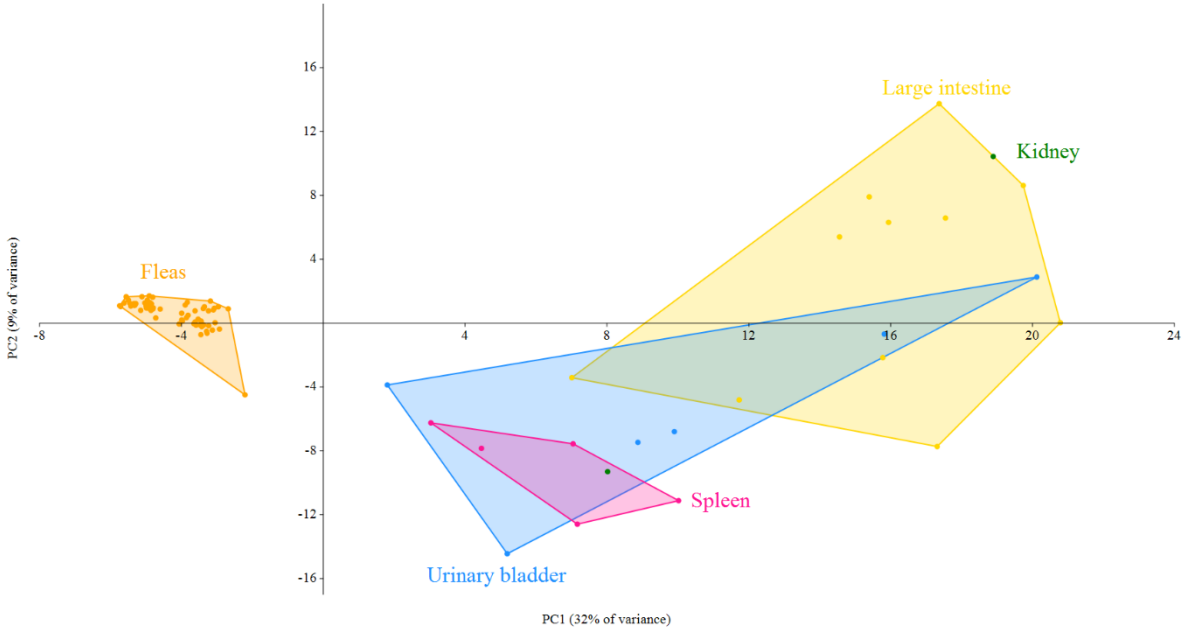
As mentioned above, a total of 96 samples (fleas, n = 69; mouse organs, n = 27), were analyzed to investigate the composition of the bacterial microbiota. Samples were used to generate deep

V4 16S rRNA gene profiles. A total of 6,987,491 high-quality reads were obtained, with an average of 72,786.365 ± 29,879.598 sequences per sample. The overall number of OTUs detected was 1,433 based on a 97% nucleotide sequence identity between reads. The number of reads per sample ranged from 259 to 134,956. After subsampling 1,000 reads/sample, 791 OTUs remained in the dataset, and two samples with fewer than 1,000 reads were excluded from further analyses.

Microbial profile analysis

PCA showed significant clustering by fleas and the different mouse organs and significant differences between large intestine and spleen clusters ($p < 0.01$, PERMANOVA) (Figure 1).

Figure 1. Two-dimensional ordination of the microbial profiles of the fleas (orange) and the mouse organs (Large intestine, yellow; Spleen, pink; Kidney, green; and Urinary bladder, blue) by principal component analysis (PCA). Significant differences; $p < 0.01$, PERMANOVA.

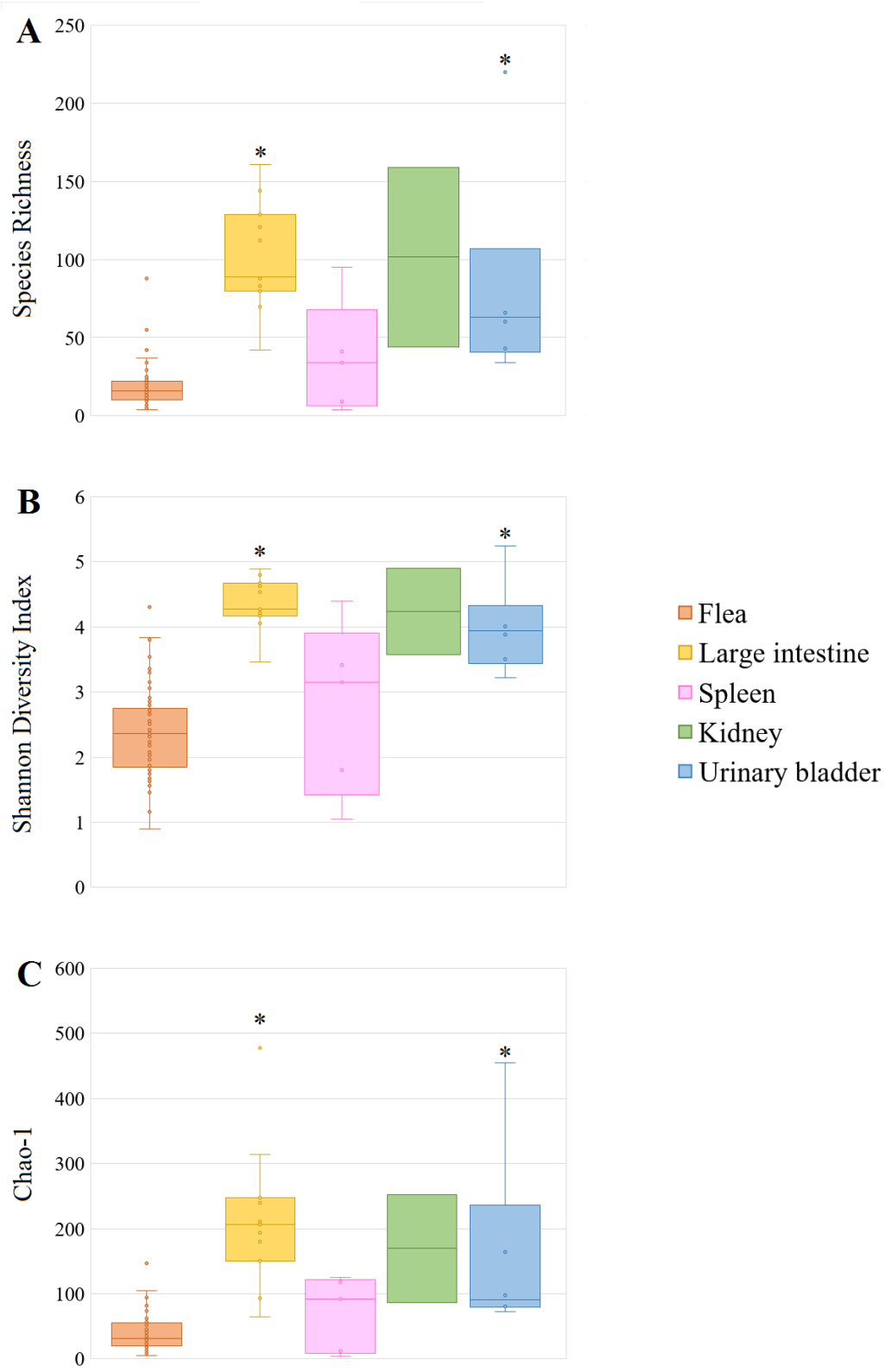


PERMANOVA

	Fleas	Large intestine	Spleen	Kidney	Urinary bladder
Fleas		p=0.001; F=30.92	p=0.001; F=13.89	p=0.003; F= 6.561	p=0.001; F=13.27
Large intestine	p=0.001; F=30.92		p=0.007; F=5.245	p= 1; F=1.02	p= 0.092; F=2.038
Spleen	p=0.001; F=13.89	p=0.007; F=5.245		p= 1; F=1.225	p= 1; F=1.447
Kidney	p=0.003; F= 6.561	p= 1; F=1.02	p= 1; F=1.225		p= 1; F=0.5185
Urinary bladder	p=0.001; F=13.27	p= 0.092; F=2.038	p= 1; F=1.447	p= 1; F=0.5185	

Microbial profiles from large intestine and urinary bladder showed significant higher values of actual species richness (number of OTUs), Shannon Diversity Index and Chao-1 than the microbial profiles from fleas ($p < 0.01$, Mann–Whitney test) (Figure 2).

Figure 2. Diversity analysis: microbial profiles of the fleas and the mouse organs (Large intestine, Spleen, Kidney and Urinary bladder). A Species richness; B Shannon Diversity Index, C Chao-1. *Significant differences; $p < 0.01$, Mann–Whitney test.



Composition of the associated bacterial communities

At the level of phylum, 17 subcategories were identified in the samples. The number of phyla found in flea were 14 and in mice were 11 (Large intestine = 8; Spleen = 10; Kidney = 8; Urinary bladder = 9), while the number of shared phyla was 8. The most predominant phylum in flea samples was *Proteobacteria* accounting for 95.33% of the bacterial communities. In all the organs *Firmicutes* was the most predominant phylum (Large intestine = 46.48%; Spleen = 57.18%; Kidney = 68%; Urinary bladder = 43.54%).

At the family level, 116 taxa were observed in the samples (Fleas = 97; Large intestine = 57; Spleen = 57; Kidney = 36; Urinary bladder = 57); however, 1.23% and 12.57% of the sequences belonging to flea and organs samples could not be identified at the family level, respectively.

At the genus level, 225 taxa were observed in the samples (Fleas = 152; Large intestine = 95; Spleen = 91; Kidney = 64; Urinary bladder = 104); however, 1.56% and 19.48% of the sequences belonging to flea and organs samples could not be identified at the genus level, respectively. The most abundant genera in fleas were endosymbionts8 (75.96%), *Wolbachia* (13.68%) and *Bartonella* (4.48%) (Figure 3). In mouse organs the genus *Bartonella* was detected in spleen (17.63%) and in urinary bladder (21.48%) (Figure 3).

Figure 3. Heat map showing the relative abundances of the most abundant genera identified in in fleas and the mouse organs (Large intestine, Spleen, Kidney and Urinary bladder) (only taxa with relative abundances of ≥ 0.3%).

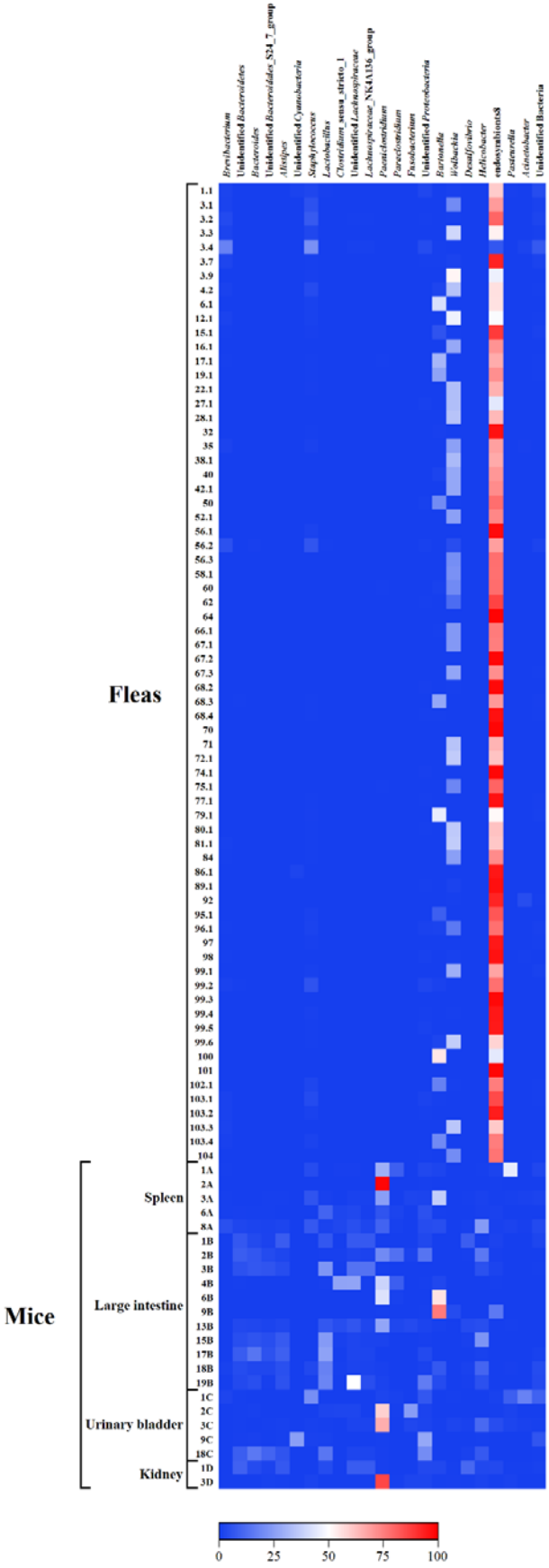
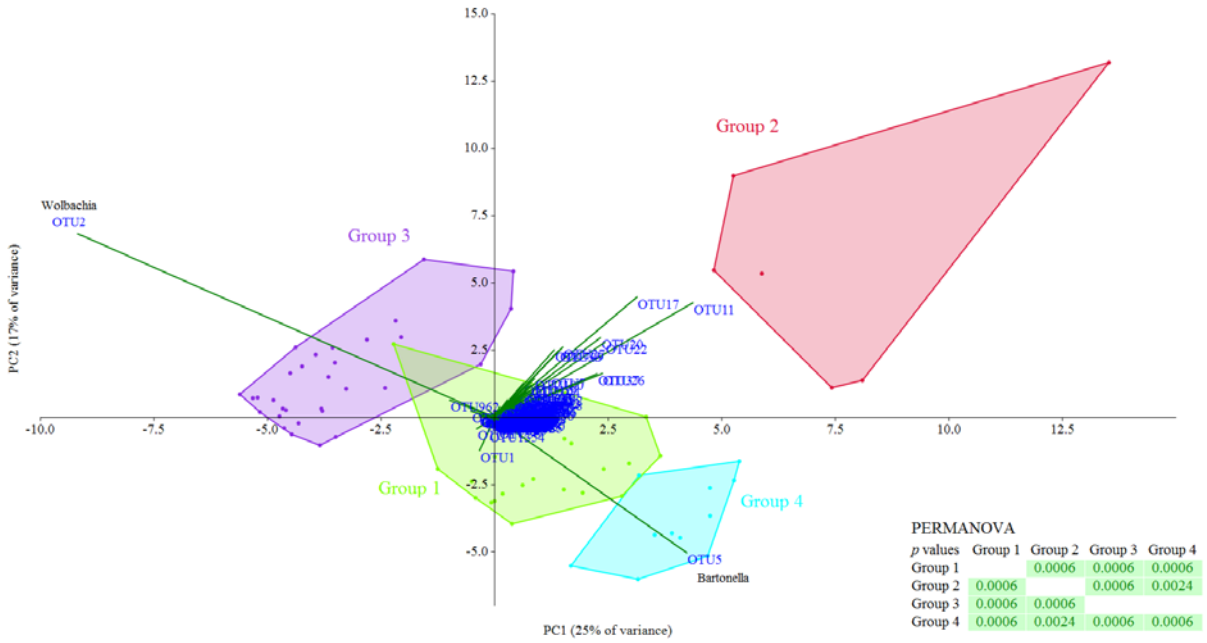


Figure 3 exposed differentiated microbial profiles in fleas regarding the genera *Bartonella*, *Wolbachia* and endosymbionts8. To confirm the differences between flea samples, we categorized them into four different groups (Group 1: <5% of the reads belonging to genera *Bartonella* and *Wolbachia* and >90% of the reads belonging to the genus endosymbionts8; Group 2: <5% of the reads belonging to genera *Bartonella* and *Wolbachia* and <90% of the reads belonging to the genus endosymbionts8; Group 3: <5% of the reads belonging to genus *Bartonella* and >5% of the reads belonging to the genus *Wolbachia* ; Group 4: >5% of the reads belonging to the genus *Bartonella* and <5% of the reads belonging to the genus *Wolbachia*). PCA showed significant clustering by the four different groups of fleas with a negative correlation between the genus *Bartonella* and the genus *Wolbachia* ($p < 0.01$, PERMANOVA) (Figure 4).

Figure 4. Two-dimensional ordination of the microbial profiles of the four different groups of fleas by principal component analysis (PCA). Significant differences; $p < 0.01$, PERMANOVA.



4. Conclusion

Understanding the ecology of the causative agents of zoonoses can be challenging because of the complex biological cycle of some of these microorganisms, especially when environmental factors influence their dynamics of spread and persistence. Wildlife and arthropod populations are particularly difficult to monitor and need to be closer investigated in order to better evaluate their significance in pathogen transmission. In the present study, we followed a closely-monitored house mouse population for 20 months and looked for the presence of zoonotic pathogens in *M. musculus* specimens and their fleas and for the potential influence of arthropod-associated endosymbionts over pathogen presence in the vector.

Currently, little is described about the prevalence and diversity of flea species among wild rodents in Switzerland. In this study, we observed that the mice population investigated was parasitized by a single flea genus *Ctenophthalmus* sp. whereas other studies investigating fleas in rodents usually find a broader diversity of parasites (Lipatova et al., 2015; Silaghi et al., 2016). *Ctenophthalmus* sp. belongs to the family *Ctenophthalmidae* that is reported to be predominantly present in the northern hemisphere and found on rodents and other small mammals like moles, pikas, and marsupials (Lewis, 1974; 1998).

Regarding the presence of zoonotic pathogens in mice and fleas, we observed no evidence for a major public health concern. Pathogenic *Leptospira* spp., *C. burnetii* and *B. burgdorferi* sensu lato were neither detected by realtime PCR nor by amplicon sequencing of the V4 region. Interestingly, *F. tularensis*, was also not detected in the samples tested by both methods. Additionally, the mortality rate in the mouse population remained normal from 2012 to 2016 which suggests that the tularemia outbreak described in 2012 (Dobay et al., 2015; Origgi et al., 2015) exhausted and that the causative agent did not persist in the population. This argues in favor of the hypothesis that some rodent species mainly develop fatal infections of tularemia and that the outbreaks are characterized by high lethality and rather low prevalence, which was the case in our population and in other studies estimating tularemia prevalence during outbreaks or in endemic regions (Zhang et al., 2006; Kaysser et al., 2008). Even if small mammals like rodents and lagomorphs represent crucial amplification hosts and potential infection sources for humans (Gyuranecz et al., 2011), the previous exposed issues lead to believe that other unidentified reservoirs might play a role in the persistence of the bacteria in the environment.

Bartonella spp. cause several emerging and re-emerging arthropod-borne diseases in human and is known to be prevalent in wild rodents and their associated fleas (Birtles, 2005; Blanco and Raoult, 2005). So far, no studies investigating the presence of *Bartonella* spp. in rodents and ectoparasites from Switzerland were published. Our results confirm the findings of other studies that detected the bacterium in *Ctenophthalmus* sp. collected on rodents in other countries (Kabeya et al., 2011; Kamani et al., 2013; Silaghi et al., 2016). The percentage of *Bartonella* spp. positive fleas by realtime PCR was 4%. Amplicon sequencing of the V4 region of the *rrs* gene revealed the genus *Bartonella* in 45/69 fleas (65.22%) accounting for the 4.48% of the total number of reads. However, by realtime PCR none of the mouse sample was positive, while by amplicon sequencing, 17.63% of the reads from spleens and 21.48% of the reads from urinary bladders belonged to the genus *Bartonella*. It is difficult to explain this discrepancy but interestingly the spleen is the organ expected to be positive for this bacterium. The urinary bladder is very small and is opened when extracted during necropsy leading to possible contamination of this organ.

Concerning *Wolbachia*, our results corroborate the relevance of the bacterium as common endosymbiont that was detected in nearly 40% of the fleas by PCR. By amplicon sequencing, *Wolbachia* was detected in 60/69 fleas (86.96%) accounting for 13.68% of the total number of reads. Interestingly, a bacterium not screened by PCR was the most abundant genus in fleas present in all the samples and accounting for the 75.96% of the total number of reads: endosymbiont8. This microorganism belongs to the *Enterobacteriaceae* and is related to a bacterium previously detected in *Irenimus aequalis*, an insect of the Family *Curculionidae* (NCBI accession number KJ494864.2).

Additionally, we could observe a significant negative correlation between genera *Bartonella* and *Wolbachia* (when one genus was present in high number of reads in a flea the other genus was absence) in the fleas belonging to this specific population of mice. *Wolbachia* has been already described as a biological control for mosquito-borne diseases (Iturbe-Ormaetxe et al., 2011; Jeffries and Walker, 2016).

In summary, *F. tularensis* was not detected in the samples tested as well as pathogenic *Leptospira* spp, *C. burnetii* and *B. burgdorferi* sensu lato. *Bartonella* spp. were detected in fleas and mice. *Wolbachia* spp. and Endosymbiont8 were the most abundant bacteria in fleas. Some discrepancies at the level of sensitivity were observed between the direct PCR assays and the

microbiota analysis. According to these results, it is not possible to determine which one of the two methods used in this study is more sensitive. This is dependent on the bacterium tested. Additionally, too many factors can influence the sensitivity of methods based on DNA amplification. The results of the microbiota analysis in fleas indicate a significant negative correlation between genera *Bartonella* and *Wolbachia* However, supplementary data including other zoonotic agents as well as various host and flea species might lead to a better global comprehension of the dynamics of flea-borne diseases.

5. References

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