CULTIVABLE BACTERIAL MICROBIOTA FROM CHOANAE OF FREE-LIVING BIRDS CAPTURED IN SLOVENIA

KULTIVABILNA BAKTERIJSKA MIKROBIOTA IZ SAPIŠČ PROSTOŽIVEČIH PTIC, UJETIH V SLOVENIJI

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ABSTRACT

Cultivable bacterial microbiota from choanae of free-living birds captured in Slovenia

We have analysed the structure of cultivable choanal microbiota from free-living birds in relation to bird diet, its richness and the relative number of opportunistic bacteria acquired from the environment. For this purpose, we have taken choanal swabs from 25 free-living birds representing 13 different bird species captured in Slovenia. From the grown cultures, 98 bacterial colonies were isolated and their 16S rRNA genes sequenced. Most of the bacteria belonged to the phylum Actinobacteria (52 %), Proteobacteria (31 %), Firmicutes (15 %) and Bacteroidetes (4 %). Thirty-two percent of sampled birds were colonized by known human opportunists and 44 % of birds by at least one known plant pathogen. Hierarchical clustering of the analyzed microbiota grouped the birds according to their predominant diet. The richness of choanal microbiota from birds feeding mainly on insects was poorer compared to the birds feeding on diverse animal and plant material. The study has shown that the free-living birds carry an important reservoir of opportunistic human and plant pathogenic bacteria in their upper respiratory tract. To get a deeper insight into its composition, a bigger pool of birds will have to be analyzed in the future.

Keywords: birds, microbiota, choanae, pathogenic bacteria, diet

IZVLEČEK

Kultivabilna bakterijska mikrobiota iz sapišč prostoživečih ptic, ujetih v Sloveniji

Sestavo kultivabilne bakterijske mikrobiote v sapiščih prostoživečih ptic smo analizirali z vidika vpliva prehrane, bogatosti mikrobiote in prisotnosti oportunističnih bakterij. Petindvajsetim prostoživečim pticam, ki so bile ujete v Sloveniji in so pripadale 13 vrstam, smo odvzeli bris sapišča. Po nacepitvi brisov na mikrobiološka gojišča in gojenju, smo izolirali 98 bakterijskih kolonij in jim določili nukleotidno zaporedje gena za 16S rRNK. Večina izoliranih bakterij je pripadala deblu Actinobacteria (52 %), Proteobacteria (31 %), Firmicutes (15 %) in Bacteroidetes (4 %). Pri približno eni tretjini ptic (32 %) smo iz sapišča izolirali vsaj eno oportunistično bakterijsko vrsto, ki lahko povzroča okužbe pri ljudeh. Pri slabi polovici ptic (44 %) pa smo v sapišču našli vsaj eno bakterijsko vrsto, ki lahko okuži rastline. Z metodo hierarhičnega združevanja smo pokazali, da imajo ptice s podobno prehrano, podobno bakterijsko mikrobioto sapišč. Ptice, ki se prehranjujejo pretežno z žuželkami so imele manj bogato mikrobioto kot ptice, ki se prehranjujejo z bolj raznoliko živalsko in rastlinsko hrano. Raziskava je tudi pokazala, da so zgornja dihala prostoživečih ptic pomemben rezervoar oportunističnih bakterij, ki lahko okužijo ljudi in rastline. Da bi dobili globji vpogled v sestavo mikrobiote zgornjih dihal, bi v prihodnosti morali povečati število analiziranih ptic.

Ključne besede: ptice, mikrobiota, sapišče, patogene bakterije, prehrana

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1 INTRODUCTION

Free-living birds are recognized vectors for spreading pathogenic bacteria across long distances with well-known transmission of various respiratory infections to humans (Murthy et al. 2008, Pan et al. 2012, Tsiodras et al. 2008). Despite of this, our knowledge on the avian respiratory tract microbiota is very limited. While some data exist for the lower respiratory tract, almost nothing is known about the bacteria living in the upper respiratory regions.

Data on microbiota of the lower respiratory tract in domestic birds have shown that it harbors potentially pathogenic bacteria. Majority of cultured bacteria found in the lungs and trachea of birds belonged to phyla Proteobacteria, Firmicutes, Tenericutes, Actinobacteria, Bacteroidetes and Chlamydia/Verrucomicrobia (Murthy et al. 2008, Charlton et al. 1993, Byrum & Slemons 1995). Additionally, culture independent analyses detected groups of fastidious or poorly represented taxons belonging to Fusobacteria, Acidobacteria, Chloroflexi, Cyanobacteria and Deinococcus -Thermus in the lower respiratory tract of poultry. Among them were also potential pathogens (*Myroides* spp., *Collinsella aerofaciens*, *Bacteroides fragilis, Enterococcus cecorum, Kurthia zopfii, Kushneria* sp. and *Bordetella* sp.) (Shabbir et al. 2015). Even though the pathogen *Riemerella* sp. has been isolated from the upper respiratory tract of some species of domestic and free-living birds (Vancanneyt et al. 1999), a deeper insight into the structure of the upper respiratory microbiota in birds is lacking. Thus far, a very limited number of research attempted to analyze the bacterial composition of the upper respiratory tract in free-living birds, besides, they used selective media for culturing specific groups of pathogenic bacteria, and thus substantially limiting the overall view on microbial diversity of the upper respiratory tract (Lamberski et al. 2003, Stenkat et al. 2014). In such a way, Lamberski et al. (2003) analyzed two species of hawks which harbored pathogens like *Salmonella* sp. and *Pasteurella* sp.. Stenkat et al. (2014) focused more on water birds and also found potential avian and human pathogens (*Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* among others).

More knowledge about the microbiota of the upper respiratory tract of birds is necessary for better understanding the influence, positive or negative, of this microbiome on animal health and the risks of spreading potential infections between free-living birds, in the environment and subsequently to humans (Waldenstrom et al. 2003, Abulreesh et al. 2007).

2 MATERIALS AND METHODS

Bird sampling, culturing of bacteria and identification

All of the 25 healthy adult birds included in this study were captured in fine mist nets for bird ringing during fall, between September 18 and December 10, 2013 in Maribor and its surroundings (Slovenia). The birds were caught in the frame of bird ringing scheme coordinated by EURING.

Choanal swabs (pre moistened with sterile saline) were immediately taken from each bird and put in a transport medium (Amies agar gel medium transport swabs – no charcoal, Copan) until further processing. All samples were sent to the laboratory within 2 to 3 hours after sampling and inoculated on nutrient agar (NA, Sigma). Inoculated plates were incubated 4 - 7 days at 30°C. After incubation, each colony morphotype per bird was isolated and stored at -80°C until further processing. Colony morphotypes were differentiated based on form, margin and pigmentation of the colonies.

Total DNA was isolated and cleaned using a commercial kit (NucleoSpin Tissue, Macherey-Nagel). Full lengths of 16S rRNA genes were amplified with PCR. The final concentrations of the PCR reaction mix contained 0.2 mM dNTP (Thermo Scientific), 1x PCR buffer with KCl (Thermo Scientific), 2.5 mM $MgCl_2$ (Thermo Scientific), 1.0 µM of forward primer (5'-AAA TTG AAG AGT TTG ATC ATG GC-3'), 1.0 µM of reverse primer (5'-AAG GAG GTG ATC CAG CCG CA-3') and 0.025 units/µL of *Taq* polymerase (Thermo Scientific). Amplicons were obtained using the following PCR protocol: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. The PCR product was purified with a commercial kit (GeneJET PCR Purification Kit, Thermo Scientific) and sequenced (Eurofins Genomics). The obtained sequences were compared to EMBL/GenBank/DDBJ databases and identified using BLAST. The closest hits to type strains, with 98.7 or higher % similarity, were

identified at species level. In case of two or more different hits with similarity score above 98.7 %, the isolate was identified at the genus level.

Hierarchical clustering

Ward method with Euclidian distances was used for the clustering of the choanal microbiota of the investigated birds, for which the data on the presence or absence of bacterial species were included in the analysis.

Statistical analysis

To test the differences in the presence of microbial groups between different species of birds, we used Fisher's exact test, where $P < 0.05$ was considered significant. Where the differences in the frequencies of microbial groups were significant, odds ratio was calculated ($P < 0.05$).

The differences in species richness between the groups of birds were tested with Student - T test, $P < 0.05$ was considered significant.

3 RESULTS AND DISCUSSION

In this study we performed identification of bacteria isolated on a complex nutrient agar medium from birds' choanae with the aim to assess microbial diversity from this specific niche and find a possible correlation with birds' diet. For this, we have sampled 25 birds, from which 98 bacterial colonies were isolated and sequenced. The number of different bacterial species per bird ranged from 12 (Song thrush (*Turdus philomelos*) to 1 (Willow warbler (*Phylloscopus trochilus*)) (Table 1).

Table 1: Richness of choanal microbiota.
Preglednica 1: Bogatost mikrobiote sapišč.

Bird Species	Number of birds	Average number of different isolates per bird species
European robin (*Erithacus rubecula*)	4	2.8 ± 2.0[a]
Garden warbler (*Sylvia borin*)	1	4
Common reed bunting (*Emberiza schoeniclus*)	1	3
Willow warbler (*Phylloscopus trochilus*)	1	1
Dunnock (*Prunella modularis*)	4	2.5 ± 1.1[a]
Common redstart (*Phoenicurus phoenicurus*)	1	5
Common chiffchaff (*Phylloscopus collybita*)	1	3
Yellowhammer (*Emberiza citrinella*)	1	4
Eurasian blackcap (*Sylvia atricapilla*)	2	2.5 ± 1.5[a]
Song thrush (*Turdus philomelos*)	1	12
Pigeon (*Columba livia*)	4	3.3 ± 2.3[a]
Common chaffinch (*Fringilla coelebs*)	1	10
Eurasian tree sparrow (*Passer montanus*)	3	5.7 ± 1.9[a]

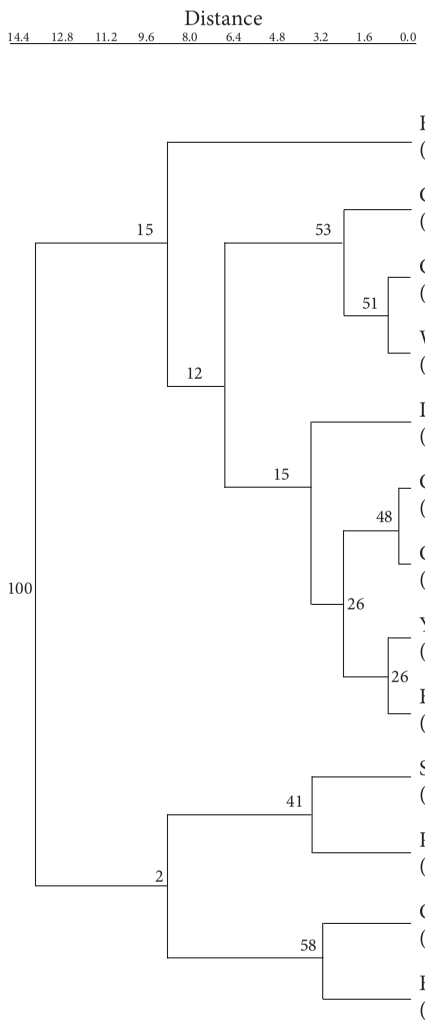
[a], standard deviation

Majority of isolates belonged to phyla Actinobacteria (52 %) and Proteobacteria (31 %). The phyla Firmicutes and Bacteroidetes were represented by only 15 % and 4 %, respectively. The isolates belonged to 22 families. The majority constituted families Microbacteriaceae (36 %), Pseudomonadaceae (11 %), Enterobacteriaceae (10 %), Micrococcaceae (7 %), Flavobacteriaceae, Xanthomonadaceae and Staphylococcaceae (all 4 %). Other families were found only sporadically. Of 13 different bird species, which have been sampled, 85 % were colonized with members of Microbacteriaceae. Pseudomonadaceae, Nocardiaceae and Enterobacteriaceae were found in 38 %, 31 % and 31 % of bird species, respectively. Xanthomonadaceae, Moraxellaceae, Staphylococcaceae were found in 23 % and Micrococcaceae in 15 % of analyzed bird species. Other isolates were found only per single bird. Although two previous studies analyzed choanal swabs from birds, there are some substantial differences in experimental approaches in comparison to our study and also in the birds analyzed. Lamberski et al. (2003) analyzed choanal swabs from captive and free-living red-tailed and Cooper's hawks, but the samples were grown on blood and MacConkey media. In this way they found the microbiota to be composed of *Bacillus* sp., *Corynebacterium* sp., *Escherichia* sp., *Salmonella* sp., *Pasteurella* sp., *Streptococcus* sp. and coagulase positive and negative staphylococci. Since we have performed the isolation on a complex nutrient medium in order to detect a wider range of environmental bacteria our results only partially overlapped. We have also isolated the genus *Bacillus* sp. and coagulase negative staphylococci, but otherwise the choanal microbiota of our birds greatly differed. This can be explained also by the fact that we have sampled different species of birds, with different diets (Cooper's hawk feeds exclusively on small and mid-sized birds and red-tailed hawk is opportunistic carnivorous feeder) and in different geographical locations (Slovenia vs. Unites States). The other group, Stenkat et al. (2014) used blood, MacConkey and Brilliant green agar to investigate pharyngeal bacterial microbiota in water rails, spotted crakes, barn swallows, mute swans, reed warblers and black cormorants, and found numerous ubiquitous bacteria belonging predominantly to Enterobacteriaceae, Pseudomonadaceae, Aeromonadaceae, Bacillaceae, Staphylococcaceae and Streptococcaceae which are frequently present in the environment and on food. We have also found members of the forementioned bacterial families, except the family Aeromonadaceae, which is more associated with water habitats and the family Streptococcaceae, which was absent in our study, possibly due to different growth media (nutrient agar as opposed to blood agar).

Out of 98 isolates from choanal swabs, 13 (13.3 %) have been known to cause opportunistic infections in humans. Species previously described as being associated with human infections were *Acinetobacter calcoaceticus* (Nonaka et al. 2014), *Cellulosimicrobium funkei* (Petkar et al. 2011), *Curtobacterium citreum* (Rivera et al. 2012), *Curtobacterium flaccumfaciens* (Francis et al. 2011), *Exiguobacterium sibiricum* (Tena et al. 2014), *Hafnia alvei* (Gunthard & Pennekamp 1996), *Microbacterium oleivorans* (Kim & Lee 2012), *Microbacterium resistens* (Panackal 2013), *Pantoea agglomerans* (Rezzonico et al. 2010), *Pseudomonas aeruginosa* (Yamazaki et al. 2012), *Serratia grimesii* (Kumar et al. 2013), *Staphylococcus epidermidis* (Vuong & Otto 2002) and *Staphylococcus gallinarum* (Tibra et al. 2010). Eight out of 25 (32 %) sampled birds carried one or more human opportunistic bacteria in their choanae. Five out of 25 (20 %) birds were colonized by one, two birds were simultaneously colonized by two opportunists and the song thrush (*Turdus philomelos*) by three different putative pathogens. Pigeons also seemed to be frequent carriers of potential pathogens. Three out of four sampled pigeons were colonized by *Staphylococcus gallinarum* (in our study found only in pigeons) and the fourth bird was colonized by *Acinetobacter calcoaceticus*. Two out of three sampled eurasian tree sparrows which, as pigeons, also live in close proximity to humans, also carried opportunists (*Curtobacterium citreum*, *Curtobacterium flaccumfaciens* and *Exiguobacterium sibiricum*) in choanal microbiota.

In addition to human opportunistic bacteria, 7 potential plant pathogens were also isolated from choanae of 11 (44 %) sampled birds. These were *Agrobacterium larrymoorei* (Bouzar & Jones 2001), *Clavibacter michiganensis* (Xu et al. 2010), *Curtobacterium flaccumfaciens* (Francis et al. 2011), *Plantoea agglomerans* (Rezzonico et al. 2010), *Pseudomonas aeruginosa* (Yamazaki et al. 2012), *Pseudomonas flavescens* (Fett, Cescutti & Wijey 1996) and *Rhodococcus fascians* (Crespi et al. 1992). Ten (40 %) birds were colonized by one plant pathogen and only one tree sparrow by two (*Agrobacterium larrymoorei* and *Curtobacterium flaccumfaciens*). The most frequently isolated plant pathogen was *Rhodococcus fascians*, which was isolated from four different birds belonging to four different species (Eurasian tree sparrow, Common chaffinch, Yellowhammer and Dunnock) with different feeding habits (seeds/insects, seeds/insects, insects and insects), respectively. This suggests that it is commonly present in bird population.

Previous investigations have shown that the composition of intestinal microbiota in birds depends on



	DIET (winter)	DIET (breeding season)	DIET (summer)	DIET (autumn)
European robin (*Erithacus rubecula*)[a]	insects / seeds / fruit	insects	insects	insects
Garden warbler (*Sylvia borin*)[b]	berries	insects	berries	berries
Common reed bunting (*Emberiza schoeniclus*)[c]	seeds	invert.[f]	seeds	seeds
Willow warbler (*Phylloscopus trochilus*)[b]	insects	insects	insects	insects / berries
Dunnock (*Prunella modularis*)[a]	insects /seeds	insects	insects	insects
Common redstart (*Phoenicurus phoenicurus*)[a]	insects	insects	insects	insects
Common chiffchaff (*Phylloscopus collybita*)[b]	insects	insects	insects	insects
Yellowhammer (*Emberiza citrinella*)[c]	seeds	invert.[f]	seeds	seeds
Eurasian blackcap (*Sylvia atricapilla*)[b]	berries	insects	berries	berries
Song thrush (*Turdus philomelos*)[a]	invert.[f]	invert.[f]	invert.[f] / fruit	invert.[f] / fruit
Pigeon (*Columba livia*)[d]	invert.[f] / seeds / artif.[g]	invert.[f] / seeds / artif.[g]	invert.[f] / seeds / artif.[g]	invert.[f] / seeds / artif.[g]
Common chaffinch (*Fringilla coelebs*)[e]	seeds	invert.	seeds	seeds
Eurasian tree sparrow (*Passer montanus*)[e]	invert.[f] / seeds / berries	invert.[f] / seeds / berries	invert.[f] / seeds / berries	invert.[f] / seeds / berries

Figure 1: Clustering of sampled bird species based on bacterial (family level) composition of choanal microbiota. Top: Euclidian distance. Numbers at each node designate bootstrap value (bootstrap number 100). a (Cramp 1988); b (Cramp et al. 1992); c (Cramp & Perrins 1994); d (Cramp 1985); e (Cramp 1994); f (invertebrates); g (artificial man-made food).

Slika 1: Hierarhično združevanje vzorčenih vrst ptic glede na sestavo bakterijske (na nivoju družin) mikrobiote v sapiščih. Zgoraj: Evklidska razdalja. Številke pri razvejitvah so bootstrap vrednosti (število ponovitev 100). a (Cramp 1988); b (Cramp et al. 1992); c (Cramp & Perrins 1994); d (Cramp 1985); e (Cramp 1994); f (nevretenčarji); g (prehrana človeškega izvora).

Table 2: Identified bacterial isolates from choanae of free-living birds. The birds were grouped based on choanal microbiota composition with hierarchical clustering on group 1 (predominantly insectivorous birds) and group 2 (birds with mixed diet of invertebrates and seeds).
Preglednica 2: Identificirani bakterijski izolati iz sapišč prostoživečih ptic. Ptice smo s hierarhičnim združevanjem združili v dve skupini, skupina 1 (pretežno žužkojede ptice) in skupina 2 (ptice z mešano prehrano sestavljeno iz nevretenčarjev in semen).

Bacterial isolates found in group 1	Bacterial isolates found in group 2	Bacterial isolates found in group 1 and 2
Aeromicrobium ponti/ *A. tamlense*	*Agrobacterium larrymoorei*	*Acinetobacter calcoaceticus*
Aeromicrobium sp. nov.[a]	*Agromyces terreus*	*Chryseobacterium indoltheticum*
Agrococcus versicolor	*Agromyces* sp. nov.[b]	*Frigoribacterium faeni*
Agromyces allii	*Arthrobacter aurescens*	*Microbacterium hydrocarbonoxydans*/ *M. phyllosphaerae*
Bacillus aryabhattai	*Arthrobacter nitroguajacolicus*/ *A. aurescens*	*Microbacterium phyllosphaerae*
Citrobacter gillenii	*Arthrobacter oxydans*	*Micrococcus* sp.
Clavibacter michiganensis	*Brochothrix campestris*	*Paenibacillus xylanexedens*/ *P. amylolyticus*/ *P. tundra*
Curtobacterium plantarum	*Cellulosimicrobium funkei*	*Pseudomonas flavescens*
Enterococcus plantarum	*Chryseobacterium daecheongense*	*Rathayibacter festucae*
Hafnia alvei	*Chryseobacterium* sp. nov.[c]	*Rhodococcus fascians*
Microbacterium hominis	*Curtobacterium citreum*	*Stenotrophomonas rhizophila*
Microbacterium oleivorans	*Curtobacterium flaccumfaciens*	
Microbacterium oxydans	*Exiguobacterium sibiricum*	
Microbacterium sp.	*Leucobacter exalbidus*	
Microbacterium xylanilyticum	*Microbacterium hydrocarbonoxydans*	
Micrococcus yunnanensis	*Microbacterium resistens*	
Ochrobactrum thiophenivorans	*Microbacterium testaceum*	
Pantoea agglomerans	*Oerskovia* sp.	
Pantoea anthophila	*Okibacterium fritillariae*	
Plantibacter flavus	*Pantoea agglomerans*	
Pseudomonas aeruginosa	*Pseudoclavibacter helvolus*	
Pseudomonas moraviensis	*Pseudomonas cedrina*	
Pseudomonas orientalis	*Pseudomonas extremorientalis*	
Pseudomonas psychrotolerans	*Pseudoxanthomonas koreensis*	
Sanguibacter keddieii	*Sphingobacterium faecium*	
Serratia grimesii	*Staphylococcus gallinarum*	
Staphylococcus epidermidis	*Staphylococcus* sp.	
Stenotrophomonas chelatiphaga		
Variovorax paradoxus		

[a,b,c,] potentially new species isolated from garden warbler (*Sylvia borin*)[a], song thrush (*Turdus philomelos*)[b] and Eurasian tree sparrow (*Passer montanus*)[c]

various factors, among them being the host species and feeding patterns. The differences extend to functional properties such as the greater capacity for amino acid metabolism and energy harvest in carnivores compared to herbivores (Waite & Taylor 2014). Therefore, since the gastrointestinal and respiratory tracts are connected, it is reasonable to assume that these factors also influence the composition of the respiratory microbiota. To assess the differences in the choanal microbiota of the sampled birds, we have performed hierarchical clustering which grouped the birds into two groups (Fig. 1). Choanal microbiota of the birds with similar diet grouped together. Birds which feed predominantly on insects or have more monotonous

diet clustered in one group, those that have more mixed diet of animals (invertebrates) and seeds throughout the year formed a separate group (Fig. 1). The number of bacterial species was used to assess the difference in choanal microbiota richness between the two groups. The first group which contains the birds predominantly feeding on insects, or which have a more monotonous diet in general, had a significantly lower average number of species (2.9 ± 1.6) in comparison to birds enjoying a more mixed diet of animals and plants throughout the season (7.8 ± 3.4) (P = 0.0002) (Table 1). The choanal microbiota differed between the two groups not only in terms of species richness, but also in terms of bacterial composition. Majority of isolates were found in only one of the two groups of birds (29 – the first group, 27 – the second group) and only 11 bacterial species colonized choanae of birds belonging to both groups (Table 2).

Stenkat et al. (2014) have previously found correlations between certain bacterial families and feeding habits, although they targeted specific pathogenic groups of bacteria. Enterobacteriaceae and Aeromonadaceae were correlated to piscivores, Staphylococcaceae and Streptococcaceae to aerial insectivores, and Pseudomonadaceae and Bacillaceae to herbivores. Our findings corroborate this, as hierarchical clustering grouped the choanal microbiota of the sampled birds into two groups based on the bird diet.

When comparing the presence or absence of individual bacterial species, pigeons showed to be far more likely colonized with *Staphylococcus gallinarum* than other sampled birds (Fisher's exact test (P = 0.013); odds ratio (pigeons/other birds) = 31.5, P = 0.012). Furthermore, the presence of the genus *Staphylococcus* sp. was indicative of the birds with a more diverse diet throughout the season; these birds also clustered in one of the two groups (Fig. 1, Fisher's exact test (P = 0.040); odds ratio (second group/first group) = 12.0; P = 0.044).

Apart from finding numerous human and plant opportunists, we have also isolated one novel species from garden warbler (*Sylvia borin*) (*Aeromycrobium choanae* sp. nov.) (Ber et al. 2017), and two potentially novel species from song thrush (*Turdus philomelos*) (*Agromyces* sp., 16S rRNA gene sequence similarity < 97 %) and Eurasian tree sparrow (*Passer montanus*) (*Chryseobacterium* sp., 16S rRNA sequence similarity < 98.7 %). Their description is part of ongoing research.

4 CONCLUSIONS

Our study has shown that the choanal microbiota of free-living birds with a diet composed predominantly of insects, or with a generally monotonous diet, was poorer in terms of species richness, compared to birds with a more diverse diet during the year. Previously, correlation between selected bacterial families and diet has been determined, however our analyses have shown that the differences in microbiota extend beyond selected bacterial families. Hierarchical clustering of bacteria showed a correlation between the birds feeding patterns and the upper respiratory microbiota composition. Our study has also shown that free-living birds carry a wide array of known human and plant pathogens in their upper respiratory tract, but also possible novel species. Given the impact microbiota has on the bird´s health and bird´s potential for spreading pathogens in the environment, it will be necessary to extend the analysis of choanal microbiota and factors that shape its structure, on more free-living bird species.

5 POVZETEK

Da bi ocenili mikrobno diverziteto v sapiščih prostoživečih ptic, smo 25 pticam odvzeli brise sapišč, ki smo jih nacepili na hranilni agar. Po gojitvi smo izolirali 98 bakterijskih kolonij in jih na podlagi nukleotidnega zaporedja za 16S rRNK identificirali. Število različnih bakterijskih izolatov pri posamezni ptici se je gibalo med 12 (cikovt, *Turdus philomelos*) in 1 (severni kovaček, *Phylloscopus trochilus*). Večina izolatov je pripadala deblom Actinobacteria (52 %), Proteobacteria (31 %), Firmicutes (15 %) in Bacteroidetes (4 %). Izolati so večinoma pripadali družinam Microbacteriaceae (36 %), Pseudomonadaceae (11 %), Enterobacteriaceae (10 %), Micrococcaceae (7 %), in Flavobacteriaceae, Xanthomonadaceae in Staphylococcaceae (vse 4 %). Največ ptic (11) je bilo koloniziranih z bakterijami, ki so pripadale družini Microbacteriaceae, nato Pseudomonadaceae (pet ptic), Nocardiaceae (štiri ptice), Enterobacteriaceae (štiri ptice), Xanthomonadaceae (tri ptice),

Moraxellaceae (tri ptice), Staphylococcaceae (tri ptice) in Micrococcaceae (dve ptici). Ostale družine bakterij smo detektirali le pri posamezni ptici.

Od skupno 98 bakterijskih izolatov, smo našli 13 (13,3 %) takih, ki lahko povzročajo okužbe pri ljudeh: *Acinetobacter calcoaceticus, Cellulosimicrobium funkei, Curtobacterium citreum, Curtobacterium flaccumfaciens, Exiguobacterium sibiricum, Hafnia alvei, Microbacterium oleivorans, Microbacterium resistens, Pantoea agglomerans, Pseudomonas aeruginosa, Serratia grimesii, Staphylococcus epidermidis* in *Staphylococcus gallinarum*. Pri osmih pticah (32 %) smo v sapišču našli vsaj eno oportunistično bakterijo. Petina ptic je bila koloniziranih z eno, dve ptici z dvema, cikovt pa hkrati s tremi oportunističnimi vrstami bakterij. Tudi ptice urbanih okolij (golob in domači vrabec) so bile kolonizirane s človeškimi oportunisti. Golobi s *Staphylococcus gallinarum* in *Acinetobacter calcoaceticus*, vrabci pa s *Curtobacterium citreum*, *Curtobacterium flaccumfaciens* in *Exiguobacterium sibiricum*.

Poleg oportunističnih bakterij, ki povzročajo okužbe pri ljudeh, smo pri 44 % vzorčenih ptic našli bakterije, ki so patogene za rastline: *Agrobacterium larrymoorei*, *Clavibacter michiganensis, Curtobacterium flaccumfaciens, Plantoea agglomerans, Pseudomonas aeruginosa, Pseudomonas flavescens* in *Rhodococcus fascians*. Največkrat smo detektirali bakterijo *Rhodococcus fascians*, ki je bila prisotna pri štirih različnih vrstah ptic (domači vrabec, ščinkavec, rumeni strnad in siva pevka). Prvi dve vrsti se prehranjujeta z raznovrstno hrano sestavljeno iz semen in žuželk, zadnji dve pa pretežno z žuželkami, kar bi lahko pomenilo, da je bakterija med pticami splošno prisotna.

Na sestavo in delovanje črevesne mikrobiote pri pticah vplivajo različni dejavniki, kot sta vrsta gostitelja in vrsta hrane (mesojedci/rastlinojedci). Ker so prebavila in dihala povezana, ti dejavniki verjetno vplivajo tudi na sestavo in delovanje mikrobiote v dihalih. Z metodo hierarhičnega združevanja smo ptice na podlagi sestave bakterijske mikrobiote sapišč združili v dve skupini. V prvi skupini so bile pretežno žužkojede ptice, v drugi pa ptice z bolj raznovrstno prehrano rastlinskega in živalskega izvora. Tudi bogatost mikrobiote, ki smo jo ocenili na podlagi števila prisotnih bakterijskih vrst, je med obema skupinama ptic bila različna. Pri žužkojedih pticah smo zaznali manjše število vrst (2,9 ± 1,6) v primerjavi s pticami, ki se hranijo z bolj raznovrstno hrano živalskega in rastlinskega izvora (7,8 ± 3,4) ($P = 0{,}0002$). Obe skupini ptic sta imeli tudi različno sestavo mikrobiote, saj smo večino bakterijskih vrst našli le pri eni ali drugi skupini (29 bakterijskih vrst pri žužkojedih pticah, 27 bakterijskih vrst pri pticah z raznoliko prehrano) in le 11 bakterijskih vrst smo detektirali pri obeh skupinah ptic. Pri vrtni penici (*Sylvia borin*), cikovtu (*Turdus philomelos*) in vrabcu (*Passer montanus*) smo v sosledju našli tudi novo in dve domnevno novi vrsti bakterij; *Aeromycrobium choanae* sp. nov., *Agromyces* sp. in *Chryseobacterium* sp..

Z metodo hierarhičnega združevanja smo pokazali, da imajo ptice s podobno prehrano, podobno bakterijsko mikrobioto sapišč. Ptice, ki se prehranjujejo pretežno z žuželkami, so imele manj bogato mikrobioto kot ptice, ki se prehranjujejo z bolj raznoliko živalsko in rastlinsko hrano. Raziskava je tudi pokazala, da so zgornja dihala prostoživečih ptic pomemben rezervoar oportunističnih bakterij, ki lahko okužijo ljudi in rastline, in tudi novih vrst bakterij. Da bi dobili globji vpogled v sestavo mikrobiote zgornjih dihal, bi v prihodnosti morali povečati število analiziranih ptic.

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