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Ekologické aspekty ovlivňující mikrobiotu trávicího traktu ptáků

Ecological aspects affecting microbiota in the digestive tracts of birds

Disertační práce

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Abstrakt

Střeva obratlovců jsou obydlena taxonomicky i funkčně různorodým společenstvem bakterií a jiných mikroorganismů. Pokroky v sekvenačních technologiích odhalily vliv střevní mikrobioty (dále jen SM) na fyziologii, imunitu i chování hostitele. Naše současné vědomosti jsou však založené zejména na studiu modelových organismů, jejichž SM se od volně žijících obratlovců významně liší. Předložená práce je proto zaměřena na studium SM pomocí sekvenování druhé generace u volně žijích pěvců (Passeriformes) a srovnává jejich SM s ostatními obratlovci, zejména s nejvíce studovanými savci. Na vnitrodruhové i mezidruhové úrovni se předložená práce věnuje faktorům, které složení SM ovlivňují, mezi něž patří například vnitřní regulační mechanismy hostitele (například genotyp, imunitní systém či fyziologie hostitele), sociální kontakty či environmentální faktory (včetně potravy). Dále se tato práce zabývá stabilitou SM v čase a změnami SM během ontogeneze.

Z výsledků této práce vyplývá, že u pěvců jsou v SM dominantní bakteriální kmeny Proteobacteria, Firmicutes, Actinobacteria, Tenericutes, Bacteroidetes a Chlamydia, což naznačuje složení SM odlišné od SM savců. Mezidruhová variabilita ve složení SM pěvců je ovlivněna zejména fylogenezí hostitele, efekt geografické vzdálenosti mezi lokalitami sběru vzorků je méně výrazný. Zatímco u savců jsou ekologické znaky hostitele v čele s potravou významným faktorem ovlivňujícím složení SM, u pěvců mají ekologické faktory společně s potravou nesrovnatelně menší vliv na mezidruhovou i vnitrodruhovou variabilitu ve složení SM. U vlaštovky obecné (*Hirundo rustica*) data naznačují vliv sociálních kontaktů u sociálních párů či velmi slabý transgenerační přenos SM od sociální matky na mláďata. Zároveň jsme pozorovali rozdílnou SM mláďat a dospělců, ale u mláďat v hnízdě jsme nepozorovali dramatické změny SM s věkem, které jsou známé od savců. Detekovali jsme ovšem část SM, která se zdá být stabilní v čase, a tedy vhodná pro další podrobnější studium. Výsledky této práce ukazují důležitost studia SM u dalších skupin obratlovců, nežli jsou savci. Pěvci mají nejen jiné taxonomické složení SM, ale zdá se, že i faktory ovlivňující složení SM jsou jiné než u savců.

Abstract

Vertebrate intestines are inhabited by taxonomically and functionally diverse communities of bacteria and other microorganisms. While recent advances in sequencing technology have revealed the influence of gut microbiota (hereinafter GM) on the physiology, immunity and behaviour of the host; our current knowledge is based mainly on the study of model organisms that will have a different GM composition than that of wild vertebrates. The work presented here focuses on the study of wild songbird (Passeriformes) GM using next generation sequencing, and compares their GM with that of other vertebrates, including mammals, which have been studied far more than birds. This work focuses mainly on factors that affect GM composition at the inter- and intra-specific levels, including intrinsic regulatory mechanisms of the host (such as genetic, immunological and/or physiological mechanisms), social contacts and environmental factors (including diet). In addition, the stability of GM over time and any changes during ontogeny were also assessed.

The results indicate that songbird GM is dominated by the bacterial phyla Proteobacteria, Firmicutes, Actinobacteria, Tenericutes, Bacteroidetes and Chlamydia, a community that differs from that of mammals. At the interspecies level, GM composition is mainly influenced by phylogeny and less so by sampling site. While ecological factors, and especially diet, are important GM predictors in mammals, the effect of ecological factors, including diet, have a much smaller effect on inter- and intraspecific variability in songbird GM composition. Our data from barn swallows (*Hirundo rustica*) suggest an effect of social GM transmission among adults forming social pairs, as well as trans-generational transmission from a social mother to her own nestlings. At the same time, we observed differing GM in adults and nestlings, with no dramatic change in GM with nestling age, a process previously recognised in mammals. Part of the GM detected appears to remain stable over time, and is therefore suitable for further detailed study. To conclude, our studies demonstrate that songbird GM differs from that of mammals and that the factors influencing GM composition appear to differ from those in mammals, thereby highlighting the importance of studying multiple vertebrate groups (not just mammals) when assessing the effects on GM composition.

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1. Úvod

Společenstvo symbiotických bakterií v trávicím traktu může, co do počtu buněk, převyšovat nebo být srovnatelné s počtem buněk svého hostitele (Sender, Fuchs & Milo, 2016). Zároveň však počet genů, které toto společenstvo obsahuje, může být až o dva řády vyšší než počet genů v genomu hostitele (Qin *et al.*, 2010). Je dobře známo, že střevní mikrobiota (dále jen SM) a hostitel spolu vzájemně interagují na mnoha úrovních a tyto interakce přináší hostiteli celou řadu důležitých výhod. SM pomáhá hostiteli trávit pro něj jinak nestravitelné složky potravy, jako je například rostlinná celulóza (review v Bäckhed *et al.*, 2005; Thursby & Juge, 2017; Valdes *et al.*, 2018). Pomocí SM jsou rovněž syntetizovány některé vitamíny (review v Thursby & Juge, 2017), jako například vitamín B12 (LeBlanc *et al.*, 2013). SM dále ovlivňuje správný vývoj a funkci trávicího traktu (Reikvam *et al.*, 2011) a také pomáhá s ochranou proti patogenům (Koch & Schmid-Hempel, 2011; review v Ubeda *et al.*, 2012). Například u lidí bylo pozorováno, že přítomnost bakterií rodu *Bacillus* ve střevě vyloučila přítomnost *Staphylococcus aureus* (Piewngam *et al.*, 2018; Chung & Raffatellu, 2019), což je potenciálně nebezpečný lidský patogen (Liu, 2009). SM rovněž stimuluje a podporuje vývoj imunitního systému hostitele a ovlivňuje jeho specificitu (review v Macpherson & Harris, 2004; Wu & Wu, 2012; Ost & Round, 2018). Nezanedbatelný je také podíl SM na funkci mozku a chování (Cryan & Dinan, 2012; Matthews & Jenks, 2013; Strandwitz, 2018). Na druhou stranu, odchýlení SM od normálního stavu může být pro hostitele škodlivé nebo dokonce fatální (Brucker & Bordenstein, 2013; Wang *et al.*, 2015; review v Das & Nair, 2019). Kupříkladu tzv. germ-free myši (tedy laboratorní myši bez mikrobioty) nebo myši ošetřené širokospektrálními antibiotiky mají porušenou morfologii a funkci střev, která se opět obnoví po kolonizaci střev přirozenou SM (Smith, McCoy & Macpherson, 2007; Reikvam *et al.*, 2011). U lidí je popsáno několik nemocí, které patrně souvisí s patologickými změnami SM. V řadě případů však není jisté, zda je patologie SM důsledek nebo příčina nemoci (review v DeGruttola *et al.*, 2016; Sarkar & Banerjee, 2019). Konkrétně je patologie SM spojována například s obezitou, zánětlivým onemocněním střev, rakovinou tlustého střeva a konečníku a také s neurologickými poruchami (Ley *et al.*, 2006; Frank *et al.*, 2007; Ahn *et al.*, 2013; Zackular *et al.*, 2016; Tremlett *et al.*, 2017). Není proto překvapivé, že se v průběhu evoluce vyvinula celá řada genetických, fyziologických, morfologických anebo behaviorálních znaků, kterými hostitel může optimalizovat složení SM ke svému prospěchu (Lombardo, 2008; Benson *et*

al., 2010; McKnite *et al.*, 2012; Archie & Tung, 2015; Ohbayashi *et al.*, 2015, 2019; Lanan *et al.*, 2016; Suzuki *et al.*, 2019). Tyto interakce mezi hostitelem a jeho SM mohou hrát rovněž důležitou roli v mnoha mikroevolučních procesech, včetně evoluce výběru partnera (Sharon *et al.*, 2010; Beltran-Bech & Richard, 2014), evoluce sociálního chování (Lombardo, 2008; Archie & Tung, 2015) a speciace (Brucker & Bordenstein, 2012; Wang *et al.*, 2015).

Většina našich poznatků o SM je založena na studiu laboratorních savců a lidí. SM laboratorních zvířat i zvířat žijících v zajetí je odlišná od volně žijících zvířat (Xenoulis *et al.*, 2010; Nelson *et al.*, 2013; Kreisinger *et al.*, 2014; McKenzie *et al.*, 2017). Také byla pozorována změna složení SM u volně žijících zvířat držených nějakou dobu v zajetí (Kohl & Dearing, 2014; Kohl, Skopec & Dearing, 2014). Proto výzkum založený na jedincích ze zajetí nemusí nutně odrážet přirozené interakce mezi SM a jejím hostitelem, které se formovaly během vzájemné koevoluce. V rámci skupiny obratlovců jsou nejvíce studováni volně žijící savci, u kterých jsou Firmicutes a Bacteroidetes obvykle dominantně se vyskytující kmeny (Ley *et al.*, 2008; Nishida & Ochman, 2018), zatímco složení SM ostatních obratlovců se zdá být daleko variabilnější (Sullam *et al.*, 2012; Waite & Taylor, 2014; Hird *et al.*, 2015; Kohl *et al.*, 2017), ale zároveň méně prozkoumané. Mezi lety 1980 – 2016 bylo publikováno značné množství článků zabývajících se SM savců a lidí, nicméně studií, které se zabývaly SM u domestikovaných, či dokonce volně žijících ptáků, je o poznání méně (Grond *et al.*, 2018). Z článků zkoumajících SM, publikovaných mezi lety 2009 – 2016 se pouze 14,3 % zabývalo volně žijícími živočichy ve srovnání se zvířaty modelovými a domácími (37,5 % a 48,2 %; Pascoe *et al.*, 2017). Ve stejné publikaci bylo u volně žijících živočichů nejvíce studií SM zaměřeno na hmyz (42,5 %) a savce (30,2 %), méně pak na ostatní bezobratlé, ptáky, ryby, plazy a obojživelníky. Vzhledem k omezeným znalostem o SM volně žijících ptáků (třída Aves) jsem se rozhodla zaměřit na tuto skupinu obratlovců, a to konkrétně na řád pěvců (Passeriformes).

Všichni ptáci jsou stejně jako savci teplokrevní živočichové. Na rozdíl od většiny savců, kteří jsou živorodí (krom podtřídy vejcorodých; Prototheria), jsou všichni ptáci vejcorodí. Další podstatný rozdíl, který může ovlivňovat SM, je skutečnost, že většina savců má oddělenou trávicí, vylučovací a rozmnožovací soustavu, ale u ptáků všechny tyto soustavy ústí do kloaky a jsou tedy propojeny. Podstatné rozdíly ale existují i na úrovni fyziologie trávení potravy a vstřebávání živin (Caviedes-Vidal *et al.*, 2007; McWhorter,

Caviedes-Vidal & Karasov, 2009). Pěvci jsou monofyletický a nejpočetnější řád ptáků, který zahrnuje okolo 6000 druhů, což je přibližně 60 % všech ptačích druhů. Pěvci se od ostatních ptáků oddělili přibližně před 47 miliony let (Oliveros *et al.*, 2019). I přes relativně nedávnou diverzifikaci jsou pěvci různorodou skupinou, zejména co se týče ekologie a životních strategií. Z těchto důvodů je lze považovat za důležitou modelovou skupinu v evolučních a ekologických studiích. Oproti řadě jiných ptačích taxonů se u pěvců nachází pouze rudimentární slepá střeva. Vyvinutá slepá střeva mají důležitou roli v bakteriální fermentaci potravy. Vzhledem k tomu, že se tato práce věnuje studiu volně žijících pěvců, nebyl většinou možný odběr vzorků různých částí střev, a proto jsem se zaměřila převážně na mikrobiotu v trusu. Sběr trusu je běžně využíván jako neinvazivní metoda pro analýzy SM zejména tlustého střeva (Berlow, Kohl & Derryberry, 2020; Čížková *et al.*, 2021). Z mých nepublikovaných dat navíc vyplývá, že u ptáků mikrobiota trusu poměrně věrně odráží střevní mikrobiotu (Schmiedová *et al.*, nepublikované výsledky).

Tato práce je převážně zaměřena na složení SM a faktory, které mohou složení SM ovlivnit u volně žijících pěvců, a to jak na mezidruhové, tak i vnitrodruhové úrovni. Hlavními faktory, které mohou mít vliv na SM, jsou vnitřní regulační mechanismy hostitele, sociální kontakty a environmentální faktory. Vnitřním regulačním mechanismem hostitele je myšlena zejména geneticky podmíněná variabilita v imunitním systému (Benson *et al.*, 2010; Bolnick *et al.*, 2014). Role genotypu na SM byla studována zejména u myši jako modelového organismu, kde byl prokázán signifikantní podíl genů spojených s imunitním systémem na složení SM (Benson *et al.*, 2010; Kubinak *et al.*, 2015; Org *et al.*, 2015). U volně žijících myší (*Mus musculus*) bylo nedávno nalezeno 20 genů asociovaných s imunitním systémem a souvisejících se SM (Suzuki *et al.*, 2019). Dalším faktorem ovlivňujícím složení SM je sociální kontakt (Lucas & Heeb, 2005; White *et al.*, 2010). Konkrétně se u ptáků mohou bakterie přenášet během kopulace, při krmení mláďat rodiči nebo v rámci fyzického kontaktu mezi jedinci ze stejné kolonie, populace nebo dokonce mezi různými druhy (Lucas & Heeb, 2005; Kulkarni & Heeb, 2007; White *et al.*, 2010; Hernandez *et al.*, 2020). Vliv sociálního přenosu na složení SM byl pozorován u šimpanzů a paviánů (Tung *et al.*, 2015; Moeller *et al.*, 2016). U myší bylo pozorováno, že se sociálním kontaktem nepřenáší všechny bakterie stejně, ale některé lépe a jiné hůře (Moudra *et al.*, 2021). V neposlední řadě jsou zdrojem variability SM environmentální faktory, které zahrnují zejména potravu a obývané prostředí (Lucas

& Heeb, 2005; De Filippo *et al.*, 2010). U savců byly pozorovány signifikantní rozdíly mezi býložravci, všežravci a masožravci (Ley *et al.*, 2008). Bakterie obývající střeva obratlovců pomáhají s trávením a získáváním energie z potravy, což může vést k přizpůsobení SM dané potravě. Například fermentace potravy u býložravců je často úzce vázána na přítomnost bakterií v trávicím traktu. Studie věnující se vlivu potravy na variabilitu v SM mezi jedinci ze stejné populace jsou u volně žijících obratlovců stále vzácné (Baxter *et al.*, 2015; Li *et al.*, 2016, 2018; Phillips *et al.*, 2017; Holmes *et al.*, 2019). Specifické podmínky i specifická potravní nabídka v místě výskytu daného jedince/populace může vést k přizpůsobení SM obývanému prostředí. Struktura obývaného prostředí, například lidmi modifikovaná krajina oproti nenarušené, může ovlivnit SM, jak bylo pozorováno u guerézy stříbřitonohé (*Procolobus gordonorum*; Barelli *et al.*, 2015) a kora štětinatého (*Proechimys semispinosus*; Fackelmann *et al.*, 2021).

Stále však není zcela jasné, jak moc tyto faktory ovlivňují SM volně žijících ptáků (pěvců). Proto se v této práci snažím na mezidruhové i vnitrodruhové úrovni efekt některých faktorů objasnit trochu více. Na mezidruhové úrovni srovnávám efekt fylogeneze, ekologických faktorů a geografické vzdálenosti mezi lokalitami s použitím datového souboru zahrnujícího 51 druhů pěvců z ČR. Dále se věnuji rozdílům mezi populacemi z tropů (Kamerunu), kde byly navíc srovnávány dvě sezony (období sucha a dešťů), a z mírného pásu (ČR), kde byl také testován efekt migrace na SM. Pro tuto studii byl datový soubor z ČR rozšířen o 47 druhů z Kamerunu. Existují sice studie zabývající se analýzou SM u tropických ptáků (Hird *et al.*, 2015; Bodawatta *et al.*, 2018; Capunitan *et al.*, 2020; Bodawatta *et al.*, 2021b), ale nikdo se přímo nezabýval srovnáním SM mezi tropickými a temperátními druhy ptáků. Se zeměpisnou šířkou se mění podmínky pro život bakterií včetně patogenů v daném prostředí, jako například teplota či vlhkost prostředí nebo také množství UV záření. Bakterie z prostředí mohou přímo nebo nepřímo interagovat s imunitním systémem hostitele a tím ovlivnit i složení jeho SM. Mnoho druhů pěvců hnízdících v mírném pásu migruje mimo hnízdní sezonu do tropického pásu (Cepák *et al.*, 2008). Podmínky na zimovišti ovlivňují znaky spojené s fitness, které se mohou projevit na hnízdišti (Norris *et al.*, 2004; Saino *et al.*, 2004; Rockwell, Bocetti & Marra, 2012). Do jaké míry podmínky na zimovišti ovlivňují složení SM je zatím nejasné. Pro porovnání vlivu vnitřních regulačních mechanismů hostitele s enviromentálními faktory, které v tomto případě zahrnují i sociální kontakt, jsem využila systém hnízdního

parazitismu u kukačky obecné (*Cuculus canorus*, řád Cuculiformes). Hnízdní parazitismus je fenomén, kdy jedinec klade vajíčka do hnízd jiných druhů nebo jiných jedinců, kteří se potom starají o jejich mláďata (Davies, 2010). V práci konkrétně srovnávám mláďata kukačky obecné v hnízdech dvou různých druhů rákosníků (rákosníka velkého *Acrocephalus arundinaceus* a obecného *A. scirpaceus*) s jejich vlastními mláďaty.

Na vnitrodruhové úrovni u vlaštovky obecné (*Hirundo rustica*) se zaměřuji na efekt sociálního kontaktu (přenos v rámci hnízdních párů a z rodičů na potomky), lokality (hnízdní kolonie i hnízda) či potravy na individuální úrovni. Mimo to testuji souvislost SM a imunitní reakce pomocí kožního testu, při kterém dochází k aplikaci fytohemaglutininu do kůže (tzv. PHA test). PHA test se využívá v ekologické imunologii pro detekci buňkami zprostředkované imunity (Vinkler, Bainová & Albrecht, 2010; Vinkler *et al.*, 2012; Bowers *et al.*, 2014). Zatímco u laboratorních savců byla pozorována signifikantní interakce mezi imunitou a SM (Clavel *et al.*, 2017; Ost & Round, 2018), u volně žijících zvířat jsou naše znalosti nedostatečné.

U vlaštovky obecné jsem se zaměřila na porovnání dospělců s různě starými mláďaty v hnízdě a na variabilitu v rámci hnízdní sezony a mezi dvěma sezonami. O ptačí SM v průběhu ontogeneze a její stabilitě v dospělosti se toho mnoho neví. I v tomto případě je více prostudovaná SM u savců. Zde je potřeba vyzdvihnout velký rozdíl mezi ptačí a savčí ontogenezí. Savci, s výjimkou vejcorodých, rodí živá mláďata, která jsou během porodu v úzkém kontaktu s mikrobiotou v porodních cestách matky. U savců navíc matka po porodu mláďata na rozdíl od ptáků kojí a tím aktivně ovlivňuje jejich SM. Pěvci mají nidikolní (tzv. krmivá) mláďata, o která musejí rodiče po vylíhnutí pečovat, zejména je krmit a zahřívat. U lidí a většiny ostatních savců začíná kolonizace střeva bakteriemi krátce po porodu a možná dokonce i během těhotenství/březosti (review v Walker *et al.*, 2017). U lidí se SM mění nejvíce během prvního roku života, což je pravděpodobně spojeno s kojením a jeho ukončením. Okolo 3. roku života dítěte začíná jeho SM nabývat na stabilitě a podobat se SM dospělého člověka (Koenig *et al.*, 2011; Yatsunenko *et al.*, 2012; Bäckhed *et al.*, 2015; Mach *et al.*, 2015). U dospělého člověka a zvířat chovaných v zajetí se zdá být SM poměrně stabilní v čase (Benskin *et al.*, 2010; Schloss *et al.*, 2012; Faith *et al.*, 2013; Mehta *et al.*, 2018). Oproti tomu volně žijící zvířata podobnou stabilitu v čase obvykle nevykazují (Baxter *et al.*, 2015; Sun *et al.*, 2016). Tyto nekonzistentní výsledky u savců mě vedly ke studiu stability a ontogeneze SM u volně žijících pěvců.

1.1. Metodické přístupy

1.1.1. Sekvenační metody

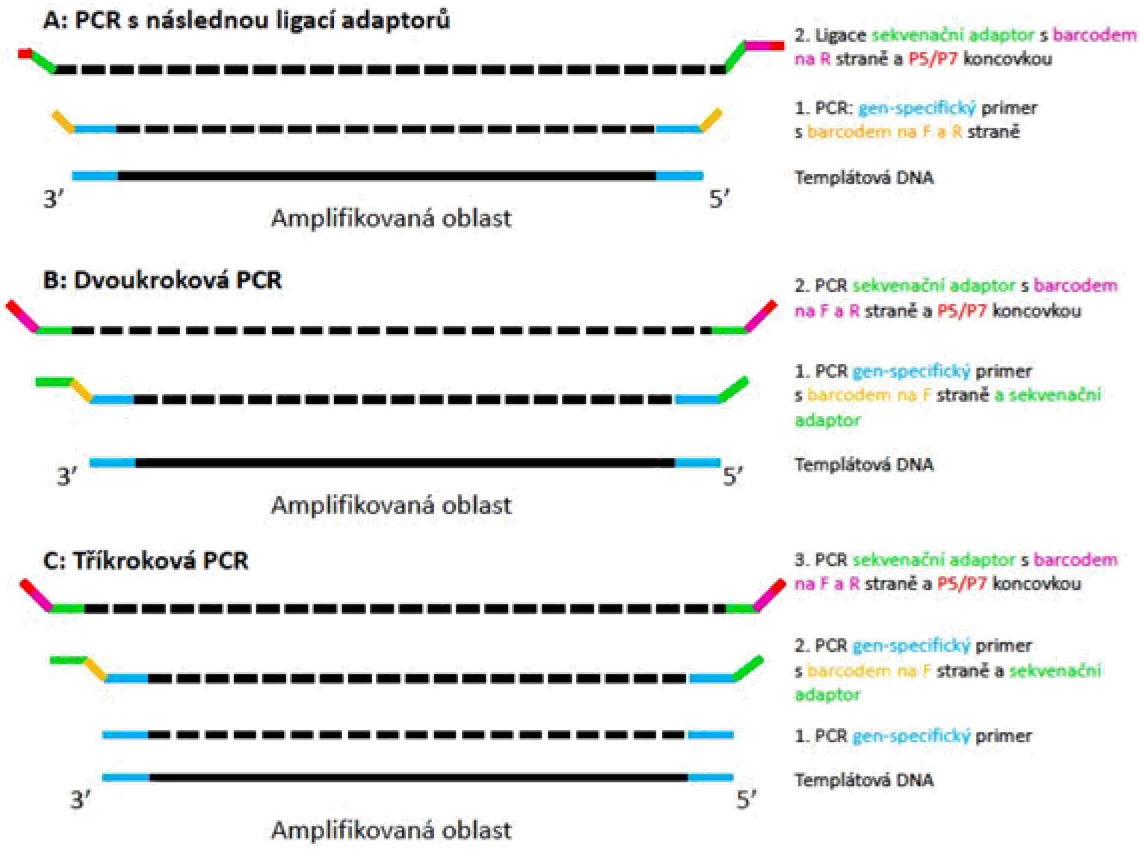
K zodpovězení kladených otázek bylo potřeba zvolit vhodnou metodu. Dříve byl podobný výzkum odkázán pouze na bakterie, které bylo možné kultivovat. Celá řada bakterií však vyžaduje tak specifické podmínky, že je jejich efektivní kultivace prakticky nemožná (Amann, Ludwig & Schleifer, 1995). I z tohoto důvodu se začaly následně využívat genetické metody, které odhalily, že v laboratoři kultivovatelné bakterie odráží pouze malou část skutečné diverzity bakterií. Nejjednodušší genetické metody jsou tzv. fingerprintové, mezi ně patří DGGE (z anglického Denaturing Gradient Gel Electrophoresis) nebo ARISA (z anglického Automated rRNA Intergenic Spacer Analysis), které však neumožňují přímou taxonomickou identifikaci bakterií. Proto se tyto metody často doplňují klonovým sekvenováním, což je ale poměrně pracné a drahé (Benskin *et al.*, 2010; van Dongen *et al.*, 2013). Až s příchodem paralelního sekvenování druhé generace se stalo studium mikrobiálních společenstev dostupnější. V současné době jsou k dispozici dva typy široce používaných genetických přístupů pro studium mikrobiálních společenstev: metagenomika a metataxonomie (nebo také metabarcoding). Metagenomika je založena na sekvenování náhodných fragmentů vyizolované DNA ke zjištění taxonomické informace a genového složení vzorku. Naproti tomu metataxonomie je založena na sekvenování specifické části genomu, která je předem amplifikována pomocí PCR (z anglického polymerase chain reaction). Tímto přístupem se dá poměrně jednoduchou a levnou cestou zjistit taxonomická diverzita, ale nelze přímo identifikovat funkční a metabolický potenciál. Metagenomický přístup je podstatně dražší, protože je potřeba o několik řádů vyšší sekvenační pokrytí, a doprovází ho i složitější bioinformatické zpracování získaných dat. Na druhou stranu data získaná metagenomickým způsobem v sobě nesou daleko více informací. Limitací metataxonomického sekvenování je výběr primerů, které nikdy nejsou zcela univerzální a mohou tudíž určité skupiny bakterií amplifikovat pouze slabě nebo vůbec. Dalším úskalím tohoto postupu je předcházející PCR amplifikace, při které dochází ke vzniku chimérických sekvencí, které uměle navyšují diverzitu společenstva, přičemž jejich identifikace a následné odstranění není dokonalé. Pomocí paralelního sekvenování je v současné době možné zaznamenat až tisíce, miliony a dokonce i biliony sekvencí najednou. V počátcích mého doktorského studia bylo na vrcholu pyrosekvenování 454

(Roche). Tato v současnosti již nedostupná platforma produkovala ve své době relativně dlouhé sekvence, avšak měla problémy detekovat přesný počet více po sobě jdoucích identických bází (tzv. homopolymerů). Dalším limitujícím faktorem byla i poměrně vysoká cena. Na podobném principu fungující sekvenační platforma, Ion Torrent, vykazuje potíže s detekcí přesného počtu stejných, po sobě jdoucích bází. Je ale levnější a produkuje ve srovnání s 454 více sekvencí. V současnosti se pro podobný typ studií, jako je tato, nejvíce využívá Illumina, a to z důvodu produkce dosud nejpřesnějších dlouhých sekvencí a menší chybovosti během sekvenování, přičemž sekvenční chyby lze použitím vhodných algoritmů efektivně odstranit. Dalším příznivým faktorem je nízká cena v přepočtu na vzorek. Nevýhodou platformy Illumina je poměrně dlouhý sekvenační proces a kratší délka výsledných sekvencí. V dnešní době jsou rovněž k dispozici dvě platformy sekvenování třetí generace: Pacific Biosciences, která využívá sekvenaci jedné molekuly DNA (SMRT z anglického single-molecule real time) a Oxford Nanopore technologie, která využívá mikroskopických pórů, ve kterých se detekují jednotlivé báze. Tyto metody produkují dlouhé sekvence na základě jednotlivých molekul. Hlavní nevýhodou obou metod je zatím značná chybovost. Předností Oxford Nanopore technologie je její malá velikost a nenáročná příprava sekvenačních knihoven, což umožňuje využití přímo v terénu. Pro více informací o sekvenačních platformách, jejich výhodách i limitacích doporučuji například review od Goodwin *et al.* (2016), Mardis (2017) nebo McCombie *et al.* (2019). První publikace zahrnutá do této disertace je založena na 454 sekvenační platformě, protože v té době byla pro nás nejdostupnější. Všechny ostatní publikace jsou založeny na sekvenování pomocí Illumina Miseq platformy. Pomocí Illumina Miseq sekvenujeme předem naamplifikovanou specifickou oblast genu pro 16S rRNA, která se běžně používá v obdobných studiích zabývajících se SM (Hird *et al.*, 2015; Suzuki & Nachman, 2016).

1.1.2. Příprava sekvenační knihovny

Většina vzorků v této disertaci byla připravena jednokrokovou PCR s následnou ligací (spojení dvou vláken DNA) sekvenačních adaptorů se specifickým P5/P7 koncem, které umožňují navázání sekvence na sekvenační čip (Obrázek 1). Pro PCR jsem používala gen-specifický primer (tj. část, která nasedne na DNA) a na forward (předním, zkráceně F) i reverse (zadním, zkráceně R) primeru byla připojena značka dlouhá 10 oligonukleotidů. Měla jsem k dispozici 11 různě značených F primerů a 12 R primerů,

což dává dohromady 132 různých kombinací. Těchto 132 vzorků bylo poté možné napipetovat dohromady do jedné zkumavky podle koncentrace. K výslednému poolu se následně ligací připojily sekvenační adaptory za použití TruSeq nano DNA library preparation kitu (Illumina, USA). Hlavní limitací tohoto přístupu je skutečnost, že během ligace adaptorů může docházet ke vzniku chimérických sekvencí zahrnujících i oligonukleotidové značky jednotlivých vzorků, což může následně vést k chybnému zařazení těchto sekvencí k jinému vzorku. V současné době proto pro přípravu sekvenačních knihoven využíváme dvou po sobě jdoucích PCR reakcí (tzv. dvoukroková metoda, Obrázek 1). V této práci je zahrnuta pouze jedna publikace s touto metodou při amplifikaci bakteriální DNA (oblast na 16S rRNA). Její varianta, tzv. tříkroková PCR (Obrázek 1), byla využita k analýzám složení potravy hmyzožravých ptáků pomocí amplikonového sekvenování oblasti na genu pro cytochrom C oxidázu I (COI). Během prvního kola PCR (1. PCR) se amplifikuje pouze oblast DNA, která nás zajímá, pomocí gen-specifického primeru a pro eliminaci amplifikace hostitelské DNA byli použity blokační primery **[detail v Publikaci H a E]** (Sottas *et al.*, 2020). Tento první krok je zde navíc oproti dvoukrokové PCR, a to z toho důvodu, že bez něj vznikalo větší množství artefaktů (zejména krátkých produktů). Při druhé PCR (2. PCR), která odpovídá první PCR v dvoukrokovém protokolu, se využívají gen-specifické primery (stejné jako během první PCR) doplněné o oligonukleotidovou značku na F primeru (5 různých značek jsem měla k dispozici, tzv. vnitřní barkód) a sekvenační adaptor. Během třetí PCR (3. PCR) jsme použili primery se sekvenačním adaptorem a barcodem pro jednotlivé vzorky (až 384 různých, vnější barkód) a specifickým P5/P7 koncem, který umožňuje nasednutí sekvencí k sekvenačnímu čipu.



***Obrázek 1**: Schematické znázornění A: PCR s následnou ligací adaptorů; B: Dvoukroková PCR a C: Tříkroková PCR.*

2. Cíle a otázky

I. **Jaké je složení SM u pěvců a jak se liší od ostatních obratlovců (zejména savců)? [Publikace A–I]**

II. **Jaká je variabilita SM u pěvců na mezidruhové úrovni a jaké jsou hlavní faktory, které tuto variabilitu ovlivňují?** Využila jsem velký komparační datový soubor (319 vzorků, 51 druhů), abych zjistila, do jaké míry ovlivňuje variabilitu SM fylogeneze hostitele, ekologie (včetně životních strategií) a lokalita sběru vzorků. **[Publikace A]** Poté jsem tento datový soubor nasbíraný v mírném pásu (ČR) rozšířila o vzorky z tropické oblasti (Kamerunu, 205 vzorků, 47 druhů), aby bylo možno porovnat SM mezi druhy z tropické a temperátní klimatické zóny. U populace z mírného pásu jsem si navíc kladla otázku, zda je SM ovlivněna migrací. Navíc u dvou druhů trans-saharských migrantů jsem přímo srovnávala jejich SM v tropech během období sucha (zimoviště) a v temperátu během hnízdní sezony (hnízdiště). **[Publikace B]** U slavíků obecného a tmavého (*Luscinia megarhynchos* a *L. luscinia*) jsem testovala souvislost mezi reprodukční bariérou a mezidruhovou divergencí SM. **[Publikace C]**

III. **Jaké jsou hlavní faktory ovlivňující vnitrodruhovou variabilitu SM?** U populace vlaštovky obecné jsem se zaměřila na srovnání SM mezi různými lokalitami a mezi hnízdními sezonami. **[Publikace D]** U tohoto druhu jsem také studovala, jestli je SM ovlivněna potravou jedince. **[Publikace E]** Dále jsem pomocí stimulace zánětlivé reakce u mláďat vlaštovky testovala souvislost SM s imunitním systém. **[Publikace F]**

IV. **Jaký je potencionální vliv sociálního přenosu na podobnost SM mezi jedinci?** Studovala jsem podobnost kloakální mikrobioty mezi hnízdními páry na vnitroduhovém datovém souboru u vlaštovky obecné. **[Publikace G]** Navíc jsem u vlaštovky obecné také analyzovala podobnost mikrobioty v trusu mezi mláďaty a jejich sociálními rodiči a také mezi mláďaty pocházejícími ze stejného hnízda. **[Publikace D]**

V. **Je SM stabilní v čase a rezistentní vůči environmentálním změnám?** U vlaštovky obecné jsem studovala změny v čase u opakovaně vzorkovaných mláďat a dospělců. **[Publikace D]** Dále jsem studovala vliv hnízdního prostředí

na složení SM u mláďat kukaček obecných vychovávaných dvěma různými druhy rákosníků jako sociálními rodiči. **[Publikace H]**

VI. **Jak se mění SM během ontogeneze?** U populace vlaštovky obecné jsem porovnávala SM mezi dospělci a mláďaty, jakož i změny SM s věkem mláďat. **[Publikace D]**

VII. **Jak se mění mikrobiota napříč trávicím traktem?** U slavíků obecného a tmavého jsem porovnávala mikrobiotu mezi třemi částmi tenkého střeva. **[Publikace C]** U sýkory koňadry (*Parus major*) jsem srovnávala mikrobiotu proximální (výtěr zobáku) a distální (trus) části trávicího traktu. **[Publikace I]** U kukačky obecné jsme srovnávali trus a zastrašující sekret, který je pravděpodobně původem ze slepého střeva. **[Publikace H]**

3. Publikace zahrnuté do disertační práce

A. **Kropáčková, L.**, Těšický, M., Albrecht, T., Kubovčiak, J., Čížková, D., Tomášek, O., Martin, F.-P.J., Bobek, L., Králová, T., Procházka, P. & Kreisinger, J. (2017)

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B. **Schmiedová, L.**, Kreisinger, J., Kubovčiak, J., Těšický, M., Martin, F.-P.J., Tomášek, O., Kauzálová, T. Sedláček, O. & Albrecht, T. Gut microbiota differentiation between tropical and temperate passerine birds and due to migration strategy. *Rukopis před odesláním do časopisu ISME Journal*

C. Sottas, C., **Schmiedová, L.**, Kreisinger, J., Albrecht, T., Reif, J., Osiejuk, T. S., & Reifová, R. (2021). Gut microbiota in two recently diverged passerine species: Evaluating the effects of species identity, habitat use and geographic distance. *BMC Ecology and Evolution*, *21*(1), 41. doi: 10.1186/s12862-021-01773-1

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ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Codiversification of gastrointestinal microbiota and phylogeny in passerines is not explained by ecological divergence

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Abstract

Vertebrate gut microbiota (GM) is comprised of a taxonomically diverse consortium of symbiotic and commensal microorganisms that have a pronounced effect on host physiology, immune system function and health status. Despite much research on interactions between hosts and their GM, the factors affecting inter- and intraspeci-fic GM variation in wild populations are still poorly known. We analysed data on faecal microbiota composition in 51 passerine species (319 individuals) using Illu-mina MiSeq sequencing of bacterial 16S rRNA (V3–V4 variable region). Despite pro-nounced interindividual variation, GM composition exhibited significant differences at the interspecific level, accounting for approximately 20%–30% of total GM varia-tion. We also observed a significant correlation between GM composition diver-gence and host's phylogenetic divergence, with strength of correlation higher than that of GM vs. ecological or life history traits and geographic variation. The effect of host's phylogeny on GM composition was significant, even after statistical control for these confounding factors. Hence, our data do not support codiversification of GM and passerine phylogeny solely as a by-product of their ecological divergence. Furthermore, our findings do not support that GM vs. host's phylogeny codiversifi-cation is driven primarily through trans-generational GM transfer as the GM vs. phy-logeny correlation does not increase with higher sequence similarity used when delimiting operational taxonomic units. Instead, we hypothesize that the GM vs. phylogeny correlation may arise as a consequence of interspecific divergence of genes that directly or indirectly modulate composition of GM.

KEYWORDS
birds, cophylogeny, metagenomics, microbiome, neutral/adaptive evolution

1 | INTRODUCTION

The digestive tracts of animals are inhabited by a taxonomically and functionally diverse community of microorganisms, dominated by mutualistic and commensal prokaryotes, viruses and fungi (Costello et al., 2009; Muegge et al., 2011). The cell and active gene count of this community may equal or even exceed that of the host (Qin et al., 2010; Sender, Fuchs, & Milo, 2016). It is well known that gut microbiota (GM) can interact with a broad range of host physiologi-cal systems and provide valuable ecosystem services to their host, ranging from increased digestion and vitamin synthesis efficiency, protection against pathogens and immune and nervous system regu-lation (Cryan & Dinan, 2012; Jumpertz et al., 2011; Koch & Schmid-Hempel, 2011), while GM absence or dysregulation can be

detrimental, or even lethal, to the host (Brucker & Bordenstein, 2013; Wang et al., 2015). On the other hand, individuals, populations and species all show substantial variability in GM composition, as do individuals over time (David et al., 2014; Kreisinger et al., 2017). Hosts have developed a broad range of mechanisms enabling them to fine-tune or adjust microorganism community composition. Numerous candidate host genes and physiological, morphological and behavioural traits that shape the composition of associated microbial communities have now been identified (Benson et al., 2010; Bode, 2009; Lombardo, 2008; McKnite et al., 2012; Ohbayashi et al., 2015; Salminen, Gibson, McCartney, & Isolauri, 2004). At the same time, GM have developed properties that facilitate effective proliferation within the host while reducing any potential adverse effect on host fitness, as shown by differences in the genomic regions of bacterial strains colonizing animal hosts and their environmental counterparts (Lee et al., 2013). Interactions between hosts and their GM are believed to play an important role in many microevolutionary processes, including evolution of social behaviour (Lombardo, 2008), mate choice and mating systems (Sharon et al., 2010) and speciation (Brucker & Bordenstein, 2013; Wang et al., 2015).

It is possible to distinguish two different modes of GM evolution, both of which are linked to differing degrees of "intimacy" in the host–GM relationship. The first is a trans-generational mode of evo-lution, involving transfer of GM from parents, or other community members, into progeny over many generations, including over speci-ation events (Brucker & Bordenstein, 2013; Clark, Moran, Baumann, & Wernegreen, 2000; Falush et al., 2003; Ochman, Elwyn, & Moran, 1999). A number of morphological and physiological adaptations facilitating trans-generational GM transfer have been discovered in invertebrates (Douglas, 1998). Inoculation of newborn viviparous vertebrates with maternal microbiota during delivery has been shown to have a long-term effect on their GM (Salminen et al., 2004) and, as such, is the most likely mechanism of trans-genera-tional transfer. In addition, physical contact during parental care, as well as physical contact with family members, may also represent a plausible mechanism for GM transfer between generations in both viviparous and nonviviparous vertebrates, including birds (Falush et al., 2003; Kreisinger, Cížková, Kropackova, & Albrecht, 2015; Kreisinger et al., 2017; Tung et al., 2015). It follows that trans-genera-tional transferred GM shares its evolutionary history with the host, leading to codiversification of the host and its GM. This codiversifi-cation can be driven by selectively neutral processes such as random loss of microbial taxa or a random increase in the abundance of new taxa generated by mutation. In at least some host taxa, however, there is suggestive evidence that GM evolve under selection (Brucker & Bordenstein, 2013). In this case, codiversification is expected between GM and the host traits/genes linked to the respective selective pressure(s). The second GM evolution mode involves transfer of microbes from the environment; that is, the source of microbial variability differs from trans-generational transfer, being based on acquisition of microbes with closer evolutionary links to other organisms and/or environmental microbes. Evolution through environmental transfer can be selectively neutral, which would result in a GM composition stochastically reflecting the available environmental microbial pool. Assuming microbial environmental pools to be spatially related, neutral evolution through environmental transfer would result in codiversification between GM and host geography (Linnenbrink et al., 2013). On the other hand, if selective evolution prevails, one would expect patterns simi-lar to those for codiversification of trans-generational GM and the host; that is, GM composition would reflect variability between interacting host traits and genes. Selective GM evolution through environmental transfer is theoretically faster than trans-generational evolution. By acquiring novel functions from the environment, host GM can change and react to sudden changes in selective pressure (Kikuchi et al., 2012).

Thus far, more attention has been paid to codiversification of host and GM patterns in mammals and insects. In this study, we assess the general validity of GM evolutionary patterns found in mammals and insects by identifying codiversification patterns in an avian model system, passerine birds. Phylogenetically, passerines are the most diverse group of modern birds, harbouring more than 50% of avian species richness (Jetz, Thomas, Joy, Hartmann, & Mooers, 2012). Despite relatively recent diversification, passerines exhibit a broad variation in ecology, reproductive biology, life history traits and distribution patterns. Detailed knowledge of many aspects of passerine biology, together with a robust phylogeny (Albrecht et al., 2013; Jetz et al., 2012; Kolecek & Reif, 2011; Wilman et al., 2014), has led to this group becoming an important model for research into correlated evolution in ecological and life history traits. Surprisingly, however, little effort has been aimed at integrating host vs. GM interactions into otherwise well-established ecological and evolutionary research on passerines.

Most previous studies focusing on avian GM have only considered a limited number of species sparsely distributed across the avian phylogeny (Lewis, Moore, & Wang, 2016; Waite & Taylor, 2014; Yang, Deng, & Cao, 2016). The only exception is the study by Hird, Sanchez, Carstens, and Brumfield (2015), who focused on the GM of Neotropical birds, including passerines. Tropical birds differ considerably from their temperate counterparts in many ecological traits, however, and there may also be pronounced divergence in the environmental bacterial pool interacting with the host in tropical and temperate biomes.

Here, we analyse faecal microbiota structure in more than 300 temperate passerine specimens sampled across 51 species using high-throughput sequencing of 16S rRNA amplicons, using these data as a proxy for passerine GM. In doing so, we assess to what extent interspecific differences in GM contribute to overall GM variation in terms of total diversity and taxonomic and predicted functional composition (e.g., Langille et al., 2013). Second, using unified analytical toolbox, we test for evidence of codiversification between GM and phylogeny in the species sampled, and whether the codivergence signal is explainable either through phylogenetically fixed divergence in host ecology or through geographic GM variation between sampling sites.

2 | METHODS

2.1 | Field sampling of GM

All faecal samples used in this study (319 samples from different individuals covering 51 passerine species; Table S1) were obtained during the 2014 breeding season (April–July) from sampling sites spread over 68,444 km^2 of the Czech Republic (Fig. A1 in Appendix S1). Species included in our analysis represented 51% of all passerines, covering 21 families of the 28 breeding in the Czech Republic (Table S2). Birds were caught using mist nets and placed in paper bags for approx. 15–20 min, after which they were ringed using aluminium rings following standard Czech Bird Ringing Center regulations and released. Faecal samples were harvested from the bag using sterile microbiological swabs (minitip FLOQSwabs, Copan, Italy), especial care being taken to avoid sampling the faecal layer in direct contact with the bag and to avoid any direct contact between the swab with the bag's surface. Swab tips coated with faecal mate-rial were subsequently transferred to sterile cryotubes (Simport, Canada) and filled with DNA/RNA stabilizing solution mimicking RNAlater (protocol available upon request). The samples were then cooled to 80°C within 5 days and stored until further analysis.

2.2 | Species ecological traits and phylogeny

Based on a literature review, we compiled a database comprising information on eight ecological and life history traits for the species sampled (Table S2) that can directly or indirectly affect GM content as explained more in depth in Supporting information, Appendix S1. Data on diet preferences (classified on a continuous scale, ranging from full herbivory/granivory to full insectivory/carnivory) were taken from Wilman et al. (2014). Preferred habitat was classified on a continuous scale, ranging from [1] closed forest to [7] open habitats without solitary trees or shrubs (Bohning€-Gaese & Oberrath, 2003; Kolecek & Reif, 2011). Data on longevity were obtained from www.euring.org. We noted a significant correlation between log-scaled longevity and log-scaled number of ringed individuals per spe-cies (sum of ringing totals from 15 European countries available at www.euring.org; Pearson's r = .3764, p = .0039). To account for this potential sampling bias, we used residuals from a linear regression between longevity and number of ringed individuals (both log-scaled) for all further analysis. Average clutch size, average number of breeding attempts per year, length of breeding season and average body weight were extracted from Stastny and Hudec (2011). We estimated migration length as the straight-line distance from the centre of the Czech Republic to the middle of the wintering range, using Google Earth and maps on species distribution provided in Cramp and Perrins (1993).

For further comparative analysis, we used the phylogenetic tree available at http://birdtree.org/ (Jetz et al., 2012). To account for uncertainties in phylogenetic resolution, we downloaded a random subset of 1,000 Bayesian trees, corresponding to the species sampled. If necessary, consensus phylogeny for these alternative topologies was constructed using the majority consensus method in Dendroscope (Huson et al., 2007).

2.3 | GM analysis: wet laboratory procedures

Faecal metagenomic DNA was extracted in a laminar flow cabinet using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., USA). The samples were homogenized using a MagNA Lyzer (Roche, Switzerland) for 30 s at 6,000 rpm to optimize DNA isolation efficiency, whereupon the extracted DNA was eluted to 50 ll with elution buffer. Following the recommendations of Klindworth et al. (2013), primers covering the V3–V4 variable region on bacterial 16S rRNA (i.e., S-D-Bact-0341-b-S-17 [CCTACGGGNGGCWGCAG] and S-D-Bact-0785-a-A-21 [GAC-TACHVGGGTATCTAATCC]) were used during the polymerase chain reaction (PCR) step. Both forward and reverse primers were tagged with 10-bp barcodes designed by TAGGD software (Costea, Lundeberg, & Akan, 2013). For PCR, we used 8 ll of KAPA HIFI Hot Start Ready Mix (Kapa Biosystems, USA), 0.37 lM of each primer and 7 ll of DNA template. PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles each of 98°C (20 s), 61°C (15 s) and 72°C (40 s), and a final extension at 72°C (5 min). The PCR product, together with negative controls (PCR products for blank DNA isolates), was run on 1.5% agarose gel, the concentration of the PCR product being assessed based on gel band intensity using GENOSOFT software (VWR International, Belgium). The samples were subsequently pooled at equimolar concentration and run on 1.5% agarose gel, with bands of appropriate size excised from the gel and purified using the High Pure PCR product Purification Kit (Roche, Switzerland), according to the manufacturer's instructions. Sequencing adaptors were ligated using TruSeq nano DNA library preparation kits (Illumina, USA) and the resulting amplicon libraries sequenced on a single MiSeq run (Illumina, USA) using v3 chemistry and 2 9 300 bp paired-end reads. Technical PCR duplicates were sequenced for individual DNA samples. As there was high consistency in both GM composition (Procrustean correlation: r = .97, p < .0001) and GM diversity (Pearson's: r = .96, p < .0001) between technical replicates, we merged sequences corresponding to individual samples for downstream analysis.

2.4 | GM analysis: bioinformatic processing of sequencing data

Paired-end Illumina reads were merged using PEAR (Zhang, Kobert, Flouri, & Stamatakis, 2014) and demultiplexed using MOTHUR (Schloss et al., 2009) and custom R/Bioconductor scripts (available from the authors on request). We then used Lotus pipeline (Hilde-brand, Tadeo, Voigt, Bork, & Raes, 2014) for quality filtering of FASTQ files. Sequences were excluded if the average quality score was lower than 30 or if the average quality score within a 50-bp sliding window decreased below 25. UCHIME (implemented in the Lotus pipeline; Edgar, Haas, Clemente, Quince, & Knight, 2011) was used alongside the gold.fna database (available at http://sourceforge.net/projects/microbiomeutil/files) for detection and elimination of chimeric sequences.

For the purposes of operational taxonomic unit (OTU) definition, 16S rRNA sequences are usually clustered at the 97% similarity threshold. Note, however, that strength of codiversification between microbial communities and host phylogeny can be directly linked with the similarity threshold used for OTU delimitation, particularly if host vs. GM codivergence is driven by trans-generational transfer. In this case, the codivergence signal is expected to increase with increasing microbiota resolution (i.e., higher clustering similarity) due to the slow pace of bacterial 16S rRNA gene evolution (Ochman et al., 1999; Sanders et al., 2014). On the other hand, if codiversifi-cation is driven by different mechanisms, such as phylogenetically nested divergence in host ecology, no, or even a negative, associa-tion is expected with increasing similarity threshold for sequence clustering as divergence in ecology typically induces deep taxonomic rearrangements of GM (Ley et al., 2008). In order to assess the effect of similarity threshold during OTU delimitation on codiver-gence signal, 16S rRNA reads were clustered at 91%, 93%, 97% and 99% similarity. In addition, as the results of downstream analysis could be partly dependent on clustering procedure, we used two clustering algorithms, CD-HIT (Li & Godzik, 2006) and UPARSE (Edgar, 2013). Taxonomic assignation of representative sequences for individual OTUs was performed using RDP CLASSIFIER (Wang, Garrity, Tiedje, & Cole, 2007) and the GG_15_5 reference database (DeSantis et al., 2006). Representative sequences were further aligned using PYNAST (Caporaso et al., 2010a) and their maximum-likelihood tree constructed using FASTTREE (Price, Dehal, & Arkin, 2009). The resulting OTU tables, sample metadata, OTU trees and taxonomic annotation for individual OTUs were merged into PHYLOSEQ objects (McMurdie & Holmes, 2013) for further statistical analysis. We considered OTUs assigned as "Chloroplast" (3.2% of read after quality filtering) and those not assigned to any bacterial phylum (0.2% of read after qual-ity filtering) as diet contaminants or sequencing artefacts, respec-tively, and excluded them from all downstream analyses. The number of sequences per sample was uneven (mean = 13,318, range = 1,259–76,404), which may introduce bias to the statistical analysis. We rarefied (i.e., randomly subset) read counts in the OTU tables, therefore, in order to achieve the same number of reads per sample. Rarefaction has recently been criticized as this method of normalization may decrease the statistical power of corresponding analyses. As a result, transformation of sample-specific read counts has been proposed as an alternative (e.g., McMurdie & Holmes, 2014). However, this approach is not applicable for certain steps of our pipeline, such as OTU presence/absence-based analyses (de-tailed below). As we observed high concordance in OTU abundance analysis calculated on rarefied OTU tables or OTU tables with counts converted to sample-specific proportions, we argue that in the case of our data, rarefaction is not associated with any signifi-cant decrease in statistical power.

2.5 | GM analysis: metagenomic predictions

As direct shot-gun sequencing of metagenomic DNA was not performed, we inferred GM functional composition based on predictive models integrated in PICRUST pipeline (Langille et al., 2013). In brief, this approach utilizes 16S rRNA reads and ancestral state reconstruc-tion algorithms to predict the functional content of GM samples based on the gene content of known bacterial genomes. First, we mapped our high-quality sequences against GreenGenes reference OTUs (DeSantis et al., 2006) using 91%, 93%, 95% and 97% similarity thresholds and the closed reference algorithm implemented in QIIME (Caporaso et al., 2010b). Next, metagenomes were predicted using the default PICRUST setup and classified according to the Kyoto Encyclo-pedia of Genes and Genomes (KEGG; Kanehisa & Goto, 2000). The resulting table, including predicted KEGG category abundances for individual samples, was used for downstream analysis.

The proportion of sequences unassigned to a reference OTU in GreenGenes (i.e., unusable for metagenomic prediction) was relatively high at the 97% similarity threshold (26.5%) and relatively low at the 95—91% similarity thresholds (range = 3.6%–10.1%). Hence, we decided to use 95% similarity mapping for PICRUST predictions in order to avoid potential bias associated with a high proportion of unmappable reads. The "mean nearest sequenced taxon index" (NSTI), that is, the average branch length separating OTUs from a reference bacterial genome, was .0755 when using 95% similarity mapping. This value is lower than the NTSI for microbiomes for most nonmodel species (Langille et al., 2013). Such low NTSI values, together with the high proportion of matches against the reference database, indicate high precision in our metagenomic predictions. Downstream statistical analysis on the variation of predicted KEGG content was identical with that for OTU data (detailed below).

2.6 | GM analysis: statistical inference

A positive correlation between number of sequences per sample (log10-transformed) and number of observed OTUs (Pearson's $r = .2567$, $p < .001$) indicated that there was insufficient sequencing depth to cover complete microbial diversity. Unless otherwise stated, therefore, we used sample-specific number of observed OTUs, Chao1 estimates for total number of OTUs and phylogenetic diver-sity based on rarefied OTU tables throughout the study. In addition, all alpha diversity estimates were log10-transformed to normalize dis-tribution prior to further analysis. Using the approach described in Nakagawa and Schielzeth (2013), we calculated a "conditional R^2" corresponding to the proportion of total variance in alpha diversity explained by differences at the interspecific level using linear mixed-effect models (lmer function, R package LME4). Alpha diversity indices were included as a response variable and species identity as random intercept. These models did not include an explanatory variable as they were used to estimate the overall intercept only. Next, Pagel's λ (Pagel, 1999) was calculated using Markov chain Monte Carlo generalized mixed-effect models (R package MCMCGLMM; Hadfield, 2010) in order to assess the effect of host phylogenetic relatedness on interspecific variation in GM diversity. Pagel's λ, where values ~ 0 indicate phylogenetic independence and values ~ 1 indicate com-plete phylogenetic dependence of a given trait, is a widely used measure of phylogenetic effect. Random structure in MCMCGLMM

accounted for both phylogenetic relatedness among the passerines sampled and within-species variation in GM diversity (see Garamszegi, 2014 for details). The number of iterations for these models was set at 5,000,000, with burn-in period and thinning interval set at 10,000 and 500 steps, respectively. MCMCGLMM convergence was checked using standard diagnostic tools (Hadfield, 2010). Finally, we mapped GM diversity estimates on the passerine phylogeny using the CONTMAP function in the PHYTOOLS package (Revell, 2012). As general alpha diversity patterns did not vary markedly according to clustering parameters, here we present results based on UPARSE clustering and 97% sequence-similarity threshold only for brevity.

Analysis of interspecific variation in taxonomic and functional GM composition and codivergence between GM composition and host phylogeny relied on community dissimilarity between samples. Four community dissimilarity types were applied, each capturing different aspects of GM divergence relevant to phylogeny vs. GM correlation (Sanders et al., 2014), that is, weighted and unweighted UniFrac (Lozupone & Knight, 2005), Bray–Curtis and a binary version of Jaccard dissimilarity. Jaccard and unweighted UniFrac dissimilarity only account for OTU presence/absence and, therefore, are more sensitive than Bray–Curtis and weighted UniFrac metrics to GM changes driven by rare OTUs. In addition, both unweighted and weighted UniFrac dissimilarity take account of OTU genetic similarity and, therefore, are more sensitive to community divergence driven by phylogenetically distant bacterial groups. As UniFrac dissimilarities cannot be calculated based on KEGG data, and as KEGG proportions rather than their absence or presence are more likely to reflect functional variation of GM, only Bray–Curtis dissimilarity was used in the case of metagenomic prediction.

We used the adonis (permutational MANOVA using distance matrices; R package VEGAN) to test whether there was any difference in microbiota composition at the interspecific level, including species identity as an explanatory variable and the matrix of community dissimilarities among samples as the response. In addition, the permutation-based tests described in Sanders et al. (2014) were applied to test directly whether within-species community dissimilarity was lower than dissimilarity among species. For statistical testing, t-statistics for observed difference in within- vs. between-species dissimilarity was compared with the simulated null distribution of t-statistics obtained by random permutations of distance among sample pairs.

The Procrustean Approach to Cophylogeny (PACo; Balbuena, Míguez-Lozano, & Blasco-Costa, 2013), originally developed for assessment of host vs. parasite cophylogeny, was used to test whether divergence in GM composition was correlated with phylogenetic divergence between species. Procrustean analysis assesses the level of congruence between two ordinations of multivariate data sets. We used averages of cophenetic phylogenetic distances calculated across the set of 1,000 Bayesian trees as a response and GM distance among samples as an explanatory matrix. Both GM and phylogenetic distance were scaled using principal coordinate analysis (PCoA) prior to PACo fitting. Significance testing was based on a comparison of observed vs. permuted Procrustean sum of squares. To account for the fact that we typically analysed multiple samples for each species, species identity was reshuffled across blocks of species-specific samples during the permutation routine.

The PACo approach was also used to assess strength of association between GM variation and geographic distance between sampling sites or ecological distance between species. In our data set, principal coordinates of neighbourhood matrices (PCNM; VEGAN package in R) were used to scale geographic distance between individual samples (Borcard, Gillet, & Legendre, 2011), the resulting matrix of PCNM scores being included as the response variable in PACo. Similarly, the matrix of ecological traits was scaled using PCA (Figs A2 and A3 in Appendix S1) and the resulting PCA scores were used together with the PCoA-scaled community distances for PACo inference described above. As in the case of PACo analysis of GM vs. phylogeny codivergence, species identity and sampling location identity were reshuffled across the block of corresponding samples to account for pseudoreplications. In order to provide complementary inference to the sample-level analysis, we merged data corresponding to individual species and repeated the PACo analysis. OTU tables were rarefied prior to the merging step to ensure that all samples contributed equally to species-specific GM. We argue that this approach may provide a more robust assessment of GM vs. phylogeny/ecology codivergence than sample-level analysis as abundant taxa representing putative indigenous GM are most likely to be included in species-specific pools. Furthermore, the effect of rare and/or putative transient GM OTUs (Kreisinger et al., 2017) is likely to be suppressed during construction of species-specific profiles.

Finally, we used the varpart function (VEGAN package in R) to estimate the proportion of microbial community variation explained uniquely by the effect of phylogeny, host ecology or geography, as well as the simultaneous contribution of all three effects together. Distance-based redundancy analysis (db-RDA) and permutation-based anova.cca (both from the VEGAN package in R) were used for testing the significance of individual fractions of explained variation, as described in Borcard et al. (2011). To prevent varpart overfilling, ordination axes that contributed significantly to the proportion of explained variation in GM composition were preselected using the ordiR2step function (VEGAN package in R) and included in corresponding varpart models.

All statistical analyses were performed in R version 3.2.2 (R Core Team 2016). Benjamini and Yekutieli (2001) false discovery rate method was applied to account for potential inflation type I errors due to multiple testing.

3 | RESULTS

3.1 | Taxonomic composition of passerine microbiota

We detected presence of 33 Eubacterial and two Archeal phyla in our data set. Passerine GM was dominated by Proteobacteria (44.2% of reads), Firmicutes (20.8%), Actinobacteria (15.0%), Tenericutes (9.2%), Chlamydiae (4.0%) and Bacteroidetes (2.7%). Other Eubacterial and Archebacterial phyla were only present at low frequencies (<1%).

At lower taxonomic levels, Proteobacteria were represented predominantly by Enterobacteriaceae (9.1% of reads), Diplorickettsia (5.5%), Escherichia/Shigella (3.8%), Serratia (3.4%), Helicobacter (1.6%), Methylobacterium (1.3%), Pseudomonas (1.2%) and Aeromonas (1.1%). The most abundant Firmicutes members were Catellicoccus (3.1% of reads), Enterococcus (2.6%), Lactobacillus (1.8%), Clostridium (3.4%), Lactococcus (1.1%) and Carnobacterium (1.0%). In the phylum Acti-nobacteria, Cellulomonas (4.1%) and Microbacteriaceae (2.9%) domi-nated, while Ureaplasma (5.6%) and Mycoplasma (3.1%) dominated in Tenericutes. Taxonomic composition plots indicated pronounced GM variation at both the interspecific (Figure 1) and intraspecific levels (Figs A4 and A5 in Appendix S1; for a more detailed description of GM Taxonomic composition for individual species, see Table S3).

3.2 | Variation in GM diversity

Microbial diversity varied by two orders of magnitude between samples (Table S1), with the mean SE number of 97% UPARSE OTUs per sample being 236.7524 8.5053 (range = 21–923) and the pre-dicted number of all OTUs 336.0204 10.6847 (range 45.3112– 1256.5252; summary statistics for other sequence-similarity thresh-olds and clustering algorithms are provided in Table A1 in Appendix S1).

The proportion of alpha diversity variation explained by interspecific difference (assessed as conditional R^2) was 0.2174 for the number of observed species, 0.2496 for Chao1 and 0.2260 for phylogenetic diversity. Posterior estimates of mean Pagel's λ were moderate at 0.2656 (95% credible interval: 0.0495–0.5482) for number of observed OTUs, 0.3891 (95% credible interval: 0.0682–0.7526) for Chao 1 and 0.2948 (95% credible interval: 0.0695–0.5714) for phylogenetic diversity. Projection of Chao1 values per sample onto passerine phylogeny via CONTMAP indicates relatively labile GM diversity over evolutionary time, with closely related species (such as Passer domesticus and Passer montanus, Periparus ater and other parids, or Regulus regulus and Regulus ignicapilla) tending to exhibit marked differences in GM diversity (Figure 2). On the other hand, the same analysis suggested decreased GM diversity in the Sylvioidea clade (mean SE of observed OTUs = 73.964 4.423) compared with that represented predominantly by Passeroidea and Muscicapoidea (mean SE of observed OTUs = 117.661 5.331).

3.3 | Variation in GM taxonomic composition

Adonis revealed significant interspecific differences in passerine GM that accounted for ca. 20%–30% of total GM variation, irrespective of OTU definition similarity threshold, clustering algorithm or type of distance index used (Table A2 in Appendix S1). Comparison of intraspecific vs. interspecific GM distance using permutation-based t-tests also revealed significant variation in between-species GM composition, with highly significant differences irrespective of distance index or clustering algorithm used ($p = .001$ in all cases), although the corresponding effect size was moderate (Fig. A6 in Appendix S1).

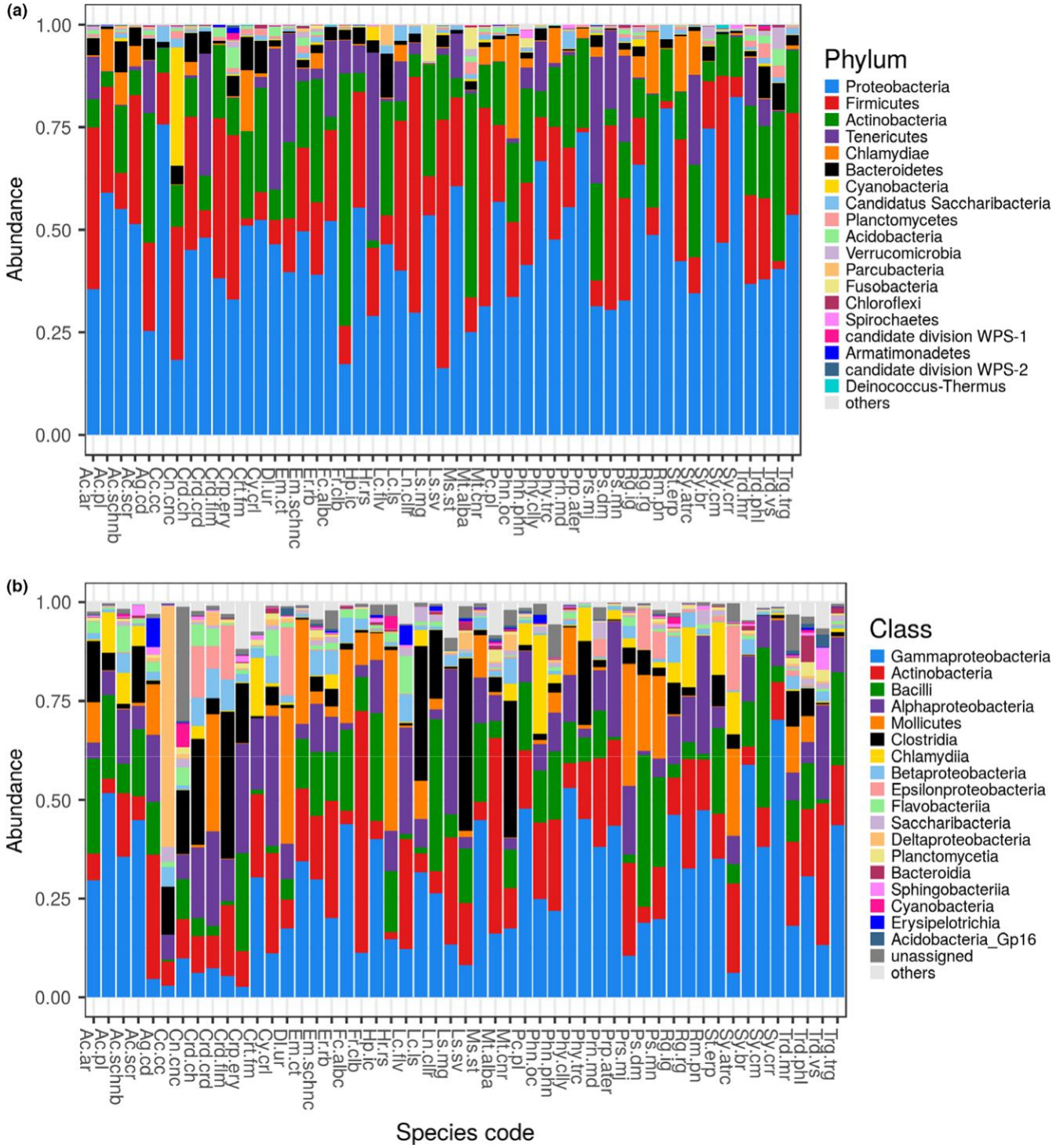
Univariate PACo analysis revealed a significant correlation between GM dissimilarity between samples and host phylogenetic divergence. While there was also a significant correlation between geographic distance between sampling sites and GM divergence, we found no significant effect of ecological divergence between hosts on GM divergence (Figure 3, and Table A3 in Appendix S1). Consequently, Procrustean correlation coefficients for GM vs. geographic divergence were ca. 25% lower and GM vs. ecological divergence ca. 35% lower than for GM vs. phylogenetic diver-gence. All forms of community distance resulted in comparable GM correlation strengths to ecological variation in the species sampled. In the case of phylogeny or geography vs. GM correla-tion, weighted UniFrac tended to exhibit lower correlation than other types of dissimilarity index. In the case of GM vs. phy-logeny or GM vs. geography, we observed no pronounced change in Procrustean correlation coefficient due to variation in sequence-similarity threshold or clustering algorithm. In the case of ecology vs. GM codivergence, however, correlation strength tended to decrease with increasing similarity threshold.

PACo analysis for species-specific GM (obtained by merging sample-specific GM profiles) provided complementary evidence for tighter correlation of GM with phylogenetic rather than ecological divergence (Figure 4; Table A4 in Appendix S1). Procrustes correlation coefficients for GM vs. phylogeny ranged between 0.7 and 0.8 across all parameters evaluated (except for weighted UniFrac dissimilarity), whereas it was only 0.5–0.4 in the case of GM vs. ecology. In addition, there was a straightforward match between ordinations for GM vs. phylogeny based on the Procrustean superimposition plots for first two ordination axes, whereas the pattern following superimposition of GM vs. ecology was unclear (Figure 4).

Consistent with the results of sample-specific PACo analysis, more complex varpart models accounting for the effect of phy-logeny, geography and variation in ecological traits simultaneously indicated the effect of phylogeny to be a stronger predictor of GM variation than that of ecology or geography. GM variation due to the simultaneous contribution of phylogeny, geography and ecology typically varied between 5% and 10% (Figure 3; Table A5 in Appendix S1). In comparison, the amount of GM variation explained exclusively by the effect of phylogeny accounted for ca. 5% of total GM variation, whereas that associated exclusively with the effect geography or ecology was considerably lower.

3.4 | Metagenomic predictions

The strength of interspecific variation in predicted metagenomes assessed using adonis ($R^2 = .2851$, $p = .001$) was comparable with interspecific divergence at the OTU level. At the same time, within-spe-cies distances calculated using predicted metagenomes were signifi-cantly lower than between-sample distances ($p = .001$) using permutation-based t-tests. PACo analysis revealed a significant correla-tion between divergence in the composition of predicted metagenomes and phylogenetic divergence (Procrustean $r = .2953$, $p = .0169$) or



F I G U R E 1 Taxonomic composition of passerine gut microbiota. Mean proportion of reads for bacterial phyla (a) and classes (b) in individual passerine species. To achieve both good taxonomic resolution and clarity of the figure, eighteen most abundant taxa are presented. Legend for species abbreviations is provided in Appendix S1

geographic distances between sampling sites (Procrustean r = .2172, p = .0001). PACo analyses, however, showed no effect of ecological divergence on metagenome composition (Procrustean r = .1923, p = .6963). According to varpart analyses, the effect of phylogeny accounted for 9.0% (p = .0001) of variation in the predicted metagenomes, while the effects of ecology and geography accounted for 0.5% ($F_{(4,300)} = 1.4423$, p = .102) and 1.3% of variation ($F_{(3,300)} = 1.1142$, p = .394), respectively. The simultaneous contribu-tion of ecology, phylogeny and geography explained 4.5% of total varia-tion in the predicted metagenomes ($F_{(11,300)} = 3.8718$, p = .001).

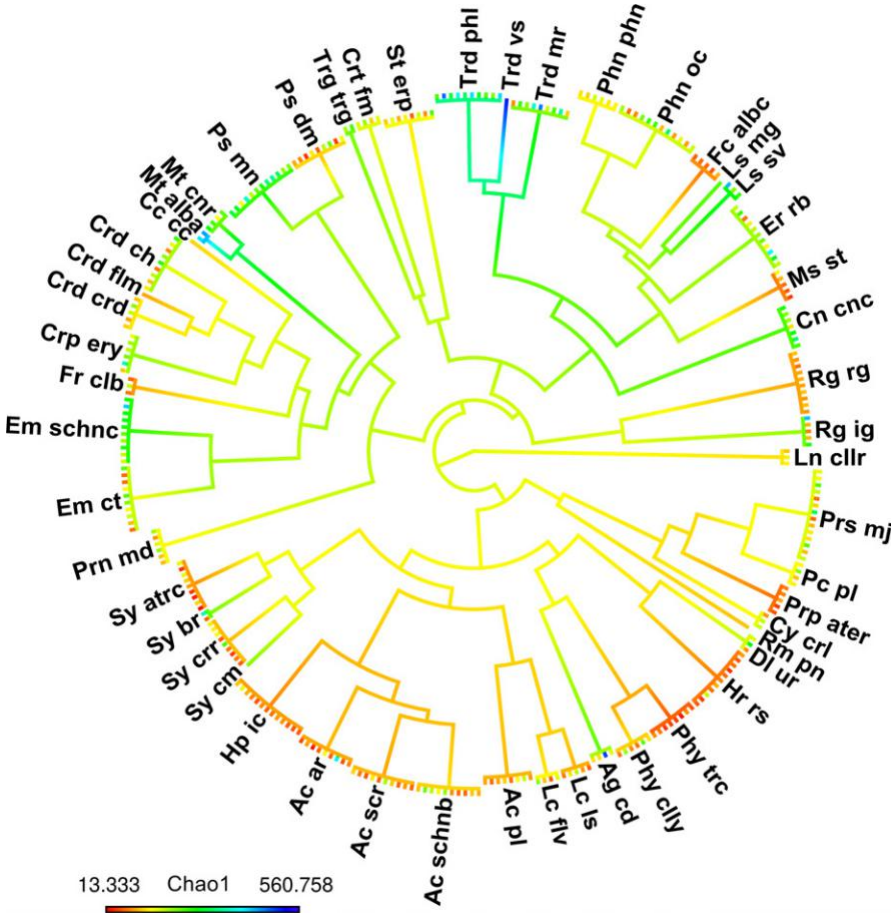


FIGURE 2 GM alpha diversity variation along Passerine phylogeny. Branch colours indicate variation in Chao1-based predictions of total GM diversity for rarefied OTU tables constructed based on UPARSE clustering with a 97% similarity threshold. Multiple samples for the same species were included as polytomies of negligible branch length. Legend for species abbreviations is provided in Appendix S1

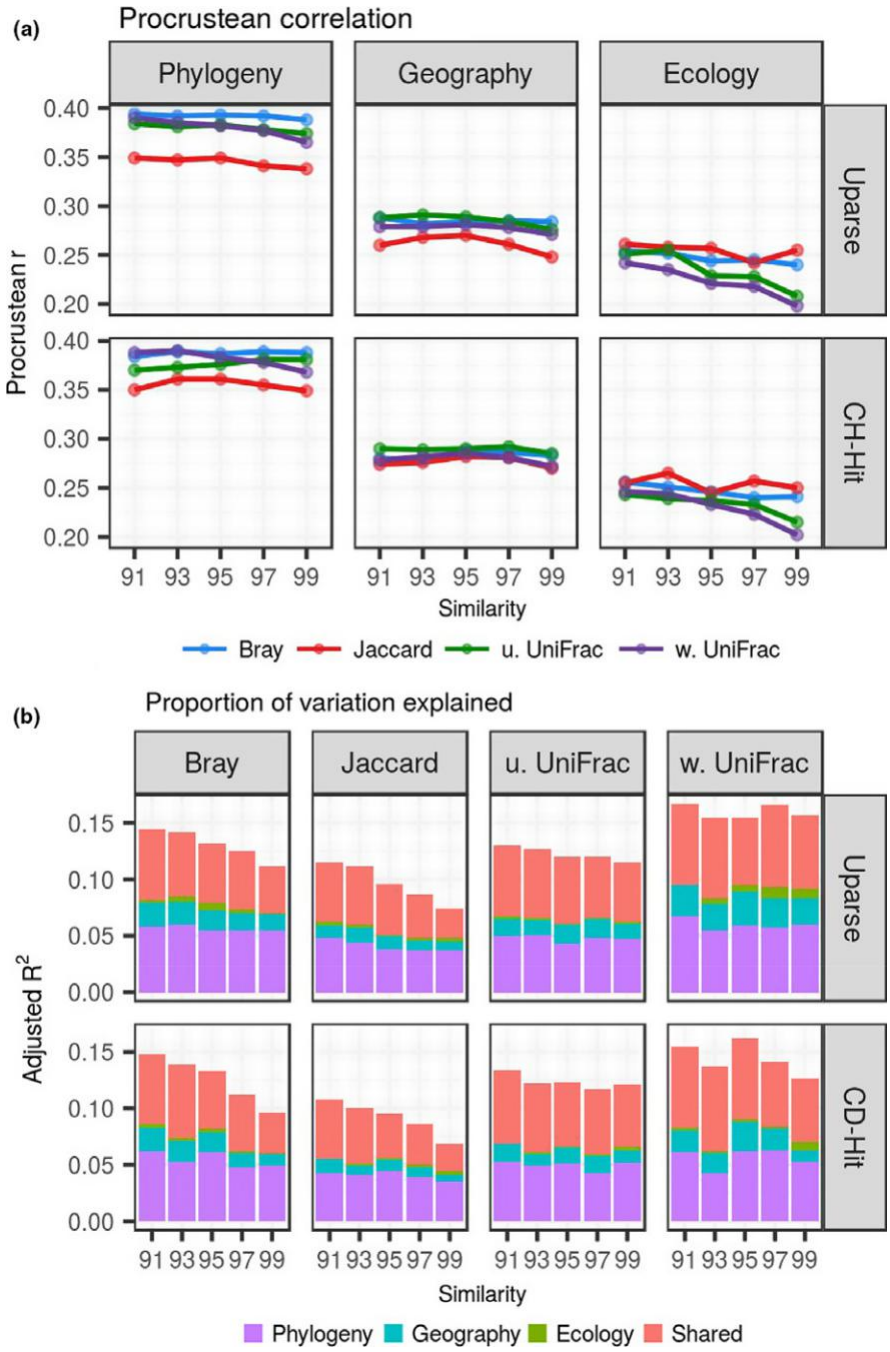
4 | DISCUSSION

Here, we provide the first comprehensive insight into interspecific and interindividual gut microbiota variation in old world passerine birds. In concordance with two recent contributions focused on Neotropical and North American passerines (Hird et al., 2015; Lewis et al., 2016), our data show that passerine GM is dominated by Proteobacteria, Firmicutes, Actinobacteria and Tenericutes (Kreisinger et al., 2015), thereby providing further support to the idea that passerine GM taxonomic composition differs markedly from that of mammals, the most intensively studied vertebrate group in this regard. Two previous comparative studies examining interspecific variation in mammalian GM (Ley et al., 2008; Muegge et al., 2011) found it to be dominated by Firmicutes and Bacteroidetes, a phylum relatively rare in passerines. Dominance of Proteobacteria, which is widespread in passerines, was only detected in a few mammalian specimens. In consequence, we believe that most current knowledge on host/GM interaction outcomes derived from mammalian models cannot be simply extrapolated to passerines or other vertebrate taxa.

However, high interindividual variation revealed systematic differences in GM among the passerine species sampled in terms of alpha diversity, as well as taxonomic and predicted functional composition, which accounted for 20%–30% of total GM variation. As in previous studies on other host taxa, these interspecific differences were correlated with host phylogeny diversification pattern; however, it provides no information on the evolutionary processes leading to it. This is particularly so with regard to neutral evolution vs. selective evolution, as the host traits linked to selection are not independent between host lineages. Furthermore, such a codiversification pattern could also arise due to both transgenerational GM evolution and evolution through environmental transfer.

Numerous studies have already shown that host ecological and life history traits codiversify with GM. Muegge et al. (2011), for example, have shown that pronounced interspecific differences in mammalian GM composition are explained through interspecific variation in preferred diet. Similarly, marked differences in the GM were found between fresh vs. salt water fish species and due to their diet preferences (Sullam et al., 2012). In order to assess the importance of selection related to host ecology and life history traits for passerine GM evolution, we scored eight ecological and life history traits for the species included in this study. To assess the importance of neutral evolution through environmental transfer, we measured the spatial distance between sampling sites.

We found that both taxonomic composition and functional GM composition were correlated with geography (according to both sample- and species-centred analyses) and host ecology (but only according to species-centred analyses). Interestingly, however, when the correlations between GM and ecology, geography or phylogeny



F I G U R E 3 Sample-level correlations between gut microbiota (GM) vs ecology, phylogeny and geography. (a) Procrustean correlation coefficients for GM variation among samples vs. ecological ("Traits"), phylogenetic ("Phylogeny") and geographic ("Geography") dissimilarities. (b) Adjusted proportion of variance in passerine GM explained by the effect of phylogeny, geography or ecology (calculations based on db-RDA and varpart). Two clustering algorithms (UPARSE, CD-HIT), five sequence-similarity thresholds (91%–99%) and four dissimilarity measures (Jaccard, Bray–Curtis, unweighted and weighted UniFrac) were used for the calculations

were compared, GM–ecology and GM–geography had ca. 50% and 30% lower correlation coefficient values, respectively, than GM–phylogeny. Moreover, multivariate varpart models revealed the independent effect of phylogeny on GM composition after statistical control for ecological and geographic disparity.

The significant effect of geography probably reflects spatial variation in environmental bacterial pools interacting with the host and is consistent with the environmental mode of evolution for passerine GM. This finding is consistent with previous studies in other vertebrates, including humans, showing a decrease in GM similarity with increasing geographic distance (Linnenbrink et al., 2013; Suzuki & Worobey, 2014).

Consistent with Hird et al. (2015), we observed relatively low effect of host ecology on GM, which is somewhat surprising, given that we focused on a broad spectrum of species with contrasting ecological and life histories. In particular, the most important gradi-ent identified based on PCA was associated with life history traits and specifically with longevity and body mass. The second PCA axis separated species according to migration behaviour and associ-ated traits such as preferred habitat and investment in reproduc-tion. Previous studies have demonstrated that ecological convergence results in GM convergence in unrelated phylogenetic lineages (Delsuc et al., 2014; Godoy-Vitorino et al., 2012). At the mechanistic level, such a convergence may evolve due to

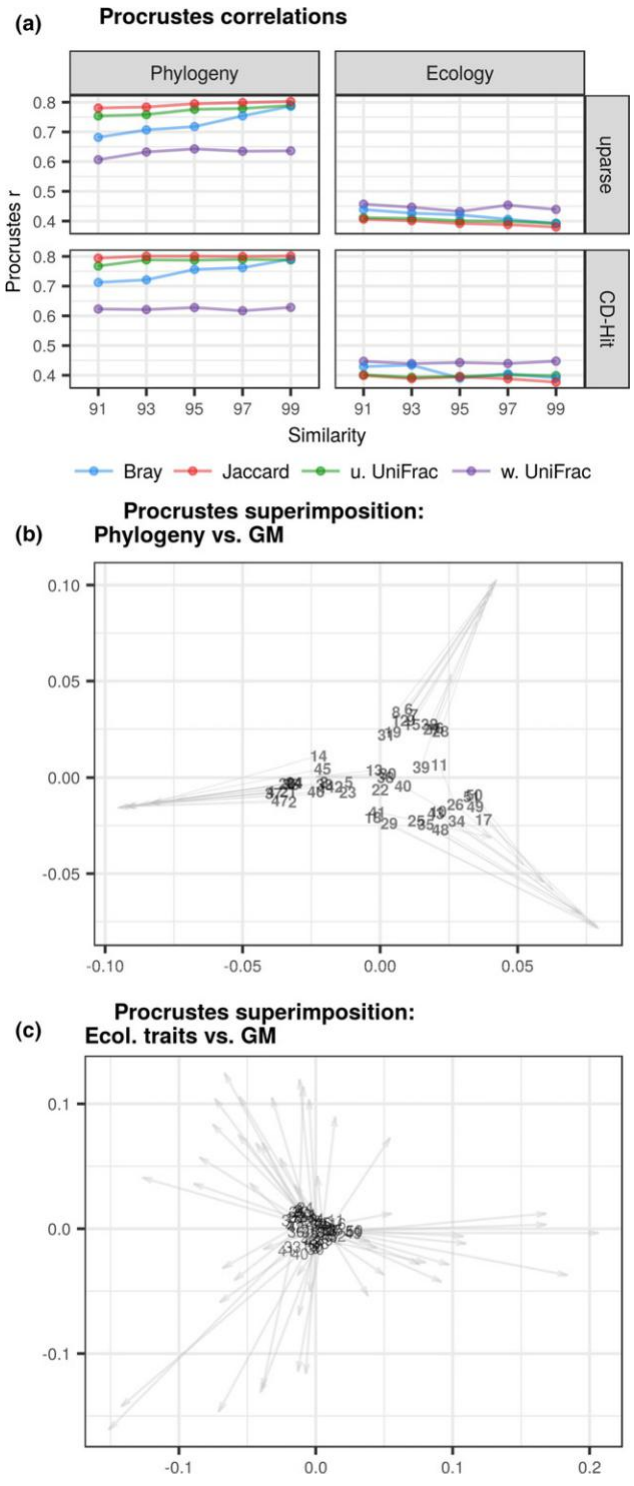


FIGURE 4 Species-level Procrustean correlation analysis. (a) Procrustean correlation coefficients for gut microbiota (GM) variation among species, and ecological ("Traits") and phylogenetic ("Phylogeny") differentiation. Two clustering algorithms (UPARSE, CD-HIT) and four dissimilarity measures (Bray–Curtis, Jaccard, unweighted and weighted UniFrac) were used for the calculations. (b) Procrustean superimposition for PCoA-scaled phylogenetic vs. GM distance and (c) ecological and GM distance. Legend for species abbreviations is provided in Appendix S1 Species codes: 1: Ac ar, 2: Ac pl, 3: Ac schnb, 4: Ac scr, 5: Ag cd, 6: Crd crd, 7: Crd ch, 8: Crd flm, 9: Crp ery, 10: Crt fm, 11: Cn cnc, 12: Cc cc, 13: Cy crl, 14: Dl ur, 15: Em ct, 16: Em schnc, 17: Er rb, 18: Fc albc, 19: Fr clb, 20: Hp ic, 21: Hr rs, 22: Ln cllr, 23: Lc flv, 24: Lc ls, 25: Ls mg, 26: Ls sv, 27: Mt alba, 28: Mt cnr, 29: Ms st, 30: Prs mj, 31: Ps dm, 32: Ps mn, 33: Prp ater, 34: Phn oc, 35: Phn phn, 36: Phy clly, 37: Phy trc, 38: Pc pll, 39: Prn md, 40: Rg ig, 41: Rg rg, 42: Rm pn, 43: St erp, 44: Sy atrc, 45: Sy br, 46: Sy cm, 47: Sy crr, 48: Trg trg, 49: Trd mr, 50: Trd phl, 51: Trd vs

evolutionary changes concerning digestive tract physiology and anatomy, which may be correlated with a shift to a new ecological niche and/or diet specialization. The anatomy of the passerine digestive tract is constrained to a large extent by natural selection in order to optimize flight ability (McWhorter, Caviedes-Vidal, & Karasov, 2009; Price, Brun, Caviedes-Vidal, & Karasov, 2015). In particular, the overall length of the gut is shorter compared to mammals (Caviedes-Vidal et al., 2007) and, consequently, food retention time is much shorter compared to mammals of similar body size (McWhorter et al., 2009). This, together with a massive reduction in those parts of the gut involved in bacterial fermenta-tion (i.e., the caecum and colon), may constrain adaptation of GM based on host ecology.

There are two opposing explanations for the major effects of phylogeny on GM evolution (independent of ecology and geography). First, it is possible that evolution of passerine GM is dominated by selectively neutral trans-generational evolution; in other words, the major portion of interspecific GM variability in passerines is not adaptive. Alternatively, selective evolution may dominate but is operating through host traits other than those defined a priori in this work. At the same time, it is possible that codiversification patterns for certain host traits are more easily traceable at the level of host genes coding for these traits. Such a trait can be, for example, host immunity. In mammals, most host genes shown to be responsible for GM regulation have been involved in immune system functioning (Benson et al., 2010; McKnite et al., 2012). Although the effect of immune genes on GM in wild populations is still poorly understood, coding sequence variation in MHC IIb has recently been shown to be a strong predictor of GM composition in sticklebacks, explaining ca. 10% of GM variation at the within-population level (Bolnick et al., 2014). Furthermore, disruption of immune gene coadaptations in wild hybrids of closely related house mouse (Mus musculus) sub-species resulted in an impaired ability to manage the GM population, resulting in an abnormal GM phenotype distinct to that in nonad-mixed individuals (Wang et al., 2015). Finally, functional variation of some immune genes, such as TLR4, is correlated with passerine phylogeny (Kralova et al. in prep.). Consequently, as a next step in research on passerine GM, we propose the inclusion of immune genes, treated in a similar manner to ecology and geography in this study.

Trans-generational evolution of bacteria, and particularly trans-fer from parents to progeny, appears to be the main cause of codiversification between GM and host phylogeny in several arthropod lineages (Brucker & Bordenstein, 2013; Sanders et al., 2014; Simon et al., 2003). However, this mechanism is unlikely to explain the

correlation between GM structure and phylogeny in passerines. First, in trans-generational evolution, concordance between GM composition and species phylogeny should increase with increasing sequence-similarity threshold used for bacterial OTU delimitation; the rationale being that higher similarity thresholds are more likely to distinguish between closely related microbes with few differ-ences in 16S rRNA, which would be expected in recently diverged host species (Ochman et al., 1999; Sanders et al., 2014). In this study, however, we failed to observe any consistent dependence between sequence-similarity threshold used for OTU selection and strength of GM vs phylogeny correlation. Similarly, ecological dis-tances accounting for phylogenetic similarities in OTUs are expected to possess a decreased ability to capture codiversification signals due to trans-generational evolution compared to its phylo-genetically uncontrolled counterparts. Again, we found no differ-ence between the performance of phylogenetically controlled and uncontrolled presence/absence distances (i.e., unweighted UniFrac and Jaccard). On the other hand, when using distances that do account for OTU abundance (i.e., Bray–Curtis), the phylogenetically controlled distance (weighted UniFrac) performed worse than the uncontrolled distance indicating environmental transfer. Overall, these patterns suggest that trans-generational evolution does not dominate in passerine GM evolution and that environmental trans-fer is more likely, at least for those microbial taxa abundant in passerine GM. It is tempting to speculate on mechanisms explaining dominance of the environmental transfer and codivergence of GM with host phylogeny observed in our study system. A possible explanation could include host traits, such as the above-mentioned immune genes, that could selectively filter microbes invading the host GM from the environmental pool.

In conclusion, we showed that passerine GM composition and evolution differ to that of mammals and insects, currently the most studied model species. Compared to mammals, we only found a limited effect of host ecology on interspecific differences in passerine GM. Similarly, we only found a limited effect of geography on GM differentiation. The major effect of phylogeny on codiversification between host and GM observed in this study, which remained significant even after statistical control for ecology and geography, suggests either unknown selective pressures or selectively neutral GM evolution. It is worth noting that despite the significance of phy-logeny and geography, most GM variation remained unexplained by these variables. Consequently, we speculate that factors operating at the within-species level may contribute to the observed GM varia-tion. As knowledge of the factors driving within-species variability in avian GM remains low (Benskin et al., 2015; Escallon et al., 2017; Kreisinger et al., 2017), however, further research in this direction is highly desirable. Compared to insects, our data fail to provide any robust evidence for the dominance of trans-generational evolution in passerine GM evolution. Instead, evolution through environmental transfer appears to prevail in the case of highly abundant microbes. Given these substantial differences, we strongly recommend that further GM studies are undertaken on wild nonmammalian organisms in order to validate general patterns of GM evolution.

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DATA ACCESSIBILITY

Raw FASTQ files: http://www.ebi.ac.uk/ena/data/view/PRJEB18750.

AUTHOR CONTRIBUTIONS

J.Kr. performed study design and data analysis. T.A., M.T., P.P., O.T., T.K. and L.B. performed field sampling. L.K., J.Ku., D.C. and J.F.M. performed laboratory analysis. J.Kr. and T.A. provided funding. J.Kr. and D.C. drafted the manuscript. All authors provided helpful comments and recommendations and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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3.2. Publikace B

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Podpis školitele

Gut microbiota differentiation between tropical and temperate passerine birds and due to migration strategy

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Abstract

Decreasing biotic diversity with increasing latitude is an almost universal macroecological pattern documented for a broad range of taxa. However, there have been few studies focused on changes in gut microbiota (GM) across climatic zones. Using 16S rRNA amplicone profiling, we analyse GM variation between temperate (Czech Republic) and tropical (Cameroon) populations of 99 passerine bird species and assess GM similarity of temperate species migrating to tropical regions with that of residents/short-distance migrants and tropical residents.

We observed no consistent GM diversity differences between tropical and temperate species. In the tropics, GM composition varied dramatically between dry and rainy

seasons and only a few taxa exhibited consistent differential abundance between tropical and temperate zones, irrespective of migration behaviour and seasonal GM changes. During the breeding season, trans-Saharan migrant GM diverged little from species not overwintering in the tropics and did not show higher similarity to tropical passerines than temperate residents/short-distance migrants. Interestingly, GM of two temperate-breeding trans-Saharan migrants sampled in the tropical zone matched that of tropical residents and converged with other temperate species during the breeding season. Consequently, our results demonstrate extensive passerine GM plasticity and the dominant role of environmental factors in its composition.

Introduction

Microbial communities associated with animal hosts have a marked effect on the host's physiology and immune system [1, 2]. In the context of these modulatory effects, the gut microbiota (hereafter GM) inhabiting the lower intestine plays a preeminent role. While GM cell counts are comparable with the number of cells in the host's body [3], functional variation within the GM, in terms of gene count, is much higher [4]. The GM has a crucial effect on the development of digestive tract morphology [5], is involved in the synthesis of essential bioactive molecules that cannot be synthesised by the host [6], stimulates the host's immune system [7, 8] and provides protection against pathogens [9]. Consequently, variation in GM composition contributes to differences in health status, body condition and other traits associated with host fitness [10–12].

Geographically segregated populations often show variations in GM [13, 14]; however, the factors driving this variation, as well as its consequences on the host, are not fully understood. Across the globe, both abiotic conditions and biotic interactions change with latitude, with potential effects on GM composition and diversity [15]. These include aerial temperature or humidity, which vary across climatic zones and can impact environmental bacterial sources of GM, but also the abundance and diversity of pathogens [16, 17], which can modulate host GM through direct or indirect interactions with the host's immune system [18, 19]. Last, but not least, variation in environmental conditions between climatic zones can impose indirect consequences on GM via selection of host phenotypic traits that affect colonisation and proliferation of bacteria within the gut. In at least one vertebrate host lineage, i.e. birds, high temporal stability and predictability of resources in tropical environments are associated with a comparatively

high life expectancy and a slower pace of life compared to phylogenetically related species inhabiting temperate climatic zones [20, 21]. This has far-reaching effects on a plethora of ecological and life-history traits that show clear latitudinal trends across birds and may also affect the GM, including reproductive investment, physiology and immunity [22–24].

Unfortunately, there is a general scarcity of empirical studies on GM variation between climatic zones. To our knowledge, most of the relevant data available focused on human GM [19, 25–27; but see 28], usually revealing marked variation in diversity and both taxonomic and functional GM composition between tropical and temperate human populations. However, these differences may have arisen as a consequence of contrasting human lifestyles in tropical and temperate zone environments, including differences in the proportion of energy-rich items in the diet [25] or the use of antibiotics and other medicaments [29], rather than latitudinal contrast in environmental factors. Further research on non-human models is needed, therefore, in order to understand the contribution of environmental factors not directly linked with variation in lifestyle on GM structure across climatic zones.

To assess how GM varies between climatic zones, we applied a comparative approach based on 16S rRNA amplicon profiling of 99 bird species covering 37 Passerine families, comprising species nesting in both temperate and tropical regions. Passerine birds represent the majority of avian species diversity and are a popular model group for research into ecological and life-history divergence between tropical and temperate organisms [20, 22, 30–32]. There is an emerging interest in microbiota associated with avian hosts, including its interaction with avian ecology and physiology [10, 33–36]. Though there have been a few studies analysing avian GM in tropical populations [37– 40], none have directly compared tropical GM with temperate populations. In general, tropical areas are characterised by highly stable environmental conditions; nevertheless, periods of high and low precipitation (i.e. rainy vs. dry season) determine periodicity in many biological processes (e.g. reproductive season, migration or moulting). This contrast between the dry and rainy seasons could also impact GM [41], though this possibility has never been addressed in birds. To fill this gap in knowledge, we included GM samples collected during both the dry and rainy seasons in our tropical samples.

Importantly, many temperate passerines migrate to tropical areas during the non-breeding season [42], whereas others spend the whole year in temperate areas. Thus, the second aim of this study was to assess how migration behaviour affects GM variation between passerine species. Environmental conditions at wintering grounds of long-distance migrants have a profound carry-over effect on a range of condition traits expressed at breeding grounds [43–45]. It is tempting to speculate that GM represents one of these carry-over effects and that exposure to environmental bacteria along migration routes and at wintering grounds could result in divergent GM structures between resident and migratory species during the breeding season. Alternatively, GM variation between migrating and resident species could be caused by ecological and physiological adaptations evolved as a consequence of migration behaviour [46–49]. Previous studies have already shown that avian GM can vary between breeding and wintering grounds [50], and that variation in migration behaviour between closely related subspecies can affect GM [51]. However, our study is the first to benefit from an extensive comparative dataset (comprising 52 species breeding in the temperate zone) that allows to search for conserved GM patterns associated with long-distance migration that discriminate migrating and non-migrating species at their breeding grounds. Furthermore, a comparison of the GM profiles of two trans-Saharan migrant species at their wintering and breeding grounds, in the context of other synoptic passerine hosts, allowed us to assess GM turnover between temperate breeding grounds and wintering grounds in tropical areas.

Methods

Filed sampling

In this study, we analysed faecal microbiota, which is shown to be a good proxy for avian GM [52, 53], with sample collection and storage as described in Kropáčková *et al.* [54]. Faecal samples of temperate passerines (405 samples from 52 species), were obtained during the 2014 breeding season (April – July) at various sampling sites in the Czech Republic (Table S1)[54]. Tropical species (205 samples from 47 species) were sampled in upland forest habitats in Cameroon (Mount Cameroon; Table S1, Fig. S1). Tropical samples were collected during both the rainy (September 2014) and dry seasons (November/December 2014), the latter corresponding to the breeding season for most tropical passerines included in our dataset. Furthermore, the migration and wintering

period of temperate trans-Saharan migrants also largely overlaps with the dry season in Cameroon. During the dry season, we also collected 25 samples from two temperate-breeding trans-Saharan migrant species, the garden warbler (*Sylvia borin*) and willow warbler (*Phylloscopus trochilus*).

Migration behaviour, diet and host phylogeny

Temperate species were categorised as trans-Saharan migrants with wintering grounds in tropical sub-Saharan Africa (n = 148 samples and 19 species; Fig. S1) or residents/short-distance migrants (n = 257 samples and 33 species) that do not fly as far as Sahara during their migration [42]. Most African species are sedentary or seasonal short-distance migrants and, while actual data on migration routes are mostly missing, it is unlikely that these species migrate over distances of more than 500 km [55]. In this study, we also consider the effect of diet as an important modulator of vertebrate GM [56], though its effect on passerine GM appears to be rather limited [54]. Data on diet for each species was extracted from the EltonTraits 1.0 database [57] and classified on a continuous scale ranging from full herbivory/granivory to full insectivory/carnivory. No other ecological variables were included in the statistical models as they are often unknown for tropical species; nevertheless, our previous research suggests a very low effect of host ecology on GM variation compared to phylogenetic relatedness and geography [54].

To account for phylogenetic co-variance, a set of 1 000 Bayesian trees with Hackett backbone was prepared for the species sampled (obtained from http://birdtree.org/) [58]. Subsequently, a maximum clade credibility tree was constructed using the maxCladeCred function in the R package phangorn [59].

Microbiota profiling

Metagenomic DNA from faecal samples was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., USA). Primers covering the V3-V4 variable region on bacterial 16S rRNA (i.e. S-D-Bact-0341-b-S-17 [CCTACGGGNGGCWGCAG] and S-D-Bact-0785-a-A-21 [GACTACHVGGGTATCTAATCC]; both tagged by 10bp barcodes) were used during the PCR step [60]. For the polymerase chain reaction (PCR), we used 8 µl of KAPA HIFI Hot Start Ready Mix (Kapa Biosystems, USA), 0.37 µM of each primer and 7 µl of DNA template. PCR conditions were as follows: initial denaturation at 95°C for 5 min followed

by 35 cycles, each of 98°C (20 sec), 61°C (15 sec) and 72°C (40 sec), and a final extension at 72°C for 5 min. The PCR products were subsequently pooled at equimolar concentration and purified using the High Pure PCR product Purification Kit (Roche, Switzerland). Sequencing adaptors were ligated using TruSeq nano DNA library preparation kits (Illumina, USA) and the resulting amplicon libraries sequenced on a single Miseq run (Illumina, USA) using v3 chemistry and 2 × 300 bp paired-end reads. We also sequenced 34 blank isolates along with the GM samples and used these for the identification of putative bacterial contaminates. A detailed description of the laboratory procedures is provided in Kropáčková *et al.* [54].

Bioinformatic processing of 16S rRNA data

Sample demultiplexing and detection and trimming of gene-specific primers were undertaken using Skewer [61]. Reads of low quality, i.e. hose with an expected error rate per paired-end read > 2, were then eliminated. Dada2 [62] was used for denoising of quality-filtered reads and subsequent quantification of 16S rRNA amplicon sequence variants (hereafter ASVs) in each sample. Chimeric ASVs were detected and eliminated using UCHIME [63] and gold.fna, a chimera-free reference database available at: https://drive5.com/uchime/gold.fa. Using the Decontam package [64], we identified and subsequently eliminated 69 putatively contaminating ASVs whose prevalence was increased in blank isolates compared to GM samples and/or showed greater representation in samples with a low concentration of metagenomic DNA (as assessed based on concentration of PCR products). Furthermore, we excluded ASVs assigned as "Chloroplast" (18.1% of reads after quality filtering), "Mitochondria" (7.2% of reads after quality filtering), or those not assigned to any bacterial phylum (<0.01% of reads after quality filtering) from all downstream analyses. Subsequently, we clustered all remaining ASVs at the 97% similarity threshold using vsearch [65] and assigned taxonomy of representative sequences for each of the 3281 resulting operational taxonomic units (OTUs) using RDP classifier (> 0.5 posterior confidence) and the Silva reference database (v.138)[66]. Representative OTU sequences were further aligned using DECIPHER [67], the maximum likelihood tree being constructed using FastTree [68]. The final dataset comprised 6.98 million high-quality sequences (median number of reads per sample = 8 353, range = 1 006 – 138 465).

Statistical analysis

GM alpha diversity was assessed using Shannon indices, the number of observed OTUs and phylogenetic diversity [69], the diversity indices being calculated for individual samples after OTU table rarefaction (i.e. random sub-setting of read counts per sample corresponding to minimal sequencing depth; 1 006 sequences/sample). To assess variation in GM composition, we calculated both Bray-Curtis and a binary version of Jaccard dissimilarity between samples. Jaccard dissimilarity only accounts for OTU presence/absence and, therefore, is more sensitive than Bray-Curtis dissimilarity to GM changes driven by rare OTUs. The resulting dissimilarity matrices were used as an input for principal coordinate analysis (PCoA), the PCoA ordination being used for visual inspection of variation in GM composition. Scores for the first two PCoA axes were later used as response variables in statistical models of variation in GM composition.

We applied a bootstrap-based tests to assess differences in GM composition between the four categories of passerine host (i.e. tropical from rainy or dry season and temperate trans-Saharan migrants or residents/short-distance migrants). Specifically, for each pair of host categories (e.g. dry vs. rainy season tropical hosts), we extracted two vectors of GM dissimilarity corresponding either to different host species from the same category or to different host species from different categories. Dissimilarities for same host species pairs were averaged. Next, we calculated the mean difference between these two vectors and corresponding 95% bootstrap-based confidence intervals using the two.boot() function in the R package simpleboot (n = 1000 resampling steps)[70]. In parallel, we analysed the effect of climatic zone, migration and diet on GM alpha diversity and composition using generalised linear mixed models (GLMM), with phylogenetic correlations fitted using the R package phyloglmm [71]. In the case of GM alpha diversity, diversity indices were used as the GLMM response, with observed OTU counts $\log_{10}$ transformed to achieve normal distribution of residuals. In the case of GM compositional variation, we considered either PCoA scores for GM dissimilarity matrices or abundances (i.e. read counts) of individual OTUs in each sample as response variables. The models estimated correlation due to host phylogeny, while systematic variation among species was modelled via random effects. All models, except those focused on OTU abundance, were assumed to have a Gaussian distribution. Models based on OTU abundance were assumed to have a negative binomial distribution, while the log-scaled total number of sequences per sample was considered as an offset to account for uneven sequencing depth. To

achieve convergence between the OTU-specific models, each model was only fitted for OTUs detected in > 20 samples and represented by > 500 sequences in total. Significance testing associated with GLMMs was based on type II sum of squares, meaning that the effect of climatic zone, migration and season was adjusted for the effect of diet and *vice versa*. In the case of OTU-level analyses, false discovery rates (FDR)[72] were calculated to account for multiple testing and only effects with an FDR < 0.05 were reported as significant. Furthermore, we applied Tukey post-hoc tests for comparisons between the four host species categories, i.e. tropical samples from dry and rainy seasons, temperate zone trans-Saharan migrants and residents/short-distance migrants. The effect of host phylogeny was assessed based on a comparison of GLMMs with and without phylogenetic correlation. Similarly, the effect of host species identity was assessed based on a comparison of models including host species as a random effect vs. a simplified model ignoring information on host species. All statistical analyses were undertaken using the R statistical environment v.4.0.3 [73].

Results

Alpha diversity variation

While the number of OTUs and phylogenetic diversity were highest in tropical birds during the rainy season, this was not true for Shannon diversity. At the same time, there was no significant difference in alpha diversity between dry season tropical birds and trans-Saharan migrants or residents/short-distance migrants sampled in the temperate zone (Fig. 1, Table 1, Table S2). Furthermore, all GM alpha diversity measurements, with the exception of Shannon index, increased with the increasing proportion of insects in the diet (Fig. S2). GLMMs on alpha diversity accounting for host phylogeny and species identity received higher support than more simplified versions lacking phylogenetic correlations and/or species identity random effects, suggesting that host species identity, as well as their phylogenetic relatedness, had an impact on GM diversity (Table S3).

Whole community GM divergence

PCoAs revealed considerable overlap in GM composition between the four passerine host groups (Fig. 2). Nevertheless, consistent with patterns in alpha diversity, subsequent GLMMs for the first two ordination axes suggested that tropical passerines from rainy season harbour the most distinct GM composition compared to other host groups (Table

2, Table S4, Fig. S3). According to taxonomic barplots (Fig. 3), this difference was largely driven by an increase in the abundance of the phylum Firmicutes during the rainy season, which represented, on average, 49.5% of reads in tropical hosts during the rainy season, 32.4% during the dry season, 34.9% in temperate trans-Saharan migrants and 32.3% in temperate residents/short-distance migrants. Aside from tropical samples during the rainy season, GLMMs revealed significant variation between the other three host groups, with the magnitude and significance of these differences being dependent on the combination of the actual GM dissimilarity index and the PCoA axis. In addition, GLMMs also uncovered a significant link between GM and diet composition (Fig. S4). The effect of phylogeny on GM composition was supported in the case of GLMMs by the first PCoA axis of Bray-Curtis dissimilarity and the second PCoA axis of Jaccard dissimilarity. Models completely ignoring both the effects of interspecific variation and phylogeny exhibited much lower performance than more complex GLMMs (Table S5).

Separate PCoAs and subsequent GLMMs for the temperate species subset revealed significant variation between trans-Saharan migrants and residents/short-distance migrants based on Jaccard, but not Bray-Curtis, dissimilarity, though the effect of diet was not significant (Table S6). On the other hand, both GM dissimilarity indices uncovered a significant effect of diet on the tropical passerine subset, as well as in the variation between rainy and dry seasons (Table S6). In addition to PCoA, bootstrap analysis was applied to elucidate the effect of climatic zone and migration on GM composition. This approach used raw dissimilarity values between host species and not PCoA ordination of all GM samples and, as such, provides more straightforward insights into effect sizes of GM differences between host categories. On the other hand, this routine does not account directly for the effect of diet and phylogeny, and does not utilise information on intraspecific GM variation. By using the bootstrap approach, we found that the highest differences in GM composition were between both temperate species categories and tropical species from the rainy season. Seasonal GM variation in the tropical zone tended to be higher than the difference between temperate and tropical GM collected during the dry season. Temperate zone residents/short-distance migrants did not exhibit higher dissimilarity to tropical birds than trans-Saharan migrants. Indeed, the opposite was true in the case of Bray-Curtis dissimilarity. These analyses also suggested low difference between trans-Saharan migrants and temperate zone residents/short-

distance migrants, with corresponding confidence intervals overlapping with zero (Fig. S5).

Bacterial OTUs involved in GM divergence

Differential abundance analysis for the 121 dominant OTUs (74% of all reads) identified 40 OTUs (41.5% of all reads) whose abundances were affected by climatic zone migration or a variation between dry vs. rainy seasons. More specifically, 19 OTUs in tropical hosts varied between the dry and rainy seasons and the abundance of three OTUs varied between trans-Saharan migrants and temperate zone residents/short-distance migrants. In addition, there was pronounced variation between temperate and tropical hosts sampled both during the dry (15 and 16 OTUs varied in trans-Saharan migrants and residents/short-distance migrants, respectively) and rainy seasons (significant difference for 25 and 23 OTUs, respectively; Fig. S6). Finally, two OTUs from the genus *Ritkettsia* and family Rhizobiales were positively correlated with the proportion of insects in the diet. OTU-level GLMM parameter estimates of contrasts between dry season tropical birds and trans-Saharan migrants were strongly correlated with contrasts between dry season tropical samples and temperate residents/short-distance migrants (Pearson correlation: $r = 0.9610012$, $p < 0.0001$), with no significant difference in their values (Paired t-test: $t = -0.80223$, $p = 0.4273$). The same held true for the comparison of rainy season tropical samples with the two groups of temperate hosts (Pearson correlation: $r = 0.8684271$, $p < 0.0001$; Paired t-test: $t = 0.80223$, $p = 0.4273$). This suggests, that tropical environment does not directly affect GM of trans-Saharan migrants during their breeding season.

As PCoA-based GLMMs revealed a pronounced effect of diet in a subset of tropical passerines, but no effect of diet in the temperate zone, we conducted additional differential abundance analyses on corresponding subsets, the results of which showed that no OTUs were associated with diet in temperate zone passerines but five OTUs that were more abundant in insectivorous passerines in the tropical zone (Figure S7).

GM of trans-Saharan migrants at wintering and breeding grounds

To assess GM changes between breeding and wintering grounds in trans-Saharan migrants, we sampled the GM of garden warblers and willow warblers in the temperate zone during their breeding season ($n = 11$ and 6, respectively) and in the tropical region

during their migration and wintering period (n = 23 and 2, respectively). PCoA suggested a dramatic effect of tropical and temperate environments on GM composition in both species. PCoA ordination showed that, while GM samples collected at their breeding grounds showed perfect overlap with other temperate zone passerines, their GM changed dramatically in the tropics. The direction of this change was congruent with the overall direction of the difference between temperate species and tropical passerines from the dry season (Fig. 4). At the same time, tropical and temperate environments had no effect on GM diversity of the two species (GLM with Gaussian distribution: $p > 0.5$ in both cases; Fig. S8X). The negative binomial models implemented in the DESeq2 package (Love, Huber and Anders 2014), which account for variation between host species, showed that the relative abundances of 16 OTUs varied between the breeding and wintering grounds (Fig. 5), with the most prominent changes detected in the case of *Serratia* and *Staphylococcus* OTUs.

Discussion

GM of tropical passerines during the rainy and dry seasons

While the tropical environment is generally considered to be relatively stable and predictable, annual fluctuations in precipitation between the dry and rainy seasons can affect a plethora of biological processes, including reproduction, migration and feather moulting [74, 75]. These periodic changes can also impact GM, as has been shown for great apes, where GM composition undergoes remarkable changes due to seasonal shifts in the diet [41]. Though several studies have been undertaken on avian GM in tropical regions [37–40], the variation between dry and rainy seasons has not yet been examined in any detail. Our comparative dataset uncovered dramatic changes in passerine GM composition and alpha diversity between rainy and dry seasons within the same geographic location, with the changes being of a comparable, or even more pronounced, effect size as GM variation between temperate and tropical host populations separated by
> 5000 km. This suggests that actual environmental conditions at a given locality have a decisive effect on passerine GM. At the proximate level, the observed changes could be linked with a range of different mechanisms, such as environmental sources of GM bacteria being affected by seasonal variation in abiotic conditions. The diet consumed is also likely to vary between the dry and rainy seasons, and thus could contribute to the differences in GM observed. Last but not least, physiological and behavioural aspects

associated with reproduction could also play some role in seasonal GM changes as reproduction of most tropical passerines in our study area mainly takes place during the dry season [76–78]. Unfortunately, we still know very little about how these factors contribute to GM composition in birds. Likewise, the role of seasonal variation of these factors in the tropical environment is not sufficiently understood. As such, further research is required to uncover the mechanistic basis of temporal fluctuations in avian GM in tropical environments.

GM variation between climatic zones

A decrease in biotic diversity with increasing latitude is a universal macroecological pattern that has been observed across a broad range of taxa [79; but see 80], including parasites and pathogens associated with animal hosts [16]. To date, however, the diversity of host-associated microbial communities has rarely been investigated in this context, with the most relevant data being based on GM profiling in human populations [19, 25– 27; but see 28]. Most of these studies provided support for an increase in GM diversity in tropical compared with temperate populations [19, 25; but see 27]. However, these differences were probably caused by contrasting lifestyles and environments between developed and developing countries. This is also supported by a recent observation that the GM of people from developed countries that stay for a long period in tropical areas and adopt a local life-style converge rapidly with those of local residents [81].

The results of our comparative study do not suggest any pronounced systematic effect of climatic zone on passerine GM alpha diversity as tropical species exhibited significantly increased GM diversity compared to temperate zone passerines during the rainy season only. Despite the lack of any clear difference in GM alpha diversity, community dissimilarity analysis revealed substantial shifts in GM composition between temperate and tropical passerines, with GM profiles from the rainy season exhibiting greater dissimilarity to temperate passerines than GM collected during the dry season. However, subsequent differential abundance analysis revealed that the divergence between temperate and tropical hosts collected during dry and rainy seasons was predominantly determined by different sets of OTUs. At the same time, only four OTUs exhibited consistent differential abundance between tropical species, regardless if sampled during dry or rainy season, and temperate species, irrespective of their migration behaviour. The OTUs exhibiting consistently higher abundance in temperate hosts comprised *Rikettsiella*

and *Rikettsia* that represents insect pathogens [82] and insect-transmitted obligate intracellular parasites of vertebrate respectively [83]. The OTU from the genus *Methylobacterium−Methylorubrum,* generally assumed to be of environmental origin [84], was also more abundant in temperate passerines. A single OTU from the genus *Bradyrhizobium,* comprising predominantly soil-dwelling species that are often involved in mutualistic interactions with plants [85], showed a significant decrease in abundance in temperate species (compared with tropical sp.), irrespective of the season in which samples were collected or host migration behaviour. As all OTUs exhibiting consistent variation between tropical and temperate species exhibited a tight association with insects or were likely of environmental origin, we assume that GM differences between climatic zones were mainly affected by a divergence in the bacterial pool present in the diet and other environmental resources. Conversely, the contribution of contrasting ecology and life history-linked phenotype traits in tropical vs. temperate passerines to GM variation between climatic zones appears to be of relatively low importance, based on our results. This does not mean that host ecology does not affect GM, however, nor that its contribution to GM variation cannot vary between climatic zones. Indeed, as differential abundance analysis detected five OTUs positively linked with the proportion of insects in diet of tropical passerines, and no OTU affected by diet in temperate hosts, our data suggests that diet has a greater effect on the GM of tropical passerines. Nevertheless, these results should be interpreted with caution. Alternative explanations for the observed patterns may lie in the fact that the dietary data we used do not reflect flexibility in foraging habits that can be greater in temperate species. Indeed, temperate zone passerines frequently change their diet opportunistically at different times of the year and according to resource availability [86]. Furthermore, the diet of temperate long-distance migrants is more dependent on insects, which further complicates statistical separation of the effect of diet and migration.

Effect of migration

In this study, we adopted two approaches to untangle how migration behaviour affects GM in passerines. First, we studied GM changes between the breeding and wintering grounds of two trans-Saharan migrant species (garden warbler and willow warbler) to assess the actual effect of climatic zone on GM. Second, we conducted a comparative analysis using GM sampled during the breeding season for temperate passerines that

varied in migration behaviour, allowing us to evaluate long-lasting pervasive effects of migration behaviour on GM.

The GM of the two trans-Saharan migrants (collected at breeding grounds in the temperate zone and at dry season wintering grounds in the tropics) exhibited substantial differences in composition, but not in alpha diversity. According to PCoA sample ordination, these changes corresponded with overall GM differentiation between temperate zone passerines and tropical passerines sampled during the dry season, but not the rainy season. Moreover, a *Staphylococcus* OTU, which contributed most to GM compositional change between wintering and breeding grounds, was also more abundant in tropical species during the dry season than during the rainy season. Altogether, our data suggest that environment is the main driver of GM variation between wintering and breeding grounds in trans-Saharan migrant species. Consistent with our results, several previous studies have reported variation in GM composition between breeding and wintering grounds, or during spring and autumn migrations in other migratory birds [35, 50, 87, 88]. However, none of these previous studies took the opportunity to contrast corresponding GM profiles with an extensive comparative dataset that included other species residing at the breeding and wintering grounds. Consequently, our study is the first to allow an assessment of whether observed changes between breeding and wintering grounds reflect GM variation in other co-occurring bird species at corresponding sites.

In addition to spatially diversified pools of environmental microbes interacting with the GM of long-distance migrants, behavioural, physiological and ecological adaptations associated with migration [46–49] could also have specific impacts on migrant GM. It is tempting to speculate that all these factors could have a long-lasting pervasive effect on the GM composition of long-distance migrants, making it distinct from that of resident species cohabiting the same geographic area.

According to our comparative analysis comprising 19 species of trans-Saharan migrants and 33 temperate residents/short-distance migrants, however, the GM of trans-Saharan migrant during the breeding season showed only a small, though still significant, difference to that of other temperate species. Furthermore, our data suggest that these differences are unlikely to be caused by bacteria incorporated into GM during overwintering and migration as alpha diversity did not increase compared to other temperate zone passerines and GM composition did not show a lower dissimilarity than

tropical hosts. Moreover, just one (genus *Lactoccus*) of three OTUs over-represented in the subset of all trans-Saharan migrants at their breeding grounds (compared to other temperate zone species) exhibited higher abundance in tropical hosts during the dry season than in temperate residents/short-distance migrants. Finally, two OTUs that exhibited a striking increase in abundance in garden and willow warblers at their wintering grounds did not vary between trans-Saharan migrants and other temperate passerines at their breeding grounds. Altogether, the results indicate that GM acquired at wintering grounds and during migration rapidly converge to a GM typical for the temperate zone following their spring arrival at the breeding grounds. This is in line with our previous study demonstrating a low level of GM stability over time in another trans-Saharan migrant, the barn swallow (*Hirundo rustica*)[34]. Similarly, Risely *et al.* [89, 90] showed that difference in GM between young non-migrating individuals and adult migrants of a non-passerine bird, the red-necked stint (*Calidris ruficollis*), tended to decline gradually after returning from their wintering grounds.

The GM of trans-Saharan migrants was characterised by an increased abundance of three OTUs of lactic acid bacteria (LAB, genus *Carnobacterium*, *Enterococcus* and *Lactococcus*) that prefer energy-rich substrates and are capable of fermenting carbohydrates under anoxic conditions [91]. The presence of some LAB is believed to be generally beneficial as they stimulate the host's immune system and produce metabolites involved in the maintenance of GM homeostasis [92]. Moreover, some LAB species contribute to the host's energy balance via improved feed conversion [93], which can be particularly beneficial for long-distance migrants adapted for energy-demanding migration, often associated with a considerable shortage of food. As the diet of migratory passerines typically comprises a higher percentage of insects, we intentionally adjusted our comparative analysis to include this confounding variable. Nevertheless, owing to the above-mentioned issues associated with dietary data, we cannot fully guarantee that our analysis precisely separated the effects of migration and diet. It is also worth noting that the OTU from the genus *Lactococcus* over-represented in our trans-Saharan migrants was also more abundant in insectivores from the tropical zone.

Conclusions

Our study provides the first insight into GM variation between tropical and temperate passerines. We show that GM composition and diversity differ dramatically between the

dry and rainy seasons in tropical hosts and, consequently, only a limited number of bacterial OTUs exhibit consistent differential abundance between tropical and temperate zones, irrespective of seasonal GM changes in the tropics. These OTUs correspond predominantly to insect-borne or environmental bacteria, suggesting a predominant impact of environment on GM differentiation across climatic zones. In trans-Saharan migrants, we observed a dramatic difference in GM between wintering and breeding grounds, consistent with overall GM differentiation of other passerines inhabiting these two regions. At the same time, systematic differences between trans-Saharan migrants and temperate residents or short-distance migrants at breeding grounds were relatively low and were probably not caused by bacteria incorporated into GM at wintering grounds or during migration. Diet had a relatively low effect and our data suggest that it has a greater effect on the GM of tropical hosts compared to temperate hosts. Altogether, our results demonstrate extensive passerine GM plasticity and the dominating role of environmental factors on its composition.

Acknowledgements

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Author Contributions

JKr, TA study design; TA, MT, OT, TK, OS field sampling; LS, JKu laboratory analysis; JKr, LS data analysis; TA, JKr, JKu funding; LS, JKr manuscript drafting; all authors provided helpful comments and recommendations and approved the final version of the manuscript.

Tables

Table 1: Parameter estimates for mixed models analysing the effect of climatic zones, season, migration and diet on GM alpha diversity. Three alpha diversity measures (Shannon diversity, Number of observed OTUs and phylogenetic diversity) were used as a response. Parameter estimates, standard error, z statistics and corresponding p values associated with individual models are shown in the table. All models included species identity as a random factors and estimated phylogenetic correlation among species.

Response	Predictor	Estimate	Standard Error	z statistic	p values
Shannon	**(Intercept)**	1.5683	0.2763	5.6760	0.0000
	Tropical (Dry S.)	-0.0740	0.1273	-0.5814	0.5609
	Temperate (Transaharan)	-0.3434	0.1425	-2.4089	0.0160
	Temperate (Resid, Short)	-0.2890	0.1429	-2.0217	0.0432
	diet	0.3273	0.2013	1.6259	0.1040
Observed	**(Intercept)**	4.2685	0.5167	8.2611	0.0000
	Tropical (Dry S.)	-0.2183	0.2444	-0.8933	0.3717
	Temperate (Transaharan)	-0.8621	0.2857	-3.0181	0.0025
	Temperate (Resid, Short)	-0.7914	0.2884	-2.7440	0.0061
	diet	0.9590	0.4001	2.3968	0.0165
Phylog. div	**(Intercept)**	2.1131	0.1850	11.4198	0.0000
	Tropical (Dry S.)	-0.1130	0.0875	-1.2908	0.1968
	Temperate (Transaharan)	-0.3605	0.1016	-3.5497	0.0004
	Temperate (Resid, Short)	-0.2979	0.1014	-2.9390	0.0033
	diet	0.3564	0.1419	2.5114	0.0120

Table 2: Parameter estimates for mixed models testing the effect of climatic zones, season, migration and diet on GM composition. Scores for the first and the second PCoA axis calculated based on two dissimilarity indexes (Bray-Curtis and Jaccard) were used as response variables. Parameter estimates, standard error, z statistics, corresponding p values associated with individual models are shown.

Response	Predictor	Estimate	Standard Error	z statistic	p values
Bray-Curtis axis 1	**(Intercept)**	-0.0097	0.0604	-0.1598	0.8731
	Tropical (Rainy S.)	0.1319	0.0164	8.0362	0.0000
	Temperate (Resid, Short)	-0.0784	0.0267	-2.9383	0.0033
	Temperate (Transaharan)	-0.0779	0.0268	-2.9108	0.0036
	diet	0.0896	0.0402	2.2270	0.0259
Jaccard axis 1	**(Intercept)**	-0.0421	0.0172	-2.4526	0.0142
	Tropical (Rainy S.)	0.1342	0.0141	9.5085	0.0000
	Temperate (Resid, Short)	-0.0496	0.0139	-3.5629	0.0004
	Temperate (Transaharan)	0.0003	0.0156	0.0210	0.9833
	diet	0.0666	0.0199	3.3468	0.0008
Bray-Curtis axis 2	**(Intercept)**	0.0105	0.0244	0.4320	0.6657
	Tropical (Rainy S.)	0.0066	0.0204	0.3241	0.7458
	Temperate (Resid, Short)	0.0534	0.0197	2.7112	0.0067
	Temperate (Transaharan)	0.0201	0.0220	0.9146	0.3604
	diet	-0.0664	0.0282	-2.3564	0.0185
Jaccard axis 2	**(Intercept)**	-0.0255	0.0420	-0.6075	0.5435
	Tropical (Rainy S.)	-0.0697	0.0156	-4.4699	0.0000
	Temperate (Resid, Short)	0.0270	0.0201	1.3385	0.1807
	Temperate (Transaharan)	0.0839	0.0201	4.1812	0.0000
	diet	0.0017	0.0307	0.0541	0.9569

Figures

Figure 1: GM alpha diversity variation for three diversity measures (Shannon diversity, number of observed OTUs and phylogenetic diversity) in tropical passerines sampled during the wet or rainy season, temperate trans-Saharan migrants and temperate residents/short-distance migrants. Different letters above bars indicate significant differences according to Tukey post-hoc tests.

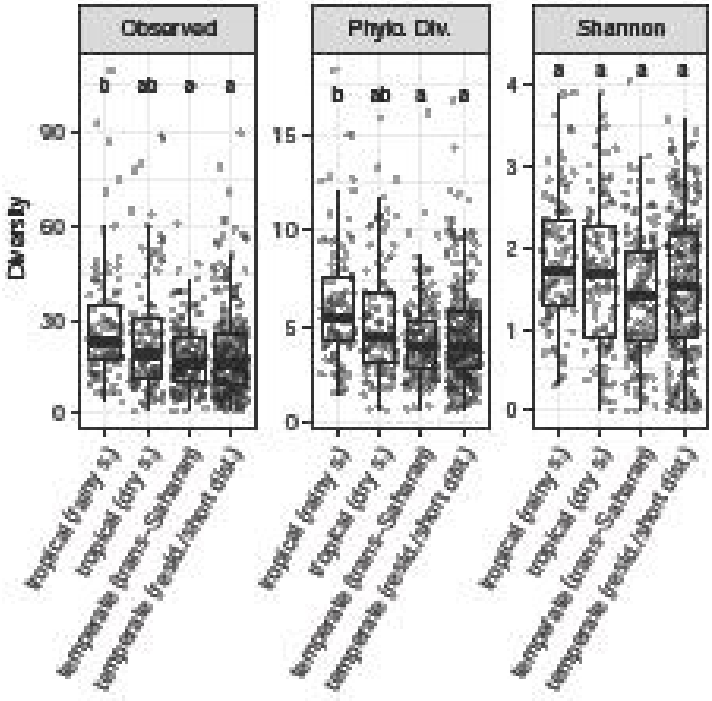


Figure 2: GM differentiation among tropical passerines collected during dry or rainy season and trans-Saharan migrants or residents/short-distance migrants collected in temperate zone according to PCoA ordination. PCoA was performed on two types of community distances (Jaccard and Bray-Curtis dissimilarities).

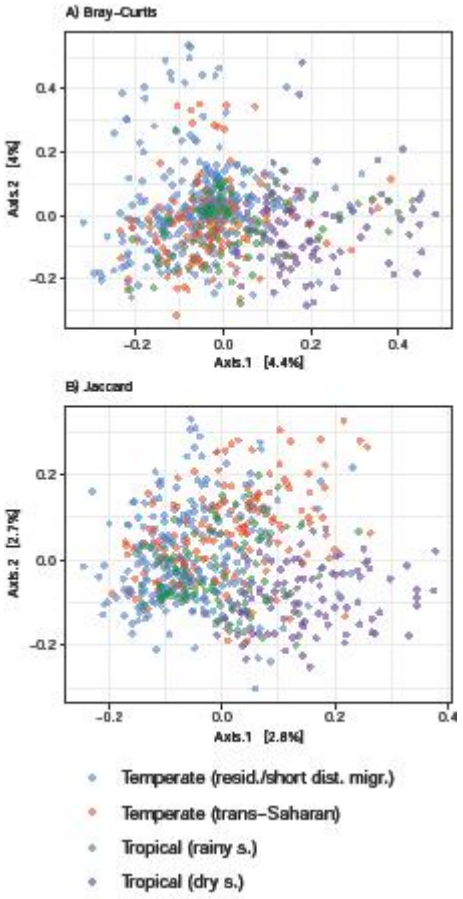


Figure 3: Average proportions of dominating bacterial phyla and classes in GM of individual passerine species ('others' indicates taxa representing < 1% of whole community).

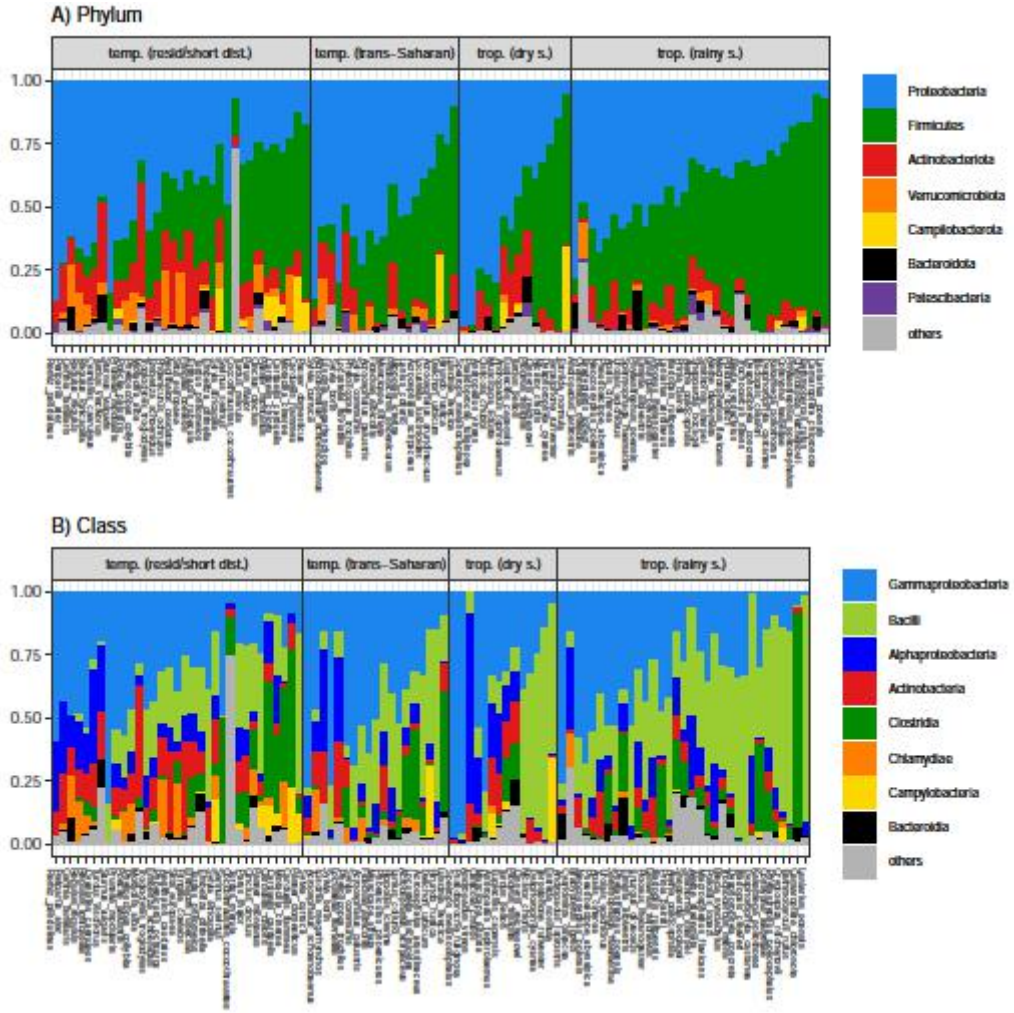


Figure 4: PCoA depicting GM changes between two trans-Saharan species (willow and garden warbler; in opaque colours) that were collected in temperate zone during the breeding period (blue) or tropical zone during the migration and wintering period (red). Samples from other species are indicated by semitransparent plotting characters.

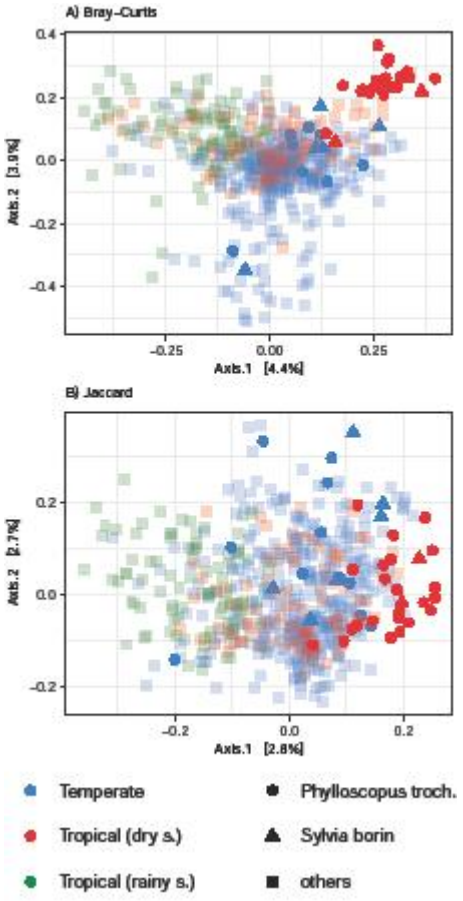
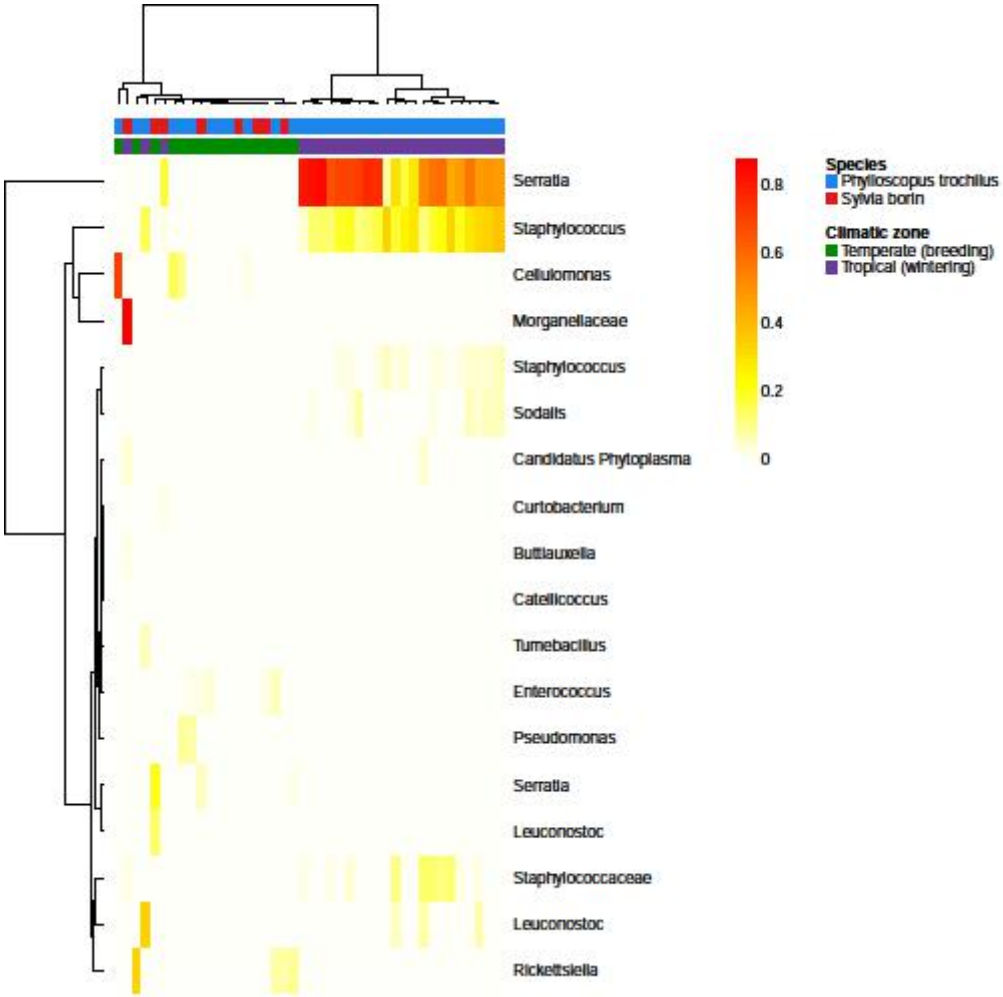


Figure 5: Heatmap for relative abundances of OTUs that exhibited significant changes between breeding vs. wintering grounds of two temperate tran-Saharan migrants. Matrix was clustered using Ward method.



Supplementary tables

Table S1: Sample metadata (code = sample identity, species, category= temperate trans-Saharan migrants [Temperate_Transaharan], temperate residents/short-distance migrant [Temperate_Resid.Short], tropical rainy season [Tropical_Wet] and tropical dry season [Tropical_Dry].**Příloha 1**

Table S2: Mixed model results testing the effect of group identity (i.e. tropical passerines collected during dry or rainy season and trans-Saharan migrants or residents/short-distance migrants collected in temperate zone) and diet on alpha diversity. Significance testing is based on Type II sum of squares. Three alpha diversity measures (Shannon diversity, Number of observed OTUs and phylogenetic diversity) were used as response. The table shows test statistics ($\chi 2$), associated degrees of freedom (Df) and probability values (P value). All models included species identity as a random factors and estimated phylogenetic correlation among species. Significant predictors (p value < 0.05) are bold.

Response	Predictor	Df	$\chi 2$	P value
Shannon	**group identity**	3	7.4465	0.0589
	diet	1	2.6436	0.1040
Observed	**group identity**	**3**	**11.9550**	**0.0075**
	diet	**1**	**5.7445**	**0.0165**
Phylog. div	**group identity**	**3**	**14.7107**	**0.0021**
	diet	**1**	**6.3072**	**0.0120**

Table S3: Effect of phylogeny and host species identity on alpha diversity. For each of the alpha diversity measures, the most complex model version (including species identity and phylogenetic correlation) were compared with simplified model versions. AIC values and corresponding model weights are shown.

	Phylogeny + Species		Species		No random	
Response	**AIC**	**weights**	**AIC**	**weights**	**AIC**	**weights**
Shannon	1491.22	0.9926	1501.00	0.0074	1522.30	0.0000
Observed	2249.18	0.9349	2254.51	0.0651	2283.24	0.0000
Phylog. Div.	1006.68	0.9490	1012.52	0.0510	1040.98	0.0000

Table S4 : Mixed model results testing the effect of group identity (i.e. tropical passerines collected during dry or rainy season and trans-Saharan migrants or residents/short-distance migrants collected in temperate zone) and diet on GM composition (i.e. PCoA scores for first two ordination axes). PCoA was conducted for Jaccard and Bray-Curtis dissimilarities. Significance testing is based on Type II sum of squares. The table shows, test statistics ($\chi 2$), associated degrees of freedom (Df) and probability values (P value). All models included species identity as a random factors and estimated phylogenetic correlation among species. Significant predictors (p value < 0.05) are in bold.

Response	Predictor	Df	$\chi 2$	P value
Bray-Curtis axis 1	**group identity**	**3**	**102.3745**	**0.0000**
	diet	**1**	**4.9594**	**0.0259**
Jaccard axis 1	**group identity**	**3**	**170.1837**	**0.0000**
	diet	**1**	**11.2011**	**0.0008**
Bray-Curtis axis 2	**group identity**	**3**	**8.4490**	**0.0376**
	diet	**1**	**5.5525**	**0.0185**
Jaccard axis 2	**group identity**	**3**	**58.8723**	**0.0000**
	diet	1	0.0029	0.9569

Table S5: Effect of phylogeny and host species identity on GM composition. GLMMs were fitted for the first two PCoA axes of Jaccard and Bray-Curtis dissimilarities. For each of these response variables, the most complex model version, including species identity and phylogenetic correlation, were compared with simplified model versions. AIC values and corresponding model weights are shown.

	Phylogeny + Species		**Species**		**No random**	
Response	**AIC**	**weights**	**AIC**	**weights**	**AIC**	**weights**
Bray-Curtis axis 1	-1144.11	0.9496	-1138.24	0.0504	-973.35	0.0000
Bray-Curtis axis 2	-789.06	0.2689	-791.06	0.7311	-762.13	0.0000
Jaccard axis 1	-1257.88	0.2689	-1259.88	0.7311	-1226.97	0.0000
Jaccard axis 2	-1148.46	0.7094	-1146.67	0.2906	-1067.92	0.0000

Table S6: Mixed model results testing differences in GM composition on the subset of passerine samples collected in tropical or temperate region. Effect of migration behaviour, season and diet was analysed. Response variables corresponded to PCoA scores for the first two ordination axes. PCoA, calculated separately for these two subsets, was conducted for Jaccard and Bray-Curtis dissimilarities. Significance testing is based on Type II sum of squares. The table shows, test statistics ($\chi 2$), associated degrees of freedom (Df) and probability values (P value). All models included species identity as a random factors and estimated phylogenetic correlation among species. Significant predictors ($p < 0.05$) are bold.

Response	Predictor	Df	$\chi 2$	P value
Bray-Curtis axis 1	**migration**	1	0.2693	0.6038
	diet	1	0.2452	0.6205
Jaccard axis 1	**migration**	**1**	**5.7479**	**0.0165**
	diet	1	0.6462	0.4215
Bray-Curtis axis 2	**migration**	1	0.4857	0.4859
	diet	1	1.9993	0.1574
Jaccard axis 2	**migration**	**1**	**16.9627**	**0.0000**
	diet	1	1.3908	0.2383
Bray-Curtis axis 1	**season**	**1**	**36.2419**	**0.0000**
	diet	**1**	**16.5502**	**0.0000**
Jaccard axis 1	**season**	**1**	**189.8693**	**0.0000**
	diet	**1**	**24.3554**	**0.0000**
Bray-Curtis axis 2	**season**	**1**	**4.9776**	**0.0257**
	diet	1	0.0126	0.9105
Jaccard axis 2	**season**	**1**	**5.2300**	**0.0222**
	diet	1	3.5069	0.0611

Supplementary figures

Figure S1: Phylogenetic tree of species included in the study. Geographic distribution and migration behaviour is indicated by different colours.

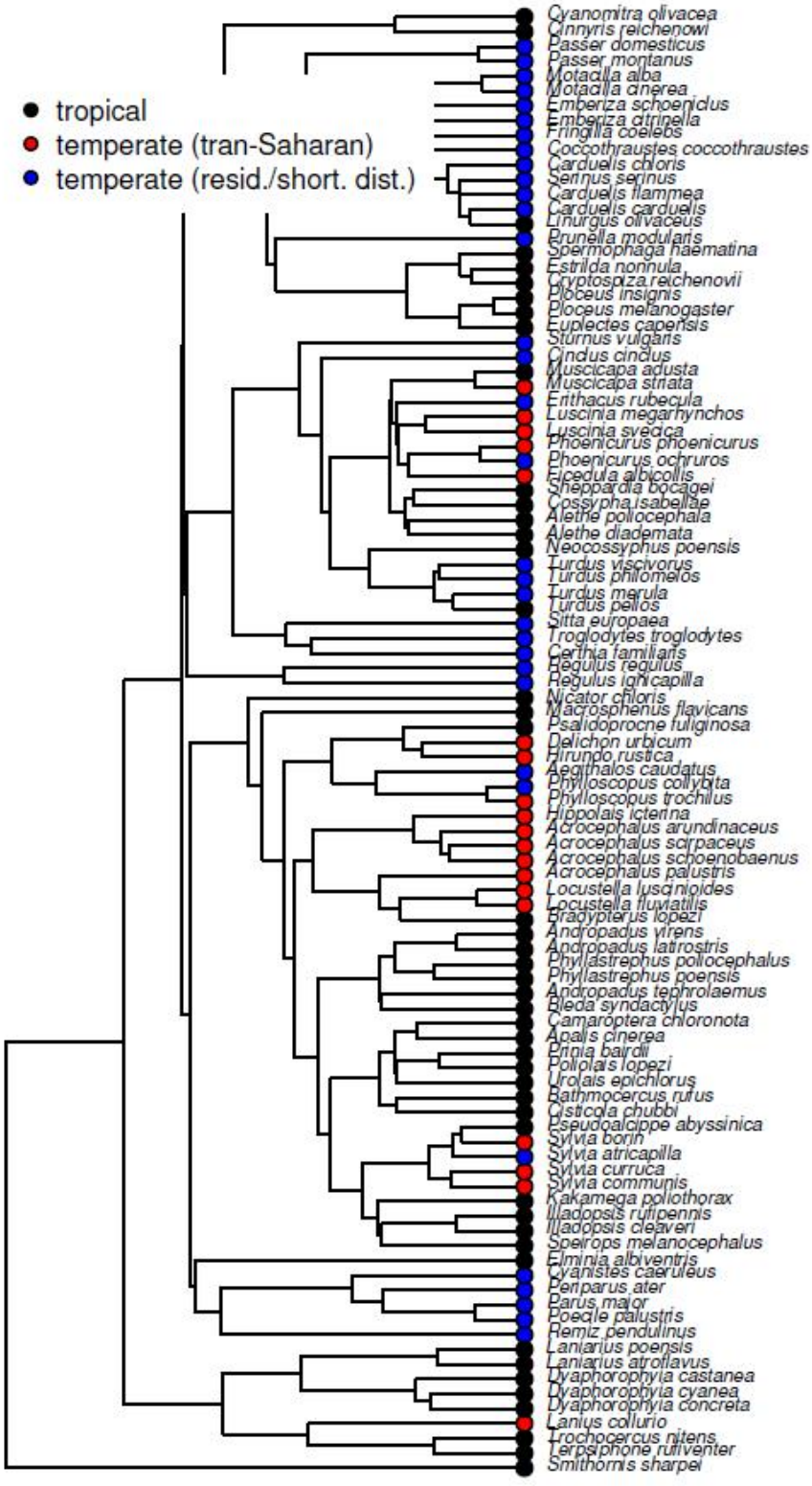


Figure S2: Association of GM alpha diversity with proportion of insects in diet. Three alpha diversity measures were analysed, namely Shannon diversity, number of observed OTUs and phylogenetic diversity. Probability values based in Type II sum of squares associated with each alpha diversity measure are shown. Parameter estimates are presented in Table S2.

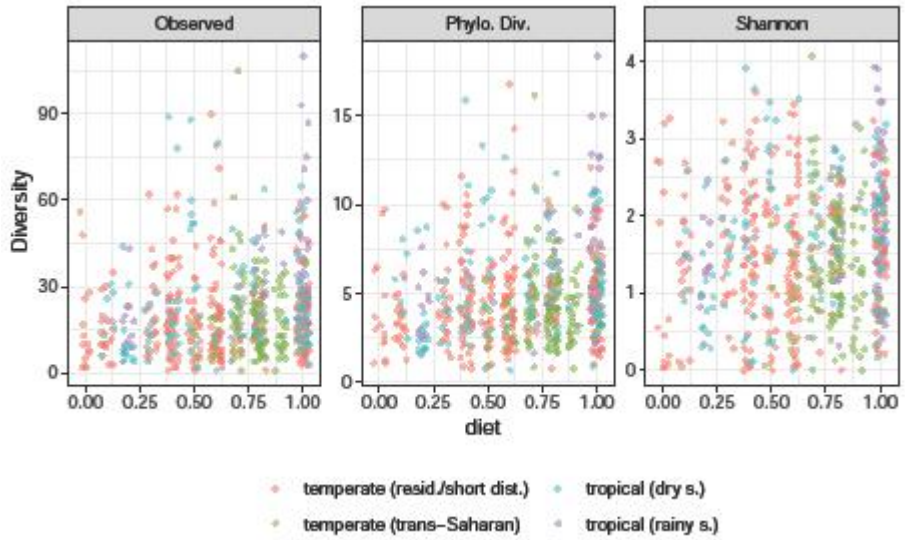


Figure S3: Variation in PCoA scores for first two ordination axes between tropical passerines collected during dry or rainy season and trans-Saharan migrants or residents/short-distance migrants collected in temperate zone. Different letters above bars indicate significant differences according to Tukey post-hoc tests.

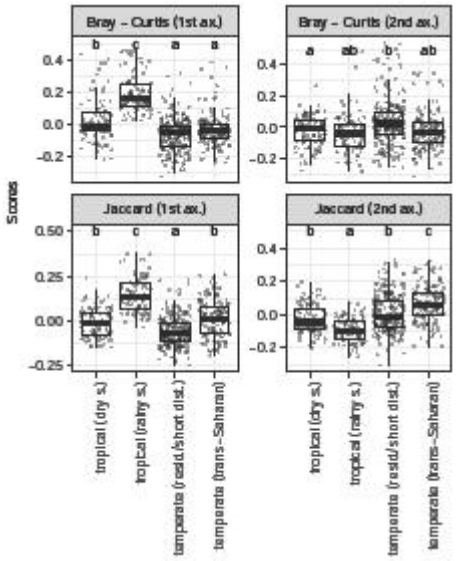


Figure S4: Association of GM composition (i.e. scores for the first two PCoA axes) with proportion of insects in diet. PCoA was conducted for Bray-Curtis and Jaccard dissimilarities. Probability values based in Type II sum of squares associated with response measure are shown. Parameter estimates are presented in Table S4X.

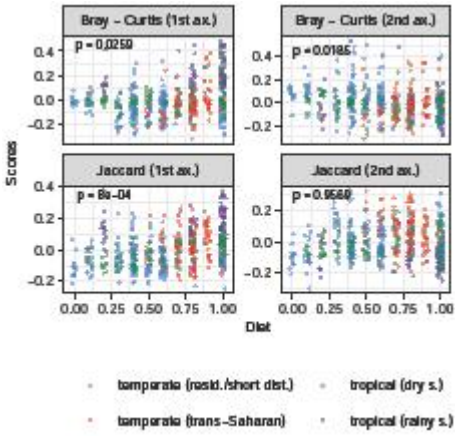


Figure S5: Average difference in interspecific dissimilarities for species belonging to the same vs. the opposite category. Error bars correspond to 95% bootstrap-based confidence intervals**.**

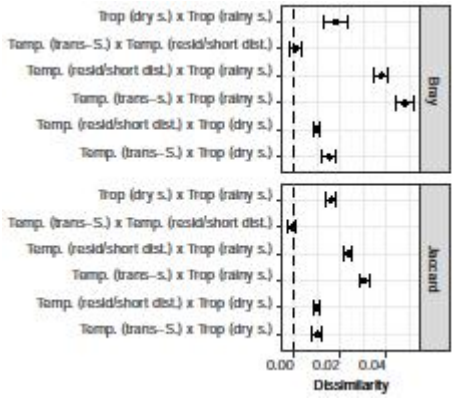


Figure S6: OTUs that exhibited significant variation between four categories of passerine hosts according to GLMMs for data with negative binomial. Only OTUs that passed multiple testing corrections (FDR < 0.05) are shown. Different letters above bars indicate significant differences according to Tukey post-hoc tests.

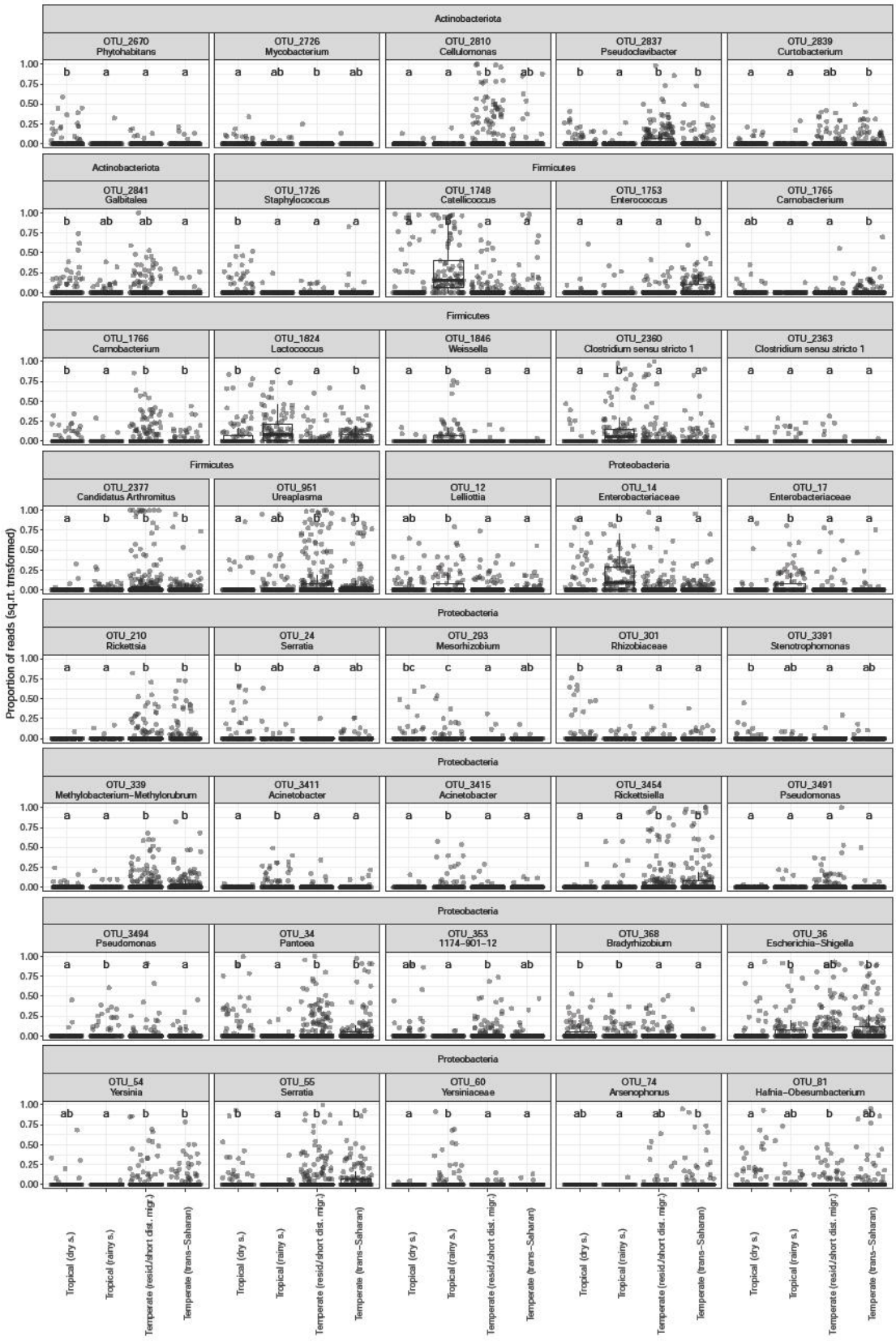


Figure S7: OTUs that exhibited significant association with diet on a subset of tropical samples according to GLMMs data with negative binomial distribution. Only OTUs that passed multiple testing corrections (FDR < 0.05) are shown.

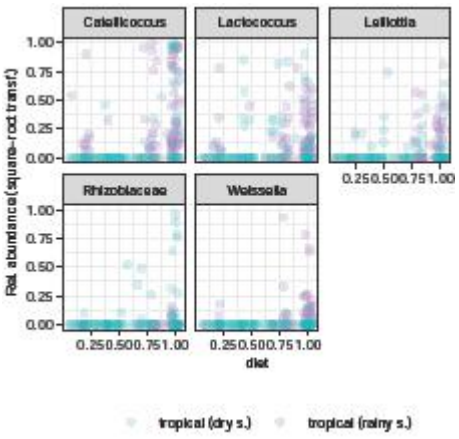
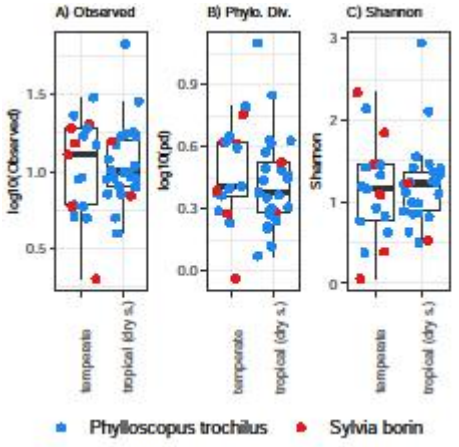


Figure S8: Alpha diversity variation between breeding vs. wintering grounds of two temperate trans-Saharan migrants.



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3.3. Publikace C

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Gut microbiota in two recently diverged passerine species: evaluating the effects of species identity, habitat use and geographic distance

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Abstract

Background: It has been proposed that divergence in the gut microbiota composition between incipient species could contribute to their reproductive isolation. Nevertheless, empirical evidence for the role of gut microbiota in speciation is scarce. Moreover, it is still largely unknown to what extent closely related species in the early stages of speciation differ in their gut microbiota composition, especially in non-mammalian taxa, and which factors drive the divergence. Here we analysed the gut microbiota in two closely related passerine species, the common nightingale (*Luscinia megarhynchos*) and the thrush nightingale (*Luscinia luscinia*). The ranges of these two species overlap in a secondary contact zone, where both species occasionally hybridize and where interspecific competition has resulted in habitat use differentiation.

Results: We analysed the gut microbiota from the proximal, middle and distal part of the small intestine in both sympatric and allopatric populations of the two nightingale species using sequencing of bacterial 16S rRNA. We found small but significant differences in the microbiota composition among the three gut sections. However, the gut microbiota composition in the two nightingale species did not differ significantly between either sympatric or allopatric populations. Most of the observed variation in the gut microbiota composition was explained by inter-individual differences.

Conclusions: To our knowledge, this is the first attempt to assess the potential role of the gut microbiota in bird speciation. Our results suggest that neither habitat use, nor geographical distance, nor species identity have strong influence on the nightingale gut microbiota composition. This suggests that changes in the gut microbiota composi-tion are unlikely to contribute to reproductive isolation in these passerine birds.

Keywords: Gut microbiome, Reproductive isolation, Diet, Habitat use, Passerines, *Luscinia*

Background

Vertebrates harbour taxonomically and function-ally diverse microbial communities in their intestines, referred to as the gut microbiota [1, 2]. It has been shown that the composition of the gut microbiota can have pro-found effects on the host's physiology and morphology, as well as behaviour [3–8]. Moreover, between-species divergence in the gut microbiota composition could play a role in the establishment of reproductive isolation between species and thus in generating species diversity [6, 9, 10]. Despite recent intensive research on variation

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in the gut microbiota composition within and between vertebrate species [1, 11–14] the factors that generate the gut microbial diversity are still not sufficiently understood, especially in non-mammalian taxa. Additionally, it is largelly unknown how often closely related species differ in the gut microbiota composition and thus how widespread the effect of the gut microbiota in speciation.

Variation in the gut microbiota composition may arise due to multiple factors including differences in the host's diet [15–18], habitat [19–21] or geographical range [22–24]. In addition, host genes involved in the manage-ment of the gut microbiota can play important roles in structuring gut microbial communities [25–27]. All these factors as well as a stable and long-lasting transfer of the gut microbiota from parents to progeny may generate divergence in the gut microbiota composition between species. However, the importance of specific factors in shaping gut microbiota diversity seems to differ among different vertebrate lineages [28].

Between-species divergence in the gut microbiota composition can contribute to the origin of reproductive isolation by multiple ways. First, host-associated micro-biota may be involved in assortative mating and thus the establishment of pre-mating reproductive barriers [6, 9]. Furthermore, interactions between the host genome and the microbiome, between different microbes of the same metagenome, or between different host's genes involved in the management of microbial communities can be disrupted in hybrids [13]. This can cause gut microbiota dysbiosis in hybrid individuals, which can reduce their fitness and contribute to postzygotic isolation [10, 12, 13].

Here we studied the gut microbiota variation in two closely related passerine bird species, the com-mon nightingale (*Luscinia megarhynchos*) and the thrush nightingale (*Luscinia luscinia*). The two species diverged approximately 1.8 Mya [29] and their breed-ing areas currently overlap in a secondary contact zone spanning across Europe [30], where they occasionally hybridize. Both species are migratory and differ in their wintering grounds in sub-Saharan Africa [31]. They both preferentially occupy dense shrubby vegetation (often close to water bodies) and feed mostly on insects [31, 32]. In allopatric regions they inhabit the same habitats, while in the sympatric region their habitat use and diet have partially differentiated, presumably to reduce interspecific competition [32–34]. Common nightingales in sympatry occur more frequently in dry habitats and feed mostly on Coleoptera, whereas thrush nightingales in sympatry prefer wet habitats and feed more often on Diptera [32]. Given that the gut microbi-ota composition can rapidly shift depending on habitat and prevailing diet [20, 35], the gut microbiota may have differentiated between the two nightingale species in sympatry.

Rarely- occurring interspecific hybrids between the common nightingale and thrush nightingale are viable, but their relative fitness compared to the parental spe-cies has not yet been evaluated thoroughly. Neverthe-less, it is known that following Haldane's rule, F-1 hybrid females are sterile while F-1 hybrid males are fertile [36–38]. It has been also documented that backcross hybrids are rarely present in the sympatric population [39] and that gene flow can occur between the two spe-cies [29, 40].

To elucidate the factors shaping the gut microbiota variation in nightingales, we analysed the gut micro-biota profiles in sympatric and allopatric populations of both species using high-throughput sequencing of bac-terial 16S rRNA. Unlike most studies on vertebrate gut microbiota based on the analyses of faecal samples as a proxy for intestinal samples, we analysed the micro-biota along the whole small intestine to obtain a more complex view of the gut microbiota composition in the two nightingale species. First, we tested whether the gut microbiota composition differs between the two species and whether there are any bacteria exhibiting host spe-cies specificity, which would suggest that the gut micro-biota could potentially contribute to the reproductive isolation between the two nightingale species. Second, we compared the level of interspecific differences in the gut microbiota composition in sympatry and in allopatry. A higher divergence in sympatry would imply a stronger effect of habitat use or diet, while a higher divergence in allopatry would indicate a stronger effect of geographical region on the gut microbiota divergence [41, 42]. Simi-lar levels of divergence in sympatry and allopatry would suggest that the divergence in host genes involved in the management of the gut microbiota and/or long-term transfer of the gut microbiota from parents to progeny may cause a divergence of the gut microbiota between the two nightingale species. To our knowledge, this study is the first to focus on the gut microbiota composition in a pair of closely related avian species with incomplete reproductive isolation, and to examine its variation in sympatric and allopatric populations. Our findings could have important implications for understanding the fac-tors affecting variation in the gut microbiota composition in birds and the possible role of gut microbiota diver-gence in avian speciation.

Results

We sequenced metagenomic DNA extracted from three sections of the small intestine in 18 individuals of the common nightingale (*Luscinia megarhynchos*, hereafter

CN) and 18 individuals of the thrush nightingales (*Luscinia luscinia*, hereafter TN). In both species, half of the individuals came from the sympatric region and half from the allopatric region. The three sections of the small intestine were: (1) the duodenum(the proxi-mal part of the small intestine, hereafter DU), (2) the jejunum (the middle part of the small intestine, here-after JE), and (3) the ileum (the distal part of the small intestine before caecal protuberances, hereafter IL). In total, 108 samples were sequenced (three gut samples for each of the 36 individuals).

After the filtering steps, which included (1) removing low -quality sequences, chimeric sequences, sequences not consistently present in both technical duplicates for a given sample, and non-bacterial sequences (includ-ing especially reads from coccidia parasites) and (2) excluding samples with less than 1000 reads after all the filtering steps above (see Material and Methods for details), we obtained a final dataset consisting of 57 samples. These included 22 samples from CN (DU= 6, JE = 6 and IL = 10, together representing 12 individu-als) and 35 samples from TN (DU = 10, JE = 10 and IL = 15, together representing 16 individuals) (Addi-tional file 1: Table S1). These samples were covered by a total of 276,676 reads. The mean sequencing depth per sample was 4035 (range = 1036–10,261) in CN and 5,369 (range = 1041–14,740) in TN. In total, 272 Operational Taxonomic Units (OTUs) were identified, and the average number of OTUs per sample was 8.33 (range: 1–46). Twelve bacteria phyla and 126 genera were detected in the gut microbiome of the two night-ingale species (Fig. 1).

The most common bacterial phyla were Firmicutes (57.95% of relative abundance, dominated by the genera *Catellicoccus, Candidatus Arthromitus* and *Clostrid-ium* sensu *stricto*), Proteobacteria (30.49%, dominated by the genera *Escherichia/Shigella* and *Rickettsiella*), Tenericutes (6.60%, dominated by the genera *Myco-plasma* and *Ureaplasma*), Actinobacteria (1.99%, domi-nated by the genera *Actinoplanes* and *Kocuria*) and Bacteroidetes (1.76%, dominated by the genus *Candi-datus Cardinium*). The relative abundance of all other bacterial phyla was less than 1% (Fig. 1). Regarding the gut sections, Firmicutes and Proteobacteria were the dominant bacterial phyla in all three-gut sections (Fig. 1). The presence of Tenericutes, Bacteroidetes, Actinobacteria and Chlamydia was largely individually specific (Fig. 1).

Differences in microbial α- diversity among gut sections, between species and regions

As estimates of microbial α-diversity, describing the diversity of the microbiome in each sample, we used the Chao1 diversity index (accounting for undetected rare OTUs), the number of observed OTUs and the Shan-non diversity index. For all three measures of α-diversity, the microbial diversity was highest in IL (Fig. 2, Table 1 and Additional file 1: Table S2a). We then used linear mixed models (LMMs) to test for the effects of the gut section (i.e. DU, JE, IL), the nightingale species (i.e. CN and TN), geographical region (i.e. allopatry and sympa-try) and the species -region interaction on the respective α-diversity indexes. The effect of individual was included as a random effect.

LMMs revealed a significant effect of the gut sec-tion on α-diversity for the log-transformed Chao1 index ($p = 0.002$, Tables 1 and Additional file 1: Table S2a, Fig. 2b) and for the log-transformed number of observed OTUs ($p = 0.004$, Tables 1 and Additional file 1: Table S2a, Fig. 2a), but not for the Shannon index (Table 1 and Additional file 1: Table S2a, Fig. 2a). Pair-wise post-hoc Tukey tests on Chao1 index and the number of observed OTUs showed that α-diversity was significantly higher in IL compared to the JE for the log-Chao1 ($p = 0.048$). All other pairwise comparisons were, however, insignificant (Fig. 2 and Additional file 1: Table S2b).

Generally, α-diversity estimates were higher in TN (mean ± standard error (se): Chao1: 9.34 ± 0.37, Shan-non: 0.96 ± 0.12, number of observed OTUs: 9.11 ± 1.44) than in CN (mean ± se: Chao1: 6.41 ± 0.30, Shannon: 0.73 ± 0.11, observed OTUs 6.18 ± 0.89). However, α-diversity was higher in TN samples compared to CN samples only in sympatry, not in allopatry (Fig. 3). Nev-ertheless, when taking into account the inter-individual variability, the effect of the species identity on α-diversity was not significant (LMMs: $p > 0.05$, Table 1). The effects of the region and the interaction between species identity were also insignificant (LMMs: $p > 0.05$, Table 1).

Differences in microbial composition (β- diversity) among gut sections

As measures of microbial composition dissimilarity between samples (β-diversity), we calculated two types of distances: the binary Jaccard distance and the Bray–Cur-tis distance. The binary Jaccard distance accounts for the presence/absence of OTUs and is thus more sensitive to gut-microbiota changes driven by rare OTUs. The Bray–Curtis distance accounts for differences in the OTUs' rel-ative abundance and is thus less sensitive to rare OTUs.

We detected within-individual correlations in micro-bial composition among the three gut sections (Man-tel test: $p < 0.05$ for both distances; range of correlation coefficients is 0.91–0.96 for Bray–Curtis distance and

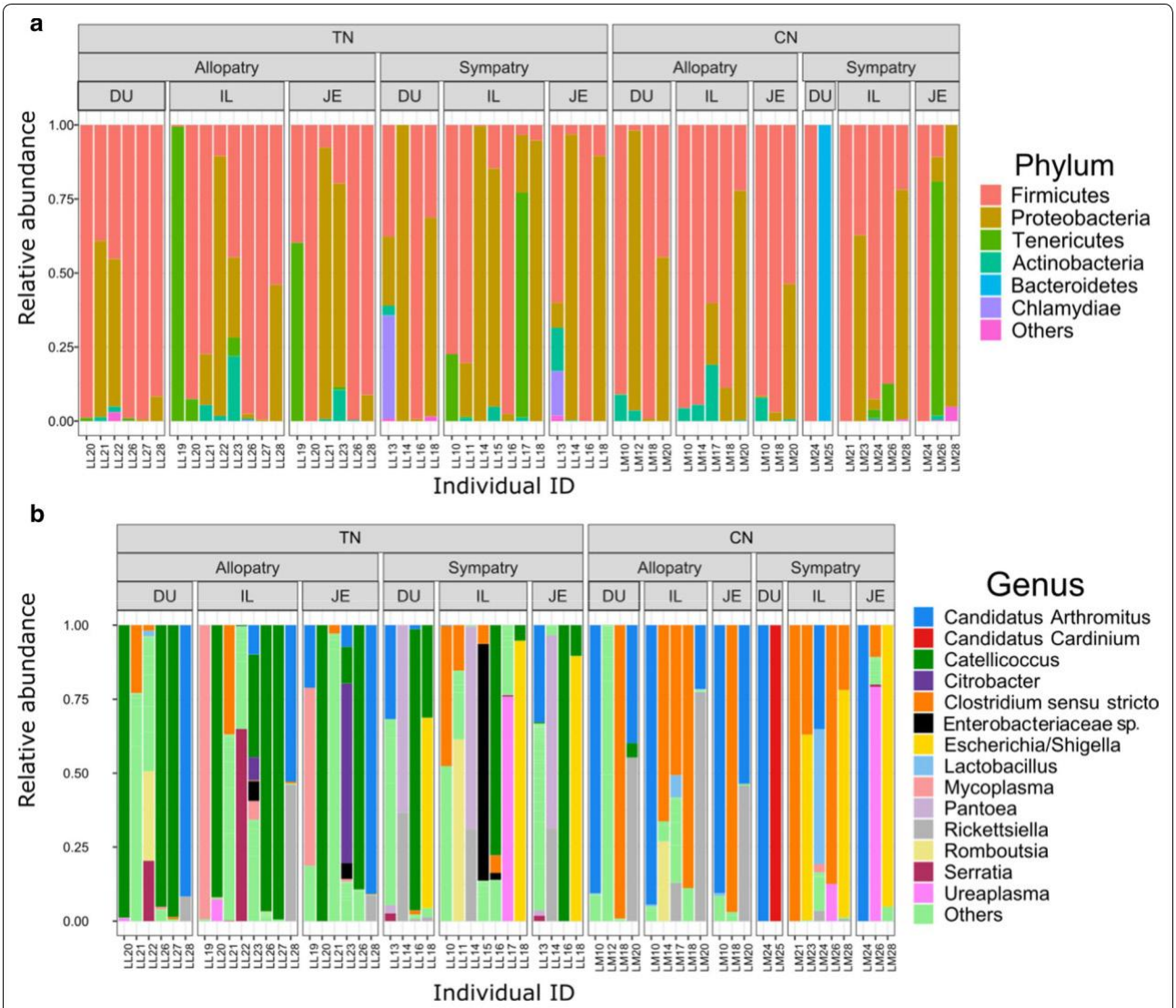

Fig. 1 Relative abundances of bacterial phyla (**a**) and genera (**b**) in common nightingale (CN) and thrush nightingale (TN) samples from allopatry and sympatry. The three gut sections (duodenum, DU; jejunum, JE; and ileum, IL) are shown separately. Only the most abundant phyla (relative abundance > 0.5%) and genera (relative abundance > 1%) are represented. Less abundant phyla and genera are included in the category 'Others'

0.32—0.70 for Jaccard distance; Additional file 1: Table S3). The db-RDA analysis revealed significant differences in the microbiota composition among the three-gut sections for the Jaccard distance ($F_{2, 54} = 0.775$, $p = 0.035$) but not for the Bray–Curtis dis-tance ($F_{2, 54} = 0.658$, $p = 0.13$). However, the variation in the gut microbiota composition explained by differ-ences among gut sections was very low both for the Jac-card distance (adjusted – $R^2 = 0.028$) and Bray–Curtis distance (adjusted – $R^2 = 0.024$) (Fig. 4a, b).

Differences in microbial composition between the two species in sympatry and allopatry

The db-RDA showed no significant effect of species identity or region on the gut microbiota composi-tion (Table 2, model complete) although it revealed a weak but significant interaction between species and regions for both distance matrices (Bray–Curtis dis-tance: $p = 0.034$ and Jaccard distance: $p = 0.043$, Table 2, model complete). This may suggest either that the spe-cies differ in microbial composition only in sympatry or allopatry, or that some differences within the species may exist between allopatric and sympatric regions. We thus tested these possibilities using db-RDA models focusing

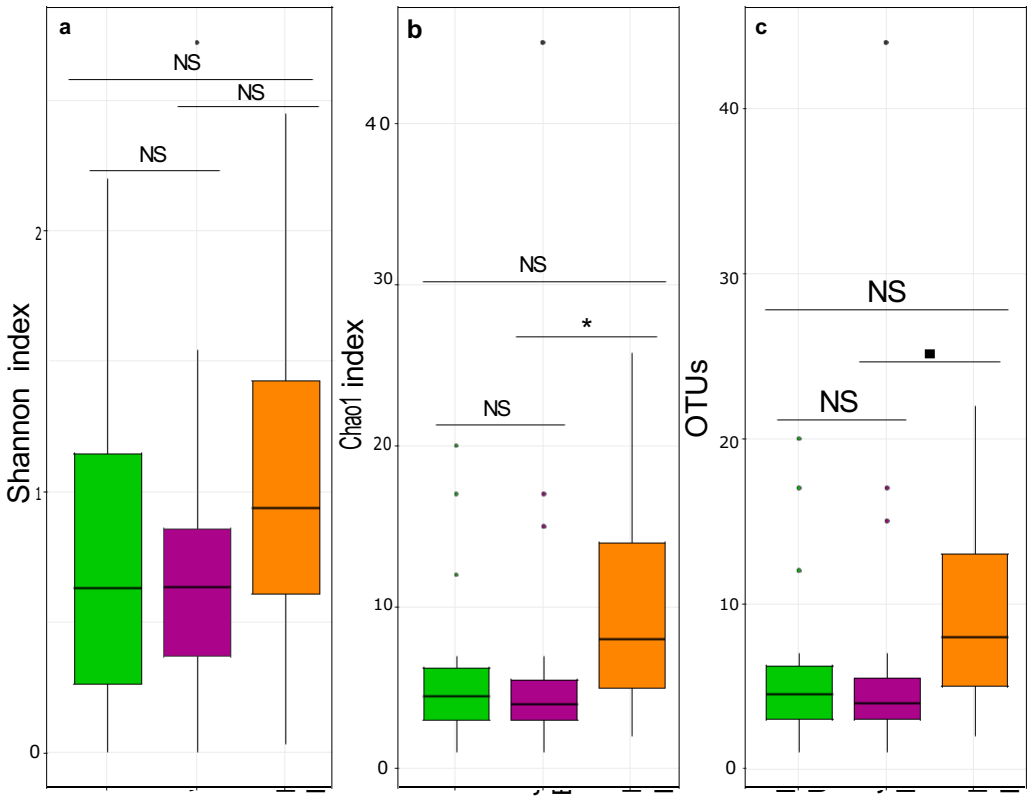

Fig. 2 α-diversity of the gut microbiota in the three gut sections across both nightingale species. α-diversity was measured by the Shannon diversity index (**a**), Chao1 diversity index (**b**) and the number of observed OTUs (**c**). To account for uneven sequencing depths, α-diversity indexes were calculated based on the rarefied OTU table (lowest number of reads = 1036)▪. DU stands for duodenum, JE for jejunum and IL for ileum. Results of pairwise post-hoc Tukey tests are shown above horizontal lines (NS $p > 0.06$, $0.05 < p < 0.06$, *$p < 0.05$). Box plots depict the median, 1.5 × interquartile range, and range

separately on each of them. However, the models did not reveal any significant differences in microbial composition between the species in sympatry (Bray–Curtis distance: $p = 0.055$; Jaccard distance $p = 0.078$; Table 2) nor in allopatry (Bray–Curtis distance: $p = 0.071$, Table 2; Jaccard distance $p = 0.051$; Table 2), although some subtle differences may exist both in sympatry and allopatry (Fig. 4c, d). Moreover, we found no significant differences in microbial composition between allopatric and sympatric regions of CN (Bray–Curtis distance: $p = 0.098$; Jaccard distance: $p = 0.096$; Table 2) despite TN showing significant differences in the gut microbiota composition between the two regions for the Bray–Curtis distance ($p = 0.014$; Table 2).

The nested.anova.dbrda function indicated that the variability in the gut microbiota composition explained by species and region was 14% for the Bray–Curtis distance and 11% for the Jaccard distance, while individual identity explained 79% (Bray Curtis distance) and 67% (Jaccard distance) of the variability in gut microbiota composition (see Table 3).

Generalized linear mixed models (GLMMs) identified one OTU belonging to *Clostridium* sensu stricto genus (Firmicutes phylum) that was significantly differentially represented in the two nightingale species in sympatry (Additional file 1: Table S4a). This OTU was more abundant in CN samples than in TN samples. No OTU was significantly differentially represented in the two species in allopatry (Additional file 1 : Table S4b). The same OTU belonging to *Clostridium* sensu stricto was also differentially represented between sympatric and allopatric regions of CN as well as TN, although in TN the difference was no longer significant after correcting for multiple testing (Additonal file 1: Table S5a, b). For both species, *Clostridium* sensu stricto was more abundant in sympatry than in allopatry (Additional file 1: Table S5).

Table 1 Effects of the gut section (i.e. duodenum, jejunum and ileum), species (i.e. common nightingale and thrush nightingale), region (i.e. sympatry and allopatry) and the interaction between species and region on α-diversity indexes assessed by linear mixed models

Response variable	Explanatory variable	Chisq	df	p-value
Chao1 index	Gut section	12.050	2	**0.002**
	Species	1.464	1	0.226
	Region	0.102	1	0.749
	Species × region	1.559	1	0.212
Shannon index	Gut section	3.890	2	0.143
	Species	1.239	1	0.265
	Region	0.035	1	0.852
	Species × region	1.625	1	0.202
No. of OTUs	Gut section	11.093	2	**0.004**
	Species	1.512	1	0.219
	Region	0.054	1	0.816
	Species × region	1.545	1	0.214

α-diversity was estimated by Chao1 and Shannon diversity indexes as well as the number of observed OTUs. Individual identity was set as a random effect. Significant p-values are marked in bold

Discussion

Microbial communities living in vertebrate gastro in-testinal tracts may affect the fitness-related phenotypic traits of their hosts [3, 43], which in turn may induce selection on mechanisms that ensure the acquisition and maintenance of beneficial microbes. This selection pressure often results in long-lasting stable associations between the host and particular gut microbiota species. As different host species can be co-adapted with differ-ent gut bacteria, it is commonly assumed that the gut microbiota can be significantly involved in reproductive isolation between species [13]. However, despite inten-sive research on various aspects of host gut microbiota interactions over the past decades, empirical evidence for the role of gut microbiota in speciation is still lim-ited and comes mainly from invertebrate taxa [14]. In this study, we examined the gut microbiota composition of two recently diverged songbirds, the common night-ingale and the thrush nightingale, in their allopatric and sympatric populations. To our knowledge, this is the first attempt to assess the potential role of gut microbiota in bird speciation.

We found no significant differences in the gut micro-biota composition between the two nightingale species, with less than 14% of the total gut microbiota variation being attributed to interspecific dissimilarities. Further-more, differential abundance analyses identified only a single OTU from the genus *Clostridium* with a signifi-cantly different representation between the two night-ingale species. Nevertheless, this OTU, as well as other highly prevalent OTUs (e.g. *Candidatus Arthromitus*) were detected in both host species, meaning that none of the OTUs exhibited species specificity. Consequently, our results do not provide support for the existence of species-specific gut microbiota components, and it is thus unlikely that the gut microbiota might be involved in reproductive isolation between the two nightingale species.

Generally, evidence for the role of the host's micro-biota in the origin of reproductive isolation is limited. In various arthropod taxa, bacterial endosymbionts are involved in cytoplasmic incompatibilities [44]. In some arthropods, divergence in the gut microbiome between species can contribute to the mortality of hybrid individ-uals [14]. It is, however, unclear whether this gut micro-biota-induced hybrid lethality arises as a consequence of host vs. gut microbiota incompatibilities or incompat-ibilities among individual microbial species or the host genes involved in the management of the gut microbiota [13]. In Drosophila, the divergence of host-associated microbiota causes assortative mating between different Drosophila lineages [6, 9], with observed changes in mat-ing preferences caused by changes in levels of cuticular hydrocarbon sex pheromones induced by symbiotic bac-teria [9]. In vertebrates, there are a few studies showing phylogenetic co-divergences between hosts and particu-lar bacterial species, typically comprising just a limited fraction of their gut microbiota [10, 12, 45]. Nonetheless, a possible contribution of this gut microbiota divergence to the origin of prezygotic or postzygotic reproductive isolation between species has not yet been demonstrated.

Changes in the host's gut microbiota can be caused by environmental changes, for example by shifts in the host's diet or habitat [16, 21, 46]. Such ecological niche shifts associated with changes in the gut microbiota could theo-retically also strengthen the degree of reproductive isola-tion between species. Our previous research documented that sympatric populations of common and thrush night-ingales in their secondary contact zone exhibited higher divergence in habitat use [33] and bill morphology [39] compared to allopatric populations. This was consistent with observed interspecific differences in the consumed diet in sympatry [32]. We expected that the greater eco-logical niche divergence in nightingale sympatric popula-tions would be associated with a higher dissimilarity of their gut microbiota in sympatric compared to allopatric populations. Nevertheless, our data did not support this expectation, as interspecific gut microbiota differences were comparable in both sympatric and allopatric popu-lations. This result corresponds to previous research that revealed a surprisingly low effect of diet and other eco-logical traits on interspecific gut microbiota variation in a set of bird species with much contrasting ecology than

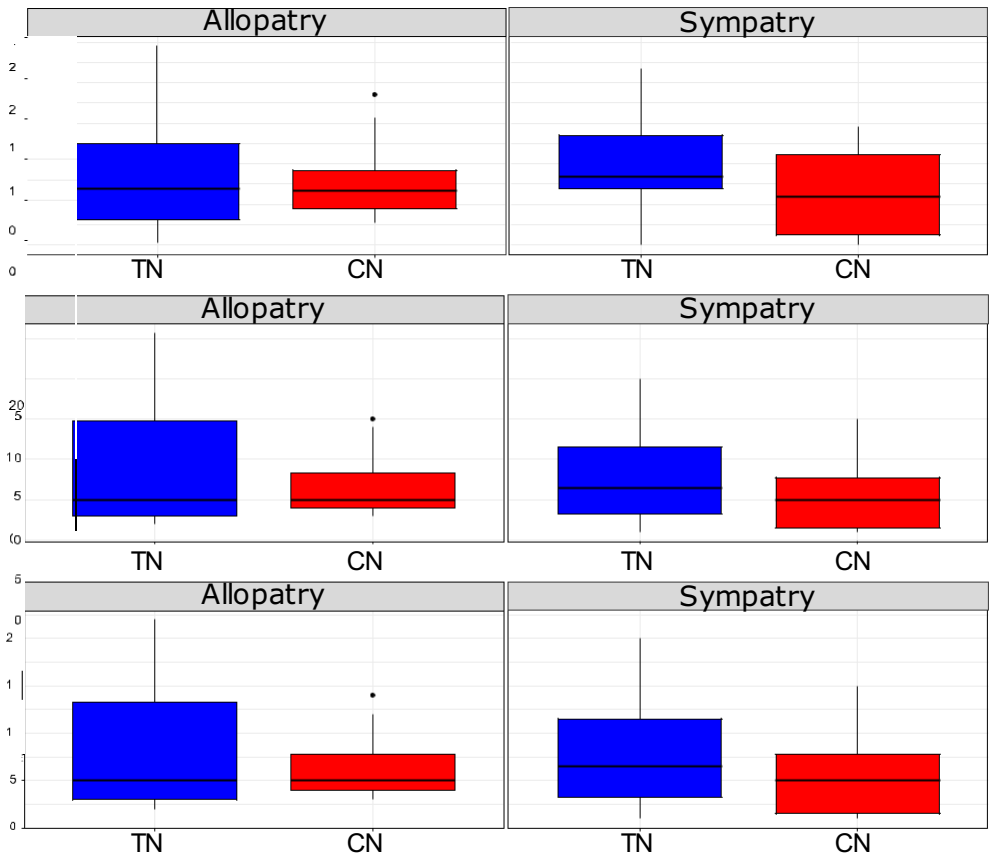

Fig. 3 Variation in α-diversity between species and geographical regions. α-diversity was estimated using the Shannon index, Chao1 index and the number of observed OTUs. The thrush nightingale (TN) is represented in blue, while the common nightingale (CN) is represented in red. To account for uneven sequencing depths, α-diversity indexes were calculated based on the rarefied OTU table (the lowest number of reads per sample was 1036). Box plots depict the median, 1.5 × interquartile range, and range

the nightingale species studied here [28, 47]. However, the absence of gut microbiota divergence between the two nightingale species in sympatry might also be a result of interspecific gene flow, as the reproductive isolation between the two species is still incomplete[29, 38, 40].

Previous studies have shown a decrease in gut microbiota similarity with increasing geographic distance in various vertebrates [42, 48, 49], suggesting that physical distance could produce barriers to bacterial dispersal. In mammals, species living in allopatry have more dissimilar gut microbiota compositions compared to sympatric species even when controlling for the diet and phylogenetic distance [41]. Our results, showing similar divergence in microbial communities between sympatric and allopatric populations of the two nightingale species, indicate that compared to mammals, geographical distance may not have such a strong effect on the gut microbiota composi-tion in passerine birds. This is generally consistent with previous studies on birds that found no or only weak associations between the gut microbiota composition and geographic distance [47, 49–51]. Nevertheless, as our study area (spanning approximately 600 km; Fig. 5) covered only a part of the two nightingale species geographic range extents, we cannot rule out that some differences in the gut microbiota composition in nightingales may exist over larger geographical distances. The weak effect of geographical distance on bird gut microbiota may be related to the fact that many species, including both our nightingale species, migrate for thousands of km each year to their wintering grounds [31]. Such migrations may be linked with higher dispersal in birds compared to non-migratory vertebrates [52]. In nightingales, natal and breeding dispersal are not known, but our unpub-lished capture-recapture data on adult birds indicate a high level of fidelity in both species. Males older than one year typically hold the same territories over multiple years. One-year-old males are more dispersive and often settle away from the site of their first breeding, but their movements are generally limited to 15 km, and we have never recorded a translocation over 20 km. Nightingales

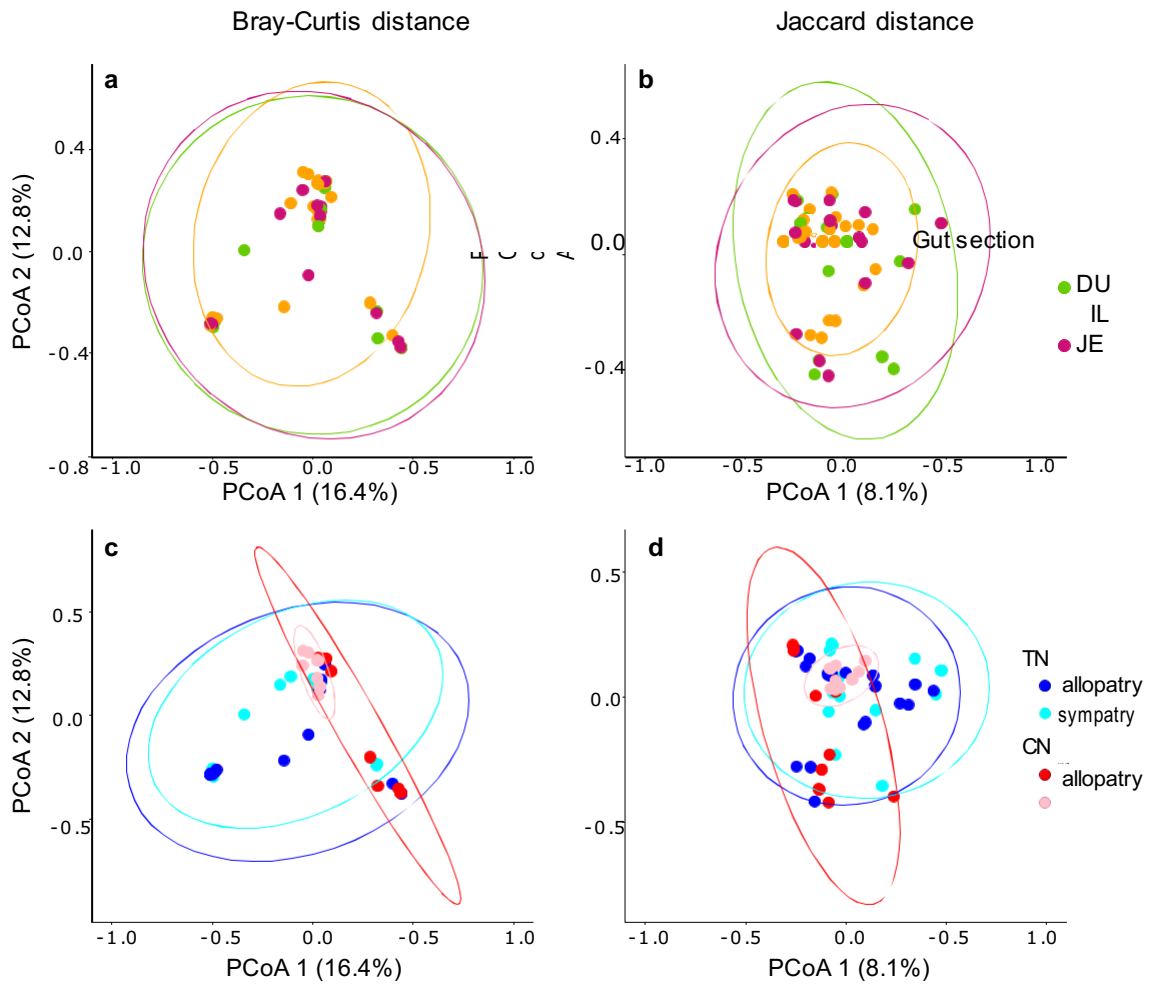

Fig. 4 Principal coordinate analyses (PCoA) based on Bray–Curtis (**a**, **c**) and Jaccard (**b**, **d**) distances comparing microbial communities among the three-gut sections (**a**, **b**) and betw een the tw o nightingale species (**c**, **d**). DU stands for duodenum, JE for jejunum and IL for ileum. TN stands for thrush nightingale and CN for common nightingale. Coloured ellipses represent 95% confidence intervals. The percentage of variability explained by each axis is show n

also show a high degree of migration connectivity [53]. Additionally, a wide variety of habitats and foods uti-lized during migration itself appears to influence the gut microbiota composition [17], which may also contribute to the weak effect of geographical distance on bird gut microbiota.

While the species identity and geographical region explained only a small amount of the variability of the gut microbiota composition in nightingales (together 14%), individual identity explained more than 79% of the variability. This finding is congruent with other stud-ies on passerine birds, with the gut microbiota typically exhibiting pronounced inter-individual variation [23, 49, 54]. The relatively high inter-individual variability in the gut microbiota composition and the small effect of diet, habitat and species identity on the gut microbiota composition in birds might be related to physiological and morphological adaptations associated with flight, as similar patterns in gut microbiota variation has been observed in bats, which also exhibit reduced intestine sizes and complexity, at least compared to other mamma-lian clades [28].

The gut microbiota composition in both nightingale species was dominated by the phyla Firmicutes (dominated by the genera *Catellicoccus*, *Candidatus Arthromitus* and *Clostridium* sensu stricto) and Proteobacteria (represented by the genera *Escherichia/Shigella*, *Rickettsiella*, and *Pantoea*) and was comparable with most passerines studies so far [55–58]. As we analysed the microbiota from three sections of the small intestine, our dataset also provides insight into gut microbiota variations along the digestive tract, which has been rarely studied in birds [51, 55]. We found significant differences in the microbiota composition among the three-gut

Table 2 Db-RDA analyses testing the effects of species identity (common nightingale vs. thrush nightingale), geographical region (sympatry vs. allopatry) and their interaction on the gut microbial composition

	Dataset	Explanatory variable	p-value	Adjusted R^2
Bray–Curtis distance	Complete	Species	0.085	0.139
		Region	0.087	
		Species × region	**0.034**	
	Sympatry	Species	0.055	0.088
	Allopatry	Species	0.071	0.090
	CN	Region	0.098	0.158
	TN	Region	**0.014**	0.049
Jaccard distance	Complete	Species	0.076	0.106
		Region	0.096	
		Species × region	**0.043**	
	Sympatry	Species	0.078	0.077
	Allopatry	Species	0.051	0.061
	CN	Region	0.096	0.101
	TN	Region	0.069	0.056

Analyses were performed on both Bray-Curtis and Jaccard distance matrices, which were the response variables. We also ran the db-RDA analyses to test for the effect of species separately in sympatric and allopatric regions and to test for the effect of region separately in each species. The significance of the models was assessed by a permutation-based ANOVA, where individual identity variation was taken into account during the permutation procedure. Significant p-values are marked in bold. CN stands for common nightingale and TN for thrush nightingale

sections in terms of the bacterial species' presence/absence, but not in terms of the relative abundances of bacterial species. Nevertheless, the variation in the gut microbiota composition explained by differences among the gut sections was very low (2–3%). The three gut sections also differed in levels of microbial α-diversity, with the ileum—the most distal part of the small intestine—showing higher α-diversity compared to the duodenum and jejunum. The ileum typically maintains a more neutral pH and is responsible for absorption of the remaining products of digestion [59]. We also detected significant within-individual correlations in microbial composition among the three gut sections. Generally, the gut microbiota profile of a particular gut section was more similar to any other gut section from the same individual than to the same gut section from a different individual, suggesting a considerable homogeneity in gut microbiota contents along the nightingale small intestine.

Conclusion

Our results suggest that neither the species identity, nor habitat, nor geographic distances have significant effects on the gut microbiota composition in the two nightingale species studied here. Instead, individual identity explains most of the observed variation in the gut microbiota composition. Our results are generally consistent with other studies in birds (e.g. [28]), and suggest that ecological factors, including diet and habitat, as well as geographical range do not have a strong influence on the avian gut microbiota composition. Altogether, this indicates that differences in gut microbiota in recently diverged bird species, especially if they are still connected by gene flow, might be usually too small to contribute to the origin of reproductive isolation. Differences in the gut microbiota composition between phylogenetically more distant avian species might arise at later stages of divergence, mostly as a consequence of the long-term independent evolution of species rather than the cause of speciation.

Methods

Study area and sampling

The sampling of common nightingales (*Luscinia megarhynchos*) and thrush nightingales (*Luscinia luscinia*) was carried out in Central Europe, in three regions (Fig. 5): an allopatric region for CN (south-western Poland), an allopatric region for TN (north-eastern Poland), and a sympatric region (central Poland) where the ranges of

Table 3 Nested analysis of variance via distance-based redundancy

		Df	Sum of squares	F	p value	Variability explained (%)
Bray–Curtis distance	Species/region	3	0.063	1.396	0.171	14
	Individual identity	24	0.364	13.611	**0.001**	79
	Residuals	29	0.032			7
Jaccard distance	Species/region	3	0.050	1.266	0.087	11
	Individual identity	24	0.316	3.641	**0.001**	67
	Residuals	29	0.105			22

Bray–Curtis and Jaccard distance matrices were the response variables, while species identity, region and individual identity were explanatory variables. The number of permutations was set at 1000. Significant p-values are marked in bold

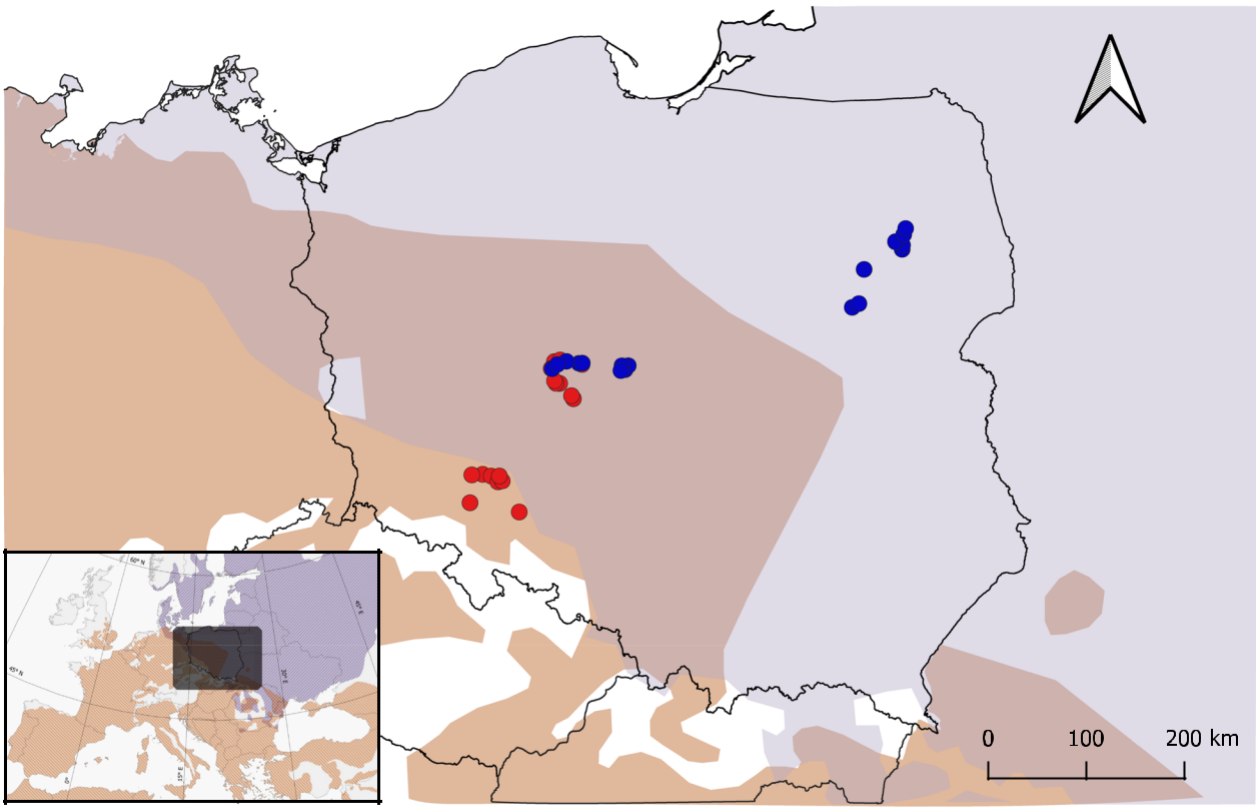

Fig. 5 Map of the sampling localities of common nightingales (red dots) and thrush nightingales (blue dots) in Central Europe. Allopatric regions for common nightingales and thrush nightingales are labelled in red and blue, respectively. The sympatric region where both species co-occur is indicated in purple. Species' ranges are redrawn from [39]

both species overlap and the species often locally co-occur [33]. The allopatric region of CN was close to the sympatric region (Fig. 5); however, according to the Polish Breeding Bird Census data analysed in [33] as well as according to our long-term field observations, no TN individuals were recorded breeding in this area. Moreover, as the south-western edge of TN's breeding range moved north-east recently (our unpublished observations), at the time of our sampling, CNs allopatric localities were not less than 100 km from the nearest breeding occurrence of TN. Both nightingale species were sampled in May 2018 at the beginning of the breeding season when territories were already established. Only male birds were caught using a mist net with a luring tape. We captured 9 CN and 9 TN males from allopatric regions and 9 CN and 9 TN males from the sympatric region. A list of the sampled birds, including their dates of sampling and GPS coordinates, is provided in Additional file 1: Table S1.

The birds were euthanized by standard cervical dislocation. Dissections started immediately; we removed the entire gastrointestinal tract from the body cavity, and gently separated intestines from the stomach. The whole gut tissue was then placed in a sterilized plastic tube (30 mL) with 99% ethanol, deep-frozen in liquid nitrogen and stored at − 80 °C until DNA extraction. The whole dissection procedure, starting with the euthanasia of the bird and finishing with the gut tissue being stored, did not exceed 8 min. All instruments used to dissect the birds (scissors, lancets) were repeatedly flame-sterilized to prevent cross-individual bacterial contamination of samples. The work with animals was approved by the General Directorate for Environmental Protection, Poland (permission no. DZP-WG.6401.03.123.2017.dl.3).

DNA extraction from the gut and 16S rRNA sequencing

From each individual's gut, we dissected three samples (each ca. 0.5 cm long) from the small intestine using sterilized dissection tools. These sections were located in: (1) the duodenum (sampled from the proximal part of the small intestine), (2) the jejunum (sampled from the middle part of the small intestine) and (3) the ileum (the distal part of the small intestine before caecal protuberances). As the passerine colon is very short [11], we were unable

to consistently dissect this gut part from all the intestine samples and thus the colon was not analysed in this study. Metagenomic DNA from each sample was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., USA). Both sample preparation and DNA extractions took place in a laminar flow cabinet. Sequencing libraries were prepared using a two-step PCR approach. The V3– V4 hypervariable region of bacterial 16S rRNA was ampli-fied using universal primers S-D-Bact-0341-b-S-17 (CCT ACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC, [60]). Both forward and reverse primers were flanked by oligonucleotides compatible with Nextera adaptors (Illumina, USA). For the first PCR round, 5 µl of KAPA HIFI Hot Start Ready Mix (Kapa Biosystems, USA), 0.2 µM of each primer and 4.6 µl of DNA template were used (final reaction vol-ume = 10 µl). PCR conditions were as follows: initial dena-turation at 95 °C for 3 min followed by 30 cycles of 95 °C (30 s), 55 °C (30 s) and 72 °C (30 s), and a final extension at 72 °C (5 min). Dual-indexed Nextera sequencing adaptors were appended to the resulting PCR products during the second PCR. The second PCR reaction consisted of 10 µl of KAPA HIFI Hot Start Ready Mix, 5 µl of H_2O, 2 µM of each primer and 1 µl of PCR product from the first PCR (final reaction volume = 20 µl) and the PCR program ran for 12 cycles with conditions being the same as during the first PCR. Products from the second PCR round were quantified by GenoSoft software (VWR International, Bel-gium) based on band intensities after electrophoresis on a 1.5% agarose gel, and mixed at equimolar concentra-tion. The final library was cleaned up using SPRIselect beads (Beckman Coulter Life Sciences, USA). Products of desired size (520–750 bp) were extracted by Pipin-Prep (Sage Science Inc., USA) and sequenced on an Illu-mina Miseq (v3 kit, 300 bp paired-end reads). Technical PCR duplicates were sequenced for all individual DNA samples.

Bioinformatic processing of the sequence data and identification of microbial taxa

Samples were demultiplexed and primers were trimmed by skewer software [61]. Using *dada2* [62], we filtered out low-quality sequences (expected number of errors per read less than 1), denoised the quality-filtered fastq files and constructed an abundance matrix represent-ing reads counts for individual haplotypes (Operational Taxonomic Units, OTUs) in each sample. Using uchime [63] and the gold.fna database (available at https://drive5. com/uchime/gold.fa), we identified chimeric sequences and removed them from the abundance matrix. Taxo-nomic assignation of haplotypes was conducted by the RDP classifier (80% confidence threshold [64]) and Silva reference database (v 132 [65]).

A large number of sequences from coccidian proto-zoa, an intracellular parasite present in the intestinal tract of vertebrates provoking Coccidiosis disease [66], were identified in TN samples (43% of the total number of reads) and in CN samples (38% of the total number of reads). These OTUs belonged to the genera *Eimeria* and *Neospora* (phylum: Apicomplexa). We removed all coccidian and other non-bacterial OTUs from the data-set. Furthermore, to eliminate PCR or sequencing arte-facts that were not corrected by dada2, we removed all OTUs that were not consistently present in both techni-cal duplicates for a given sample. Read counts for remain-ing OTUs were subsequently merged for the purpose of all later analyses. Finally, samples with less than 1,000 sequences after all the above filtering steps were dis-carded. In total, 19 samples from TN and 32 samples from CN were removed.

Statistical analyses

All statistical analyses were done using packages run-ning under R Statistical Software version 3.4.3 (R Core Team 2015). To account for uneven sequencing depth among samples, a rarefied OTU table (n = 1,036 sequences per sample, which corresponds to the minimal per-sample sequencing depth) was used in all analyses, if not stated otherwise.

Estimation and comparison of microbial α- diversity

The three α-diversity estimates, including the Chao1 diversity index, the number of observed OTUs and the Shannon diversity index, were calculated using the phy-loseq package [67]. LMMs testing the effects of gut sec-tion, nightingale species and geographical region on the respective α-diversity indexes were performed in the package lme4 [68]. To account for statistical non-inde-pendence (due to sampling of three gut sections for each individual), the effect of individual was included as a ran-dom effect. Differences between the gut sections were assessed based on Tukey post-hoc comparisons.

Dissimilarity of microbial composition (β- diversity) between samples

Two types of distances, the binary Jaccard distance and the Bray-Curtis distance, were calculated as measures of microbial composition dissimilarity between sam-ples (β-diversity) using the vegan package [69].We used a Principal Coordinates Analysis (PCoA) based on the two distance matrices to visualize the differences in microbial composition among the three gut sections across both species. Associations between gut-micro-biota composition and gut section were assessed by distance-based redundancy analyses (db-RDAs [70]) with the distance matrix as a response variable and

the gut section identity (i.e. DU, JE and IL) as explan-atory variables. The significance was assessed by a permutation-based ANOVA, with individual identity being considered as a block (i.e. 'strata') for permuta-tion. Additionally, for individuals where all three-gut sections were available (n = 11, Additional file 1: Table S1), within-individual correlations of the micro-bial composition among the three-gut sections was evaluated using a Mantel's test (R package 'ade4' [71]).

The effects of species identity (i.e. CN and TN) and region (i.e. sympatry and allopatry) on the gut-micro-biota composition were assessed via db- RDA. The dis-tance matrix was included as a response variable while nightingale species identity, region and their interaction were included as explanatory variables. The significance of explanatory variables in db-RDAs was assessed by a permutation-based ANOVA. In contrast to the analy-sis of the gut section, here explanatory variables associ-ated with each individual (i.e. region, species identity)
were reshuffled across blocks of individual specific samples during the permutation routine to account for the fact that multiple samples for each individual were analysed. To estimate the proportion of the variability explained by each factor on the gut microbiota com-position, we used a nested analysis of variance via dis-tance-based redundancy analysis (nested.anova.dbrda; package BiodiversityR [72]). The distance matrices were the response variable, while species identity, region and individual identity were explanatory variables (1000 permutations). To avoid any potential bias due to all three gut sections not being available for some indi-viduals, we also ran this analysis on the subset of indi-viduals (n=11) for which all three gut sections were available. The results were similar for both datasets and we thus present the results only for the whole dataset.

To identify specific OTUs whose abundances differed between the nightingale species in allopatric and sym-patric regions, we used generalized linear mixed models with a negative binomial distribution [73]. These analy-ses were performed on a subset of six OTUs (compris-ing 43% of all high quality reads) that were detected in at least five samples across both species and regions. The response variable was entered either as (i) the read counts for OTUs from the allopatric region or (ii) the read counts for OTUs from the sympatric region. The explanatory variable was the species identity, and indi-vidual identity was set as a random factor. Log-trans-formed total number of reads per sample was specified as the model offset. A false discovery rate method [74] was subsequently used to account for false discover-ies due to multiple tests conducted on the given set of OTUs.

Abbreviations

CN: Common nightingale; TN: Thrush nightingale; OTU: Operational taxo-nomic unit; LMM: Linear mixed model; PCoA: Principal coordinates analysis; db-RDA: Distance-based redundancy analyses; DU: Duodenum; JE: Jejunum; IL: Ileum; GLMM: General mixed model; ANOVA: Analysis of variance; PCR: Polymerase chain reaction.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12862-021-01773-1.

Additional file 1: Table S1. A list of sampled nightingale individuals with information about their species, geographical region, date of sampling and GPS coordinates. **Table S2.** α-diversity in the three gut sections across both nightingale species (a) and pairwise post-hoc Tukey tests of differ-ences in α-diversity between specific gut sections (b) **Table S3.** Within-individual correlations in the microbial composition among the three-gut sections. **Table S4.** Differences in representation of particular OTUs between the two nightingale species in sympatry (a) and in allopatry (b). **Table S5.** Differences in representation of particular OTUs between sympatric and allopatric region in the common nightingale (a) and in the thrush nightingale (b). **Table S6.** Metadata to the individual sequence samples that are available from the European Nucleotide Archive under the study accession number: PRJEB43057.

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Authors' contributions

CS assisted in the study design, performed statistical analyses, made figures and wrote the draft of the manuscript; LS performed the molecular and bioinformatics analyses; JK conceived the study design, contributed to the bioinformatic and statistical analyses and wrote parts of the manuscript; TA performed the dissections; JR and TO captured birds; RR conceived the study design and wrote parts of the manuscript. All authors contributed critically to the drafts, read and approved the final manuscript.

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Availability of data and materials

Sequencing data are available from the European Nucleotide Archive under the study accession number: PRJEB43057. Metadata to the individual sequence samples are provided in the Supplementary Material Table S6. All other data are attached as a Supplementary Material.

Declarations

Ethics approval and consent to participate

The work with animals was approved by the General Directorate for Environ-mental Protection, Poland (permission no. DZP-WG.6401.03.123.2017.dl.3).

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Temporal Stability and the Effect of Transgenerational Transfer on Fecal Microbiota Structure in a Long Distance Migratory Bird

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Animal bodies are inhabited by a taxonomically and functionally diverse community of symbiotic and commensal microorganisms. From an ecological and evolutionary perspective, inter-individual variation in host-associated microbiota contributes to physiological and immune system variation. As such, host-associated microbiota may be considered an integral part of the host's phenotype, serving as a substrate for natural selection. This assumes that host-associated microbiota exhibits high temporal stability, however, and that its composition is shaped by trans-generational transfer or heritable host-associated microbiota modulators encoded by the host genome. Although this concept is widely accepted, its crucial assumptions have rarely been tested in wild vertebrate populations. We performed 16S rRNA metabarcoding on an extensive set of fecal microbiota (FM) samples from an insectivorous, long-distance migratory bird, the barn swallow (Hirundo rustica). Our data revealed clear differences in FM among juveniles and adults as regards taxonomic and functional composition, diversity and co-occurrence network complexity. Multiple FM samples from the same juvenile or adult collected within single breeding seasons exhibited higher similarity than expected by chance, as did adult FM samples over two consecutive years. Despite low effect sizes for FM stability over time at the community level, we identified an adult FM subset with relative abundances exhibiting significant temporal consistency, possibly inducing long-term effects on the host phenotype. Our data also indicate a slight maternal (but not paternal) effect on FM composition in social offspring, though this is unlikely to persist into adulthood. We discuss our findings in the context of both evolution and ecology of microbiota vs. host interactions and barn swallow biology.

Keywords: microbiome, metagenome, symbiosis, gastrointestinal tract, barn swallow, fecal microbiota

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INTRODUCTION

The bodies of animals are inhabited by taxonomically and functionally diverse communities of symbiotic and commensal microorganisms (Qin et al., 2010; Muegge et al., 2011). Recent advances in this field have clearly shown that such host-associated microbiota provide important benefits to the host. In particular, microbiota modulate development of digestive tract morphology (Reikvam et al., 2011), enable synthesis of essential bioactive molecules that cannot be synthesized by

the host (Bäckhed et al., 2005), stimulate the host's immune system (Macpherson and Harris, 2004; Wu and Wu, 2012) and provide protection against pathogens (Koch and Schmid-Hempel, 2011). In addition, unlike host-encoded enzymes, enzymes encoded by the microbial metagenome enable cleavage of complex substrates such as plant polysaccharides. Products of these pathways can be further processed by the host's metabolism and, consequently, microbial communities positively contribute to the host's energy balance (Jumpertz et al., 2011). In addition to these benefits, however, certain species of host-associated microbiota can induce harmful effects, such as reduced diet processing capacity (Smith et al., 2013), chronic inflammation (Dapito et al., 2012), or production of toxins (Yoshimoto et al., 2013).

In vertebrates, host-associated microbiota typically exhibit pronounced variation at the inter-individual level (Baxter et al., 2015; Kreisinger et al., 2015b; Yuan et al., 2015; Lewis et al., 2016). Despite a degree of functional redundancy as regards genes encoded by individual host-associated microbiota taxa (Moya and Ferrer, 2016), such variation underlines inter-individual differences in health status and a wide range of physiological and body-condition traits (Macpherson and Harris, 2004; Koch and Schmid-Hempel, 2011; Smith et al., 2013). With respect to this inter-individual variation a number of obvious questions arise, one of the most important is to what extent does individual specificity in host-associated microbiota composition vary over time.

Hosts may exhibit a certain degree of tolerance to newly invading bacterial species; at the same time, the abundance of microbiota already present may change due to switches in host diet and physiological state (Jumpertz et al., 2011; David et al., 2014; Salonen et al., 2014; Wang et al., 2014). Such changes in microbiota over time may increase the host's ability to cope with new environmental challenges. Under certain circumstances, however, such changes in microbiota composition could have no effect or induce adverse effects on the host (DiBaise et al., 2012; Kumar et al., 2016). As a result, multicellular organisms have evolved a plethora of mechanisms aimed at maintenance of beneficial microbial taxa and suppression of potentially detrimental microbes (Salminen et al., 2004; Janson et al., 2008; Benson et al., 2010; McKnite et al., 2012). Probably the best known of these host-intrinsic factors are those genes predominately involved in regulation of the immune system, which have a long-standing effect on associate microbial communities (Benson et al., 2010; McKnite et al., 2012; Bolnick et al., 2014; Wang et al., 2015). Given that immune genes typically display high allelic variation (Sommer, 2005), they could contribute to both long-term stability and inter-individual variation of host-associate microbiota. In addition to genetic factors, trans-generational transfer of microbiota could have a long-standing effect on microbiota composition in the progeny of parental generations (Salminen et al., 2004). In some taxa, this may even result in co-divergence between microbiota and host phylogenies over their evolutionary history (Sanders et al., 2014). Both trans-generational transfer and genetic regulation of microbial communities imply some level of heritability in host-associated microbiota. Consequently, if host-associated microbiota exhibit both long-term stability and heritability, this could be regarded as an extension of host-heritable phenotype variation and serve as the substrate for natural selection (Zilber-Rosenberg and Rosenberg, 2008; Bordenstein and Theis, 2015).

Current evidence for the temporal stability and heritability of host-associated microbiota is still rather puzzling, however, and has been addressed by a surprisingly low number of studies. Furthermore, what studies there have been have tended to focus mainly on human populations and captive-bred model species (Benskin et al., 2010; Schloss et al., 2012; Faith et al., 2013; Lim et al., 2014; Salonen et al., 2014; Tap et al., 2015). Limited effort has been aimed at assessing the strength of temporal stability and mechanisms affecting host-associated microbiota establishment during ontogeny in wild populations (Waite et al., 2014; Baxter et al., 2015; Sun et al., 2016). Great care should be taken when extrapolating results obtained in captivity or from humans to microbiota vs. host interactions in wild populations. In the case of human populations, long-term host-associated microbiota stability could be affected to a large degree by long-term stability of life-style, including diet preferences, exposure to stressors modulating host-associated microbiota and other traits associated with micro-culture variation. In the case of laboratory-reared animals, the composition and functional properties of host-associated microbiota are typically distinct compared to wild populations (Xenoulis et al., 2010; Amato, 2013; Kreisinger et al., 2014). Consequently, captivity may induce large effects on the shape of interactions between host-associated microbiota and host physiology (Kreisinger et al., 2015a). In addition, environmental factors contributing to host-associated microbiota variation over time, such as variation in diet composition and environmental stressors, are typically homogeneous among individuals in breeding facilities. As a consequence, factors contributing to individual host-associated microbiota stability over time, such host genes interacting with host-associated microbiota members and/or vertical transfer of host-associated microbiota from parents to progeny, may be of higher importance in breeding facilities compared to wild populations. Last, but not least, most of our current knowledge on host-associated microbiota vs. host interaction relies on studies performed on mammals. Such taxonomic bias may affect our general view of the ecological and evolutionary factors associated with host-associated microbiota vs. host interaction as gut morphology and factors contributing to host-associated microbiota establishment and host-associated microbiota composition exhibit considerable taxon specificity in mammals. Unlike most other vertebrate taxa, mammals are typically viviparous. Physical contact between newborn young and the female's vaginal microbiota during the delivery is important for host-associated microbiota colonization and this type of transfer has a long-term effect on microbiota composition (Salminen et al., 2004).

Passerines are an important model group for evolutionary, eco-physiological, and eco-immunological research (Bennett and Owens, 2002), particularly as we have a detailed knowledge on their physiology. However, the role of microbiota is still understudied in this group. In addition, passerines have a clearly distinct composition of host-associated microbial communities

compared, to the more widely studied mammals (Hird et al., 2015; Kreisinger et al., 2015b; Lewis et al., 2016). This makes them an interesting and complementary model group for research on the ecological and evolutionary consequences of host vs. microbiota interaction. To date, the microbial community of the lower intestine, with microbiota in fecal samples typically used as a proxy, has been the most widely studied subset of animal-associated microbiota (Ley et al., 2008; Baxter et al., 2015; Hird et al., 2015; Lewis et al., 2016). Fecal microbiota (FM) differences between passerines and mammals are putatively associated with differences in gut anatomy and function. In particular, overall gut length and food retention time tends to be shorter in birds compared with mammals of similar body size (Caviedes-Vidal et al., 2007; McWhorter et al., 2009). Furthermore, those gut sections involved in bacterial fermentation, i.e., the caecum and colon, are typically reduced in passerines (Ruiz-Rodríguez et al., 2009). Unlike mammals, passerine FM may also be affected by direct contact with uric acid as the urine is conveyed to the cloaca directly by the kidney ureters (Braun and Campbell, 1989) and a number of bacterial species are capable of using uric acid as a substrate (Potrikus and Breznak, 1981; Thong-On et al., 2012). As in the case of mammals, however, microbiota may also be transferred from parents to progeny during physical contact in the course of food provisioning (Lucas and Heeb, 2005). However, little is known about the temporal stability of FM in passerines, or about the FM development during post-hatching ontogeny (Benskin et al., 2010; González-Braojos et al., 2012b).

In this contribution, we focus on temporal stability of FM in a passerine bird, the barn swallow (*Hirundo rustica*). The barn swallow is a long-distance migrant nesting in colonies with a complex social system (Petrželková et al., 2015). It has been suggested that social interaction between colony members affects the structure of host-associated microbiota in this species (Kreisinger et al., 2015b). As a species, the barn swallow forages exclusively on diverse groups of flying insects (Turner, 1980). Our study benefits from this tight specialization as FM variation due to differences in diet are likely to be relatively low compared to omnivorous taxa (Ley et al., 2008; David et al., 2014). Despite extensive knowledge on barn swallow biology (Møller, 1994), there has been just one culture-independent study aimed at characterization of microbial communities in this species based on high-throughput sequencing (Kreisinger et al., 2015b).

Here, we apply extensive repeat FM sampling of adults and juveniles from three breeding barn swallow populations. Metataxonomic approach based on high-throughput sequencing of 16S rRNA amplicones and imputation of FM functional content via PICRUSt (Langille et al., 2013) were used to get an insight into developmental trajectories of FM taxonomic and functional composition in this species. Importantly, our data set allows us to estimate the level stability in individual FM composition over time. In addition, we tested if the level of temporal stability differs among adults and juveniles and if the temporal stability is driven by abundance invariance of FM members or by invariance of their presence vs. absence. Finally, we use this dataset to assess whether social contact between parents and offspring shapes FM composition.

MATERIALS AND METHODS

Field Sampling and Parentage Assignment

Field sampling was conducted during the barn swallow breeding season (from May to August) on three populations [Šaloun farm, Lomnice nad Lužnicí (49 4'7.762"N, 14 42'36.521"E), Hamr farm, Lužnice (49 3'25.288"N, 14 46'10.82"E), and Obora (48 59'06.8"N, 14 46'48.5"E)] in the T˘rebonsko˘ Protected Landscape Area (Czech Republic, average distance between populations D 7.9 km). Adult FM was sampled during 2013 and 2014, whereas juvenile FM was only sampled during 2014, at 6, 9, and 12 days after hatching (hereafter "age-classes"). See Petrželková et al. (2015) for more details on field procedures. To collect fecal samples, adults were placed in a paper bag and young in a plastic beaker filled with paper towels, where they were kept for approx. 30 min. Feces were harvested using a sterile microbiological swab (Copan, Italy), placed in sterile DNA/RNA free cryotubes (Simport, Canada) and stored in liquid nitrogen or at 80 C for further laboratory analysis.

Individuals in each population had their polymorphic microsatellites genotyped, as described in Petrželková et al. (2015). These data, together with direct observations of individually marked adults, allowed us to determine the social parents for individual clutches and the presence of extra-pair (i.e., sired by a non-social male) or parasitic (i.e., laid by a non-social female) young within individual clutches, as described elsewhere (Petrželková et al., 2015).

We analyzed 448 barn swallow FM samples in total; 197 samples from juveniles (*n* D 99 individuals, mean no. samples per individual D 2.01) and 251 from adults (*n* D 131 individuals, mean no. samples per individual D 1.91; see **Supplementary Table S1**). Our data set also included FM samples from 31 social mother vs. offspring pairs and from 37 social father vs. offspring pairs sampled during 2014.

All field procedures were conducted in accordance with the Guidelines for Animal Care and Treatment of the European Union, and approved by the Animal Care and Use Committees at the Czech Academy of Sciences (041/2011), and Charles University in Prague (4789/2008-0).

Microbiota Genotyping

Metagenomic DNA from fecal samples was extracted in a laminar flow cabinet using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., USA). To optimize the efficiency of DNA isolation, samples were homogenized using a MagnaLyzer (Roche, Switzerland) for 30 s at 6000 rpm and the DNA extracted was eluted to 50 ml of elution buffer. Following the recommendations of Klindworth et al. (2013), primers covering the V3-V4 variable region on bacterial 16S rRNA [i.e., S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC)] were used during the polymerase chain reaction (PCR) step. Both forward and reverse primers were tagged with 10 bp barcodes designed by TagGD software (Costea et al., 2013). For the PCR, we used 8 ml of KAPA HIFI Hot Start Ready Mix (Kapa

Biosystems, USA), 0.37 mM of each primer and 7 ml of DNA template. PCR conditions were as follows: initial denaturation at 95 C for 5 min followed by 35 cycles each of 98 C (20 s), 61 C (15 s), and 72 C (40 s), and a final extension at 72 C (5 min). The PCR product, together with negative controls (PCR products for blank DNA isolates), were run on 1.5% agarose gel and the concentration of PCR product assessed based on gel band intensity using GenoSoft software (VWR International, Belgium). Samples were subsequently pooled at equimolar concentration, the pooled samples then being run on 1.5% agarose gel, with bands of appropriate size excised from the gel and purified using the High Pure PCR product Purification Kit (Roche, Switzerland) according to the manufacturer's instructions. Sequencing adaptors were ligated using TruSeq nano DNA library preparation kits (Illumina, USA) and the resulting amplicon libraries sequenced on a single Miseq run (Illumina, USA) using v3 chemistry and 2 300 bp paired-end reads. We then prepared technical PCR duplicates for individual DNA samples. As there was high consistency in both FM composition (Procrustes correlation: r D 0.98, p < 0.0001) and FM diversity (Pearson's r D 0.97, p < 0.0001) among technical replicates, we merged the sequences corresponding to individual samples for downstream analysis.

Bioinformatic Processing of 16S rRNA Data

Pair-end Illumina reads were merged using PEAR (Zhang et al., 2014) and de-mutiplexed using mothur (Schloss et al., 2009) and custom R/Bioconductor scripts (available from the authors on request). We then used Lotus pipeline (Hildebrand et al., 2014) for quality filtering of FASTQ files. Sequences were excluded if the average quality score was lower than 30 or if the average quality score within a 50 bp sliding window decreased below 25. UCHIME (implemented in the Lotus pipeline; Edgar et al., 2011) was used alongside the gold.fna database[1] for detection and elimination of chimeric sequences. The resulting 16S rRNA sequences were clustered at a 97% similarity threshold using UPARSE (Edgar, 2013) in order to define operational taxonomic units (OTUs). Taxonomic assignation of representative sequences for each OTU was performed using RDP classifier (Wang et al., 2007) and the GreenGenes reference database, version gg_13_5 (DeSantis et al., 2006). Representative sequences were further aligned using PyNAST (Caporaso et al., 2010a), the maximum likelihood tree being constructed using FastTree (Price et al., 2009). We considered OTUs assigned as "Chloroplast" (6.2% of read after quality filtering), or those not assigned to any bacterial phylum (0.6% of read after quality filtering), as diet contaminants or sequencing artifacts, respectively, and excluded them from all downstream analyses. The resulting OTU tables, sample metadata, OTU tree and taxonomic annotations for individual OTUs were merged into a phyloseq object (McMurdie and Holmes, 2013) for statistical analysis in R version 3.2.3 (R Core Team, 2015).

1 http://sourceforge.net/projects/microbiomeutil/files

Metagenomic Predictions

Functional composition of the FM was inferred based on predictive models integrated into the PICRUSt pipeline (Langille et al., 2013). In brief, this approach utilizes 16s rRNA reads and ancestral state reconstruction algorithms to predict the functional content of FM samples based on the gene content of known bacterial genomes. First, we mapped our high-quality sequences against GreenGenes reference OTUs (DeSantis et al., 2006) using 91, 93, 95, and 97% similarity thresholds and the closed reference algorithm implemented in QIIME (Caporaso et al., 2010b). Next, metagenomes were predicted using the default PICRUSt setup and classified according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2000). The resulting table, along with the predicted abundance of KEGG categories in individual samples, was used for downstream analysis.

The proportion of sequences unassigned to a reference OTU in GreenGenes (i.e., unusable for metagenomic prediction) was relatively high at the 97% similarity threshold (24.3%) and relatively low at the 95–91% similarity thresholds (range D 7.9– 1.3%). Hence, we decided to use 95% similarity mapping for PICRUSt predictions in order to avoid potential bias associated with a high proportion of unmappable reads. Althougth 95% similarity mapping may compromise PICRUSt precission, the "mean nearest sequenced taxon index" (NSTI), i.e., the average branch length separating OTUs from a reference bacterial genome, was only 0.065. This value is lower than the NTSI for the microbiomes of most non-model species (Langille et al., 2013).

Statistical Analysis

Using phyloseq (McMurdie and Holmes, 2013), we calculated sample-specific alpha diversity indices (number of OTUs observed, Chao1, Shannon index) and, using linear mixed effect models (LME) contained in the R package lme4 (Bates et al., 2015), tested whether there was any difference in alpha diversity among juveniles and adults and whether FM diversity varied among different juvenile age-classes. Individual identity was considered as a random effect and the effect of breeding colony was included as a covariate. The significance of the main effects was assessed based on deviance ratio tests.

Community-wide divergence in OTU and predicted KEGG composition was assessed using multivariate techniques based on community dissimilarity among samples. Four ecological dissimilarity types were applied, each capturing different aspects of FM divergence: weighted and unweighted UniFrac (Lozupone and Knight, 2005), Bray–Curtis and a binary version of Jaccard dissimilarity. Jaccard and unweighted UniFrac dissimilarity only account for OTU presence vs. absence; hence, they are more sensitive than Bray–Curtis and weighted UniFrac dissimilarity to FM changes driven by rare OTUs. In addition, both unweighted and weighted UniFrac dissimilarity take account of OTU genetic similarity and, therefore, are more sensitive to community divergence driven by phylogenetically distant bacterial groups. Only Bray–Curtis dissimilarity was used in the case of metagenomic prediction. In order to account for uneven sequencing depth among samples (mean per sample

coverage SE D 15773 487, range D 1112–102922), OTU and predicted KEGG counts were converted to sample-specific proportion (as recommended by McMurdie and Holmes, 2014) prior to calculation of weighted UniFrac and Bray– Curtis dissimilarity. As this form of data normalization was not applicable for the purposes of OTU absence vs. presence analysis, we rarefied the OTU data in order to achieve the same sequence coverage per sample (i.e., corresponding to minimal sequencing depth) prior to calculation of Jaccard and unweighted UniFrac dissimilarity. Principal coordinate analysis (PCoA) and distance-based MANOVA (adonis function, vegan R package) were applied to assess whether there was any difference in FM composition between adult vs. juveniles and whether FM composition changed with juvenile age-class. We used betadisper (vegan R package) to test whether interindividual variation in FM composition differed between adults and juveniles. Those OTUs and KEGGs driving FM differences among adults vs. juveniles and among juvenile age-classes through changes in abundance were identified using DESeq2 (Love et al., 2014). As adonis, betadisper, and DESeq2 are unable to effectively account for pseudo replication due to repeat sampling of the same individual through random effects, we selected a single sample per adult and juvenile collected in 2014 at random for the purpose of these analyzes. This form of data reduction produced no hint of systematic bias or any significant decrease in statistical power; adonis and betadisper giving the same results for the complete and reduced datasets, with a high degree of correlation between the DESeq2-based log2 fold changes in the reduced and full datasets (Pearson's r D 0.751 for OTU abundance and 0.879 for predicted KEGG abundance, $p <$ 0.0001 in both cases).

We also explored co-occurrence patterns between OTUs in adults and those in individual juvenile age-classes using a recently developed version of the checkerboard score index, the nc.score (Schwager et al., 2014), on individual OTU pairs. Association between OTUs was considered significant if the corresponding q value (Storey and Tibshirani, 2003) was <0.05. Significant associations were used for construction of a co-occurrence network, as implemented in qgraph (Epskamp et al., 2012). Poisson generalized linear mixed effect models (GLMM; fitted using lme4), with number of significant associations per OTU as the response variable and OTU taxonomic assignation to class level as random intercept, were used to identify taxa that were more or less likely to be involved in co-occurrence or co-avoidance associations when compared to the whole FM baseline. To account for overdispersion, individual-level (i.e., OTU-specific) random effects were also included into these models. In order to meet the requirements of computational resources (24G RAM) and maintain a reasonable number of multiple tests, we filtered out those OTUs whose $\log_{10}$ scale variance for relative abundance among samples was > 8 prior to calculation (Bourgon et al., 2010).

A series of permutation-based tests were run to assess whether dissimilarity among samples corresponding to the same adult or young individual, the same nest, breeding colony or breeding season was lower than that among samples that did not match these categories. First, the average difference in ecological dissimilarity between corresponding groups (e.g., dissimilarity for the same individual vs. a different individual) was calculated and the non-parametric Cliff's d index was used to estimate the effect size. By reshuffling dissimilarity values between sample pairs, we determined the distribution of differences expected under null hypothesis validity. The null distribution was then used for calculation of p-values. The subset of dissimilarities irrelevant for a given comparison and/or capable of biasing any observed difference was excluded prior to calculation. We also carefully specified blocking variables that define permutation constrains (a.k.a. strata) in order to obtain an unbiased null distribution (see details in **Data Sheet 1**). The same principle was used to test whether there was a higher similarity than expected among offspring vs. social parents.

To assess the contribution of individual OTUs to temporal stability at the community-wide level, we repeated the previous analysis for Bray–Curtis dissimilarity calculated for each of the FM OTUs. This dissimilarity metric was selected as proportion-based dissimilarities exhibit higher discriminatory power in whole-community analyses (see below). Furthermore, it is technically not possible to calculate UniFrac dissimilarity for individual OTUs. Each OTU was assumed to contribute significantly to FM stability at a q value threshold of 0.05. Following the procedures described in Bourgon et al. (2010), OTUs exhibiting low variation in abundance among samples were excluded from the dataset prior to multiple testing correction.

RESULTS

General Description of Barn Swallow FM

Sequencing data comprised 7.8 million high quality paired-end reads, with reads clustered in 6222 OTUs, of which 71.4% (represented by 87.5% high quality reads) were classified to family and 45.9% (represented by 66.7% high quality reads) up to genus level. Coverage indices calculated for individual samples indicated that our data captured the vast majority of FM diversity (mean Good's coverage SE D 0.994 0.0003, range D 0.950– 0.999).

The dataset included 36 Eubacterial phyla and one Archaeal phylum (Euryarchaeota). Barn swallow FM was dominated by Proteobacteria (mean SE of reads D 39.6 1.4% reads, range D 45.5–98%), Firmicutes (34.7 1.4%, range D 13.2– 99.1%), Tenericutes (12.7 1.1%, range D 0–96.0%), Bacteroidetes (6.3 0.7%, range D 0–92.2%), and Actinobacteria (4.4 0.4%, range D 0–48.7%), with other phyla present at much lower frequencies (at average <1% reads). At lower taxonomic levels, Proteobacteria were predominantly represented by unclassified Enterobacteriaceae (10.7% of all high quality reads), *Serratia* (5.1%), *Pantoea* (3.3%), *Aeromonas* (2.4%), *Pseudomonas* (1.1%), and *Rickettsia* (1.4%). The most abundant Firmicutes genera were *Enterococcus* (7.7% of all high quality reads), *Catellicoccus* (*5.8%), Lactobacillus* (4.3%), and *Lactococcus* (1.3%*). Dysgonomonas* (4.2% of reads) was the dominant genus

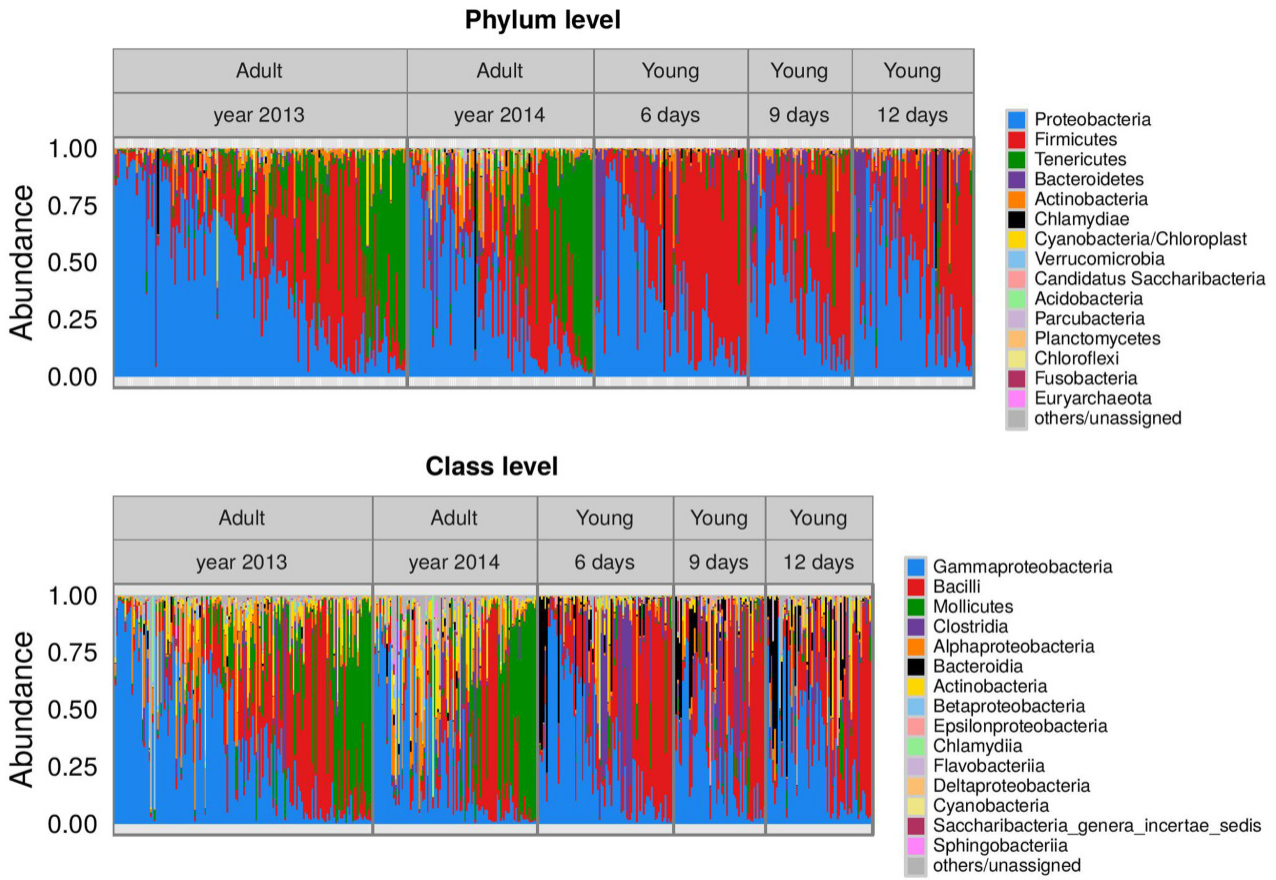

FIGURE 1 | Barplots indicating barn swallow fecal microbiota (FM) taxonomic composition. Mean proportion of reads for the 15 dominant bacterial phyla and classes detected in juvenile and adult barn swallow samples. Samples are organized according to age category (in the case of juveniles) and year of sampling (in the case of adults). Sample order within facetes correspond to scores for the first principal coordinate analysis (PCoA) axis calculated based on weighted UniFrac.

from the phylum Bacteroidetes, while the phylum Tenericutes was predominantly represented by *Mycoplasma* (9.6% of reads) and *Ureaplasma* (1.6%). The plot for taxonomic assignment of FM indicated pronounced variation at the inter-individual level (**Figure 1**) as well as consistent differences among adult and juveniles (detailed in the next section). A more comprehensive description of FM taxonomic content is provided in **Supplementary Table S2**.

FM Changes during Ontogeny

Fecal microbiota alpha diversity in terms of observed OTUs was nearly two-times higher in adults (mean SE of observed OTUs prior to rarefaction; adults D 222 11.4, young D 140 5.4). The contrast in alpha diversity between adults and young was significant for all alpha diversity indices calculated following rarefaction-based normalization of the OTU table (LME: 1DF D 1, $\$^2$ D 21.832, $p <$ 0.0001 for number of observed OTU; 1DF D 1, $\$^2$ D 29.85, $p <$ 0.0001 for Chao1 estimate; 1DF D 1, $\$^2$ D 6.944, p D 0.008 for Shannon diversity; **Figure 2**).

PCoA and adonis revealed consistent differences in FM composition among adults vs. young (**Figure 3**). Betadisper revealed lower inter-individual FM variation in juveniles for all dissimilarity index types. This difference was more pronounced in OTU presence vs. absence based methods (Jaccard and unweighted UniFrac; **Table 1**) than those based on relative abundance (**Table 1**). PCoA for predicted metagenomes indicated only slight differentiation among adults and juveniles (**Data Sheet 1**), though the differentiation was significant in adonis (**Table 1**). As in the OTU-based analyses, inter-individual variation in predicted metagenomes was higher in adults (**Table 1**).

DESeq2 analysis identified 213 OTUs (represented by 69.5% high quality reads) in which abundance varied between adults vs. juveniles, with 81 OTUs overrepresented in juveniles and 132 in adults (**Figure 4**). OTUs corresponding to the phyla Acidobacteria (genera *Gp16*, *Gp4*, and *Terriglobus*), Tenericutes (genera *Mycoplasma* and *Ureaplasma*), Verrucomicrobia, Parcubacteria, Deinococcus-Thermus (genus *Truepera*), Chloroflexi (genus *Litorilinea*), and Euryarchaeota (genus *Methanosaeta*) were more abundant in adults. The same was true for most Bacteroidetes OTUs (genera *Hymenobacter*, *Cloacibacterium*, *Flavobacterium*, *Chryseobacterium*, and *Pedobacter*) as well as most Actinobacteria OTUs (corresponding to the genera *Nocardioides*, *Iamia*, *Ilumatobacter*, *Ornithinicoccus*,

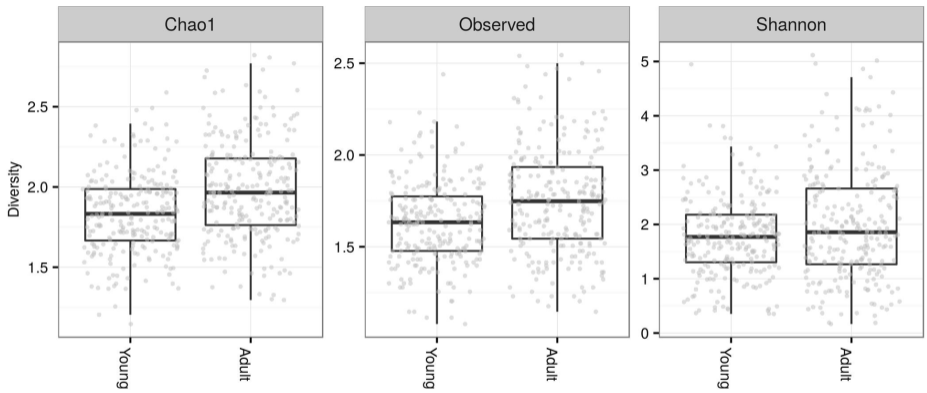

FIGURE 2 | Alpha diversity of FM in juveniles vs. adults. Number of observed operational taxonomic units (OTUs) and Chao1 estimates were log10 scaled, whereas Shannon diversities were not transformed.

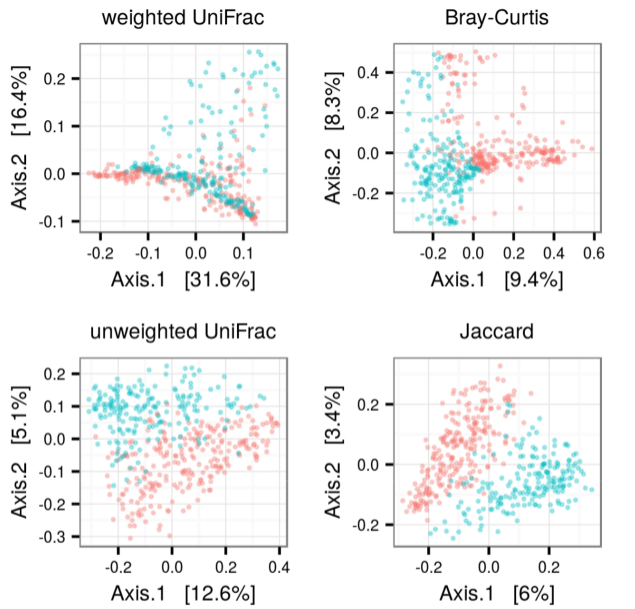

FIGURE 3 | Difference in FM composition among adult and juvenile barn swallows. Principle coordinate analysis for ecological dissimilarity among FM samples of juvenile (blue) and adult (red) barn swallows, with proportion of FM variation associated with individual axes indicated.

Actinomycetospora, Nakamurella, and *Corynebacterium*). Juvenile FM was characterized by an increase in the abundance of Chlamydiae (genus *Neochlamydia*) and several OTUs corresponding to genus *Dysgonomonas* (phylum Bacteroidetes) and *Olsenella*, *Rothia*, and *Blastococcus* (all belonging to the phylum Actinobacteria). The two dominant barn swallow FM phyla, Proteobacteria and Firmicutes, harbored a mix of OTUs that were overrepresented in either adults or juveniles. In the case of Proteobacteria, *Pseudoxanthomonas*, *Porphyrobacter*, *Luteimonas*, or *Thauera* exhibited a considerable increase in abundance in adults (log2 fold change > 5), while *Campylobacter*, *Orbus*, *Helicobacter*, *Lonsdalea*, or *Providencia* were more abundant in young. In the case of Firmicutes, *Sedimentibacter*, *Proteiniclasticum, Guggenheimella*, or *Catellicoccus* were overrepresented in adults, while abundance of *Weissella*, *Sarcina, Fructobacillus*, and most *Lactobacillus* OTUs increased in young.

Consistent with the pronounced differences observed at the OTU level, the abundance of 150 KEGG categories (DESeq2) also varied significantly between adults and juveniles, though the effect-size of these changes was low in most cases. For a summary of 34 KEGGs for which abundance varied considerably between adults vs. juveniles (absolute value of log2 fold change > 1), see **Data Sheet 1**. FM diversity tended to increase with increasing juvenile age [LME; observed OTUs (log10 transformed): slope D 1.140e 02 6.629e 03, 1DF D 1, ^{2} D 3.877, $p < 0.05$; Chao1 (log10 transformed): slope D 0.057 0.0188, 1DF D 1, ^{2} D 20.492, p D 0.0876; Shannon diversity: slope D 1.295 0.05692, 1DF D 1, ^{2} D 8.946, p D 0.0028]. On the other hand, age-dependent changes in juvenile FM composition were not significant for weighted and unweighted UniFrac according to adonis ($p > 0.1$, $R^2 < 0.02$), while only slight age-dependent differences were observed if using Jaccard and Bray–Curtis dissimilarity ($p < 0.05$, R^2 D 0.014 and $p < 0.01$, R^2 D 0.013, respectively). Inter-individual variation in FM did not change with juvenile age (betadisper: $p > 0.7$ in all cases) and no change in OTU abundance with juvenile age was detected using DESeq2 analysis. Similarly, no significant differences were observed among predicted metagenomes in juvenile age-classes ($p > 0.2$ for both adonis and betadisper).

As there was significant excess of positive nc.scores for adults and all juvenile age-classes (Wilcoxon one sample test: $p < 0.001$ in all cases), FM structure appeared to be driven predominantly by co-occurrence rather than co-avoidance interactions. Furthermore, nc.score values exhibited highly significant correlations across all juvenile age-classes and adults (Mantel's test: correlation coefficient range D 0.338– 0.391, $p < 0.0001$ in all cases) and in adults sampled in 2013 vs. 2014 (Mantel's r D 0.481, $p < 0.0001$), suggesting that among-OTU co-occurrence/co-avoidance interactions varied

TABLE 1 | Divergence between adult vs. juvenile barn swallow fecal microbiota (FM).

		Adonis						Betadisper			
Input data	**Effect**	**Df**	**SS**	**MSS**	***F***	R^2	***p***	**SS**	**MSS**	***F***	***p***
OTUs: weighted UniFrac	Adults vs. young	1	0:331	0.331	11:603	0.067	0.001	0.012	0.012	5:204	0.024
	Residuals	161	4:596	0.029		0.933		0.359	0.002		
OTUs: unweighted UniFrac	Adults vs. young	1	2:575	2.575	11:253	0.065	0.001	0.147	0.147	29:346	<0.001
	Residuals	161	36:835	0.229		0.935		0.804	0.005		
OTUs: Bray–Curtis	Adults vs. young	1	4:015	4.015	10:250	0.060	0.001	0.030	0.030	7:346	0.007
	Residuals	161	63:065	0.392		0.940		0.654	0.004		
OTUs: Jaccard	Adults vs. young	1	3:142	3.142	8:527	0.050	0.001	0.058	0.058	33:110	<0.001
	Residuals	161	59:331	0.369		0.950		0.282	0.002		
KEGGs: Bray–Curtis	Adults vs. young	1	0:070	0.070	5:949	0.036	0.003	0.015	0.015	6:166	0.014
	Residuals	161	1:902	0.012		0.964		0.401	0.002		

ANOVA tables for adonis and betadisper models testing differences in FM composition and inter-individual variation among adults and juveniles. Models were fitted for four ecological distance types (weighted and unweighted UniFrac, Bray–Curtis and Jaccard) for the OTU dataset. Shown are treatment and residual degrees of freedom (Df), sum of squares (SS), mean sum of squares (MSS), F-statistic values (F), probability values (p) and proportion of explained variance (R^2).

little over time and were independent of host age. After filtering out non-significant nc.scores, average number of co-occurrence or co-avoidance links per OTU was 0.137, 0.207, and 0.237 for six day-, nine day- and 12 day-old juveniles, respectively, and 3.437 and 5.242 for adults sampled in 2013 and 2014, respectively (**Figure 5**). The number of interactions per OTU was positively correlated with relative log-transformed OTU abundance ($p > 0.0001$ in all cases). After statistical control for this confounding effect, GLMM-based random effect estimates indicated that the number of significant per-OTU interactions was increased in Actinobacteria and Alphaproteobacteria bacteria consistently across all juvenile age-classes. The same also held true for Clostridia, with the exception of co-occurrence analysis on adults sampled in 2013 (see **Data Sheet 1**).

Temporal Invariance of FM and Divergence among Nests and Breeding Colonies

Bray–Curtis and weighted UniFrac similarity among samples from the same adults collected within individual breeding seasons was higher than that among samples from different adults (**Table 2**; **Figure 6**; **Data Sheet 1**), providing evidence for temporal stability in adult FM composition. When using unweighted UniFrac or Jaccard dissimilarity for the same test, however, we found no support for within-individual FM composition stability (**Table 2**; **Data Sheet 1**). Dissimilarity at within-individual and within-season time-scales tended to increase with increasing time-lag between collection of the corresponding samples (average time-lag SE D 34.6 1.7 days) in the case of unweighted UniFrac (LME: 1DF D 1, $\$^2$ D 4.526, p D 0.0334), Jaccard (LME: 1DF D 1, $\$^2$ D 7.731, p D 0.0054) and Bray–Curtis dissimilarity (LME: 1DF D 1, $\$^2$ D 4.560, p D 0.0327), after box–cox transformation of the response variable. This relationship was not significant for weighted UniFrac (LME: 1DF D 1, $\$^2$ D 0.404, p D 0.5253). At the between-season level, FM profiles corresponding to the same adult only exhibited higher similarity than expected by chance based on Bray–Curtis dissimilarity (**Table 2**). Temporal stability of predicted adult metagenome content was significant at the within-season time-scale, but not at the between-season time-scale (**Table 2**). Higher similarity between adults within breeding colonies than that between breeding colonies was associated with a very low effect size, despite being significant for all community distances types. Similarly, between-year variation in FM was associated with a considerable effect size in the case of Jaccard dissimilarity only (**Table 2**).

We identified 63 OTUs in the adult FM for within-season analysis and 118 OTUs for between-season analysis (represented by 30 and 20% of high-quality reads, respectively) where abundance exhibited significantly lower variation within individuals than between individuals. At the FM community level, signatures of temporal stability increased after exclusion of all but these OTUs from the dataset (mean difference between within- vs. between-individual dissimilarities D 0.125 and 0.010, Cliff 's d D 0.282 for within-season and 0.275 for between-season time-scales, $p <$ 0.001 in both cases; **Data Sheet 1**). Seventeen OTUs, assigned to Enterobacteriaceae, *Acinetobacter*, *Corynebacterium*, *Dysgonomonas*, *Tsukamurella*, *Dietzia*, *Mycoplasma*, *Streptococcus*, *Catellicoccus*, and *Lactobacillus*, exhibited significant temporal consistency both within and between seasons.

Community-wide juvenile FM temporal consistency was significant based on weighted UniFrac and Bray–Curtis dissimilarity calculated for OTU; however, there was no hint of temporal consistency based on absence vs. presence dissimilarities and KEGG predictions (**Table 3**; **Figure 7**). In addition, OTU-centered analysis identified only 16 low-abundance OTUs (represented by 1.4% high quality reads) that contributed to FM stability in juveniles. Differences in Bray– Curtis dissimilarity for sample pairs corresponding to the same vs. different juvenile were comparable when calculated for either the whole FM (**Table 3**) or for the FM subset comprising the 16 OTUs exhibiting signs of temporal stability (mean difference

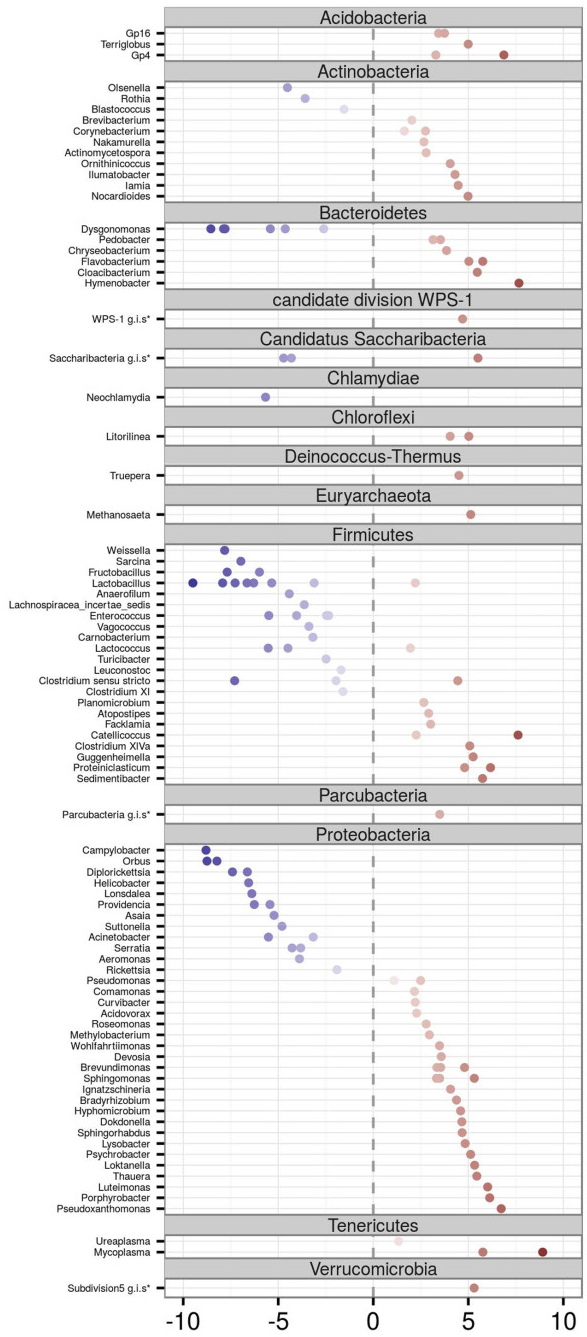

FIGURE 4 | Differential abundance analysis for FM OTUs of adult vs. juvenile barn swallows. Only those OTUs whose abundance varied significantly among adults and juveniles according to DESeq2 analyses are shown. Position on the horizontal axis corresponds to its log2 fold change. Negative (blue) and positive values (red) indicate a higher OTU abundance in juveniles and adults, respectively. Genus identity is determined by labels on the left side of the plot. OTUs are sorted according to their Phylum assignment. (g.i.s. D genera incertae sedis).

between within- vs. between-individual dissimilarity D 0.0765, Cliff's *d* D 0.125, $p < 0.01$; **Data Sheet 1**). FM similarity for juveniles of the same age at the within-clutch level was higher than that of juveniles raised in different clutches, both for OTU and predicted KEGG data and irrespective of dissimilarity index used (**Table 3**; **Figure 7**). The within-clutch similarity effect-size decreased considerably when running this analysis on FM samples from individuals of different age (Cliff's $d < 0.05$ in all cases). We also observed higher FM similarity within breeding colonies than between breeding colonies (**Table 3**; **Figure 7**), though the effect-size was low in all cases (Cliff's $d < 0.1$).

FM Similarity between Offspring and Their Parents

While juvenile FM showed greater similarity to social mothers than random adult females when using weighted UniFrac or Bray–Curtis as a measure of FM divergence, we observed no such effect when using absence vs. presence dissimilarities. We also observed no effect of social father on the FM composition of its offspring (**Table 4**; **Figure 8**; **Data Sheet 1**). These results remained unchanged after exclusion of extra-pair (*n* D 9) young from social father vs. offspring and parasitic young (*n* D 3) from social mother vs. offspring comparisons. Offspring vs. mother or father dissimilarity did not vary with juvenile age (LME: $p > 0.2$ in all cases). There was also no association between time lag in social mother vs. offspring FM sampling and FM similarity between mother and offspring (LME, $p > 0.1$ in all cases). On the other hand, social father vs. offspring Bray–Curtis and weighted UniFrac dissimilarity tended to increase with increasing time-lag between the two samples (LME, Bray–Curtis: 1DF D 1, $\$^2$ D 6.136, p D 0.0133; weighted UniFrac: 1DF D 1, $\$^2$ D 3.5994, p D 0.0570), though this effect was non-significant when using unweighted UniFrac and Jaccard dissimilarity ($p < 0.1$ in both cases). Similarity in the composition of predicted metagenomes among juveniles and social mothers or fathers was not higher than expected by chance (**Table 4**).

DISCUSSION

Age-Dependent Variation in FM Structure

Our data revealed pronounced differences in FM structure between adult and juvenile barn swallows. Adult FM alpha diversity was nearly two-times higher than that of juveniles, which, together with a slight increase in alpha diversity with juvenile age, implies that FM is gradually colonized by bacteria from external sources during the nestling period. On the other hand, six day-old juveniles had already been colonized by a rich FM consortia and the FM taxonomic composition in juveniles did not show any great variation with age. Hence, initial establishment of juvenile-specific FM appears to take place very soon after hatching and there is no evidence for a gradual succession toward adult-like microbiota due to newly invading FM species over the nestling period. In humans and other mammalian taxa, dramatic shifts in FM composition coincide with the transition between the pre- and post-weaning period due to associated nutritional changes (Schloss et al., 2012; Bäckhed et al., 2015; Mach et al., 2015). The effect of diet on mammalian host FM has also been demonstrated in numerous

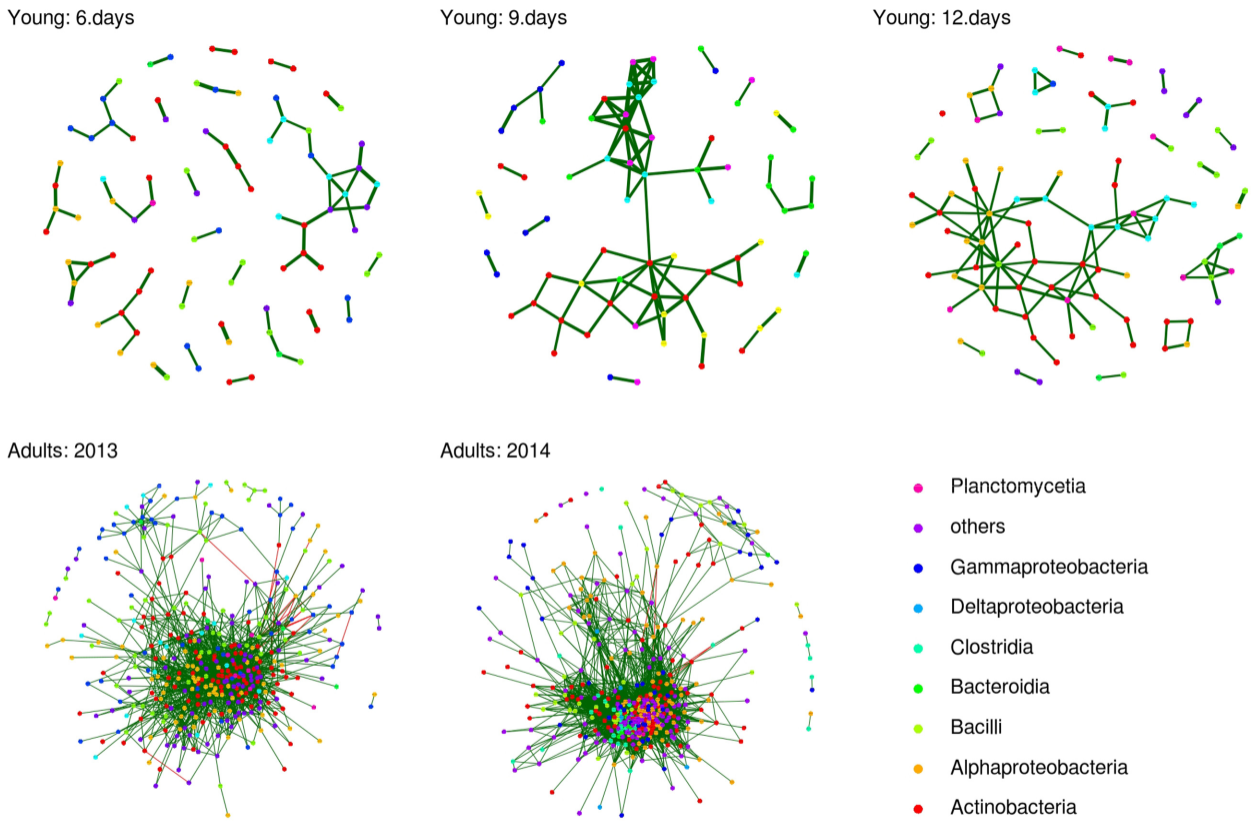

FIGURE 5 | Operational taxonomic unit co-occurrence network for adult and juvenile barn swallow FM. OTU co-occurrence network based on nc.scores for individual age-classes and for adults. Only those nc.scores passing multiple testing corrections ($q < 0.05$) are shown. Green and red lines indicate positive and negative co-associations, respectively. Class-level taxonomic assignations of individual OTUs included in the analysis are highlighted in different colors.

TABLE 2 | Effect of individual identity, breeding colony, and year on the divergence of adult barn swallow FM.

	Within vs. among individuals (within years)				Within vs. among individuals (among years)			
	Observed diff.	**95% CI range**	***p***	**Cliff's *d***	**Observed diff.**	**95% CI range**	***p***	**Cliff's *d***
OTUs: weighted UniFrac	**0.024**	**0.015 0.013**	**0.001**	**0.138**	0.011	0.016 0.016	0.118	0.046
OTUs: unweighted UniFrac	0.004	0.015 0.011	0.591	0.018	0.003	0.014 0.015	0.42	0.001
OTUs: Bray–Curtis	**0.079**	**0.022 0.027**	**<0.001**	**0.194**	**0.056**	**0.028 0.028**	**0.001**	**0.195**
OTUs: Jaccard	0.002	0.007 0.008	0.692	0.011	0.007	0.009 0.009	0.077	0.061
KEGGs: Bray–Curtis	**0.015**	**0.014 0.011**	**0.015**	**0.101**	**0.018**	**0.014 0.015**	**0.026**	**0.157**
	Within vs. among colonies				Within vs. among years			
	Observed diff.	**96% CI range**	***p***	**Cliff's d**	**Observed diff.**	**96% CI range**	***p***	**Cliff's d**
OTUs: weighted UniFrac	**0.008**	**0.002 0.002**	**<0.001**	**0.013**	**0.008**	**0.002 0.002**	**<0.001**	**0.061**
OTUs: unweighted UniFrac	**0.012**	**0.002 0.002**	**<0.001**	**0.049**	**0.012**	**0.002 0.002**	**<0.001**	**0.093**
OTUs: Bray–Curtis	**0.008**	**0.004 0.004**	**<0.001**	**0.029**	**0.008**	**0.003 0.003**	**<0.001**	**0.051**
OTUs: Jaccard	**0.01**	**0.001 0.001**	**<0.001**	**0.072**	**0.01**	**0.001 0.001**	**<0.001**	**0.127**
KEGGs: Bray–Curtis	**0.003**	**0.002 0.002**	**<0.001**	**0.009**	**0.003**	**0.002 0.002**	**<0.001**	**0.028**

Results of permutation-based tests on adult FM samples comparing dissimilarity among samples from (A) the same vs. different individuals sampled during the same breeding season and the same colony, (B) the same vs. different individuals sampled during different breeding seasons and in the same breeding colony, (C) different individuals sampled in the same vs. different breeding colony during the same breeding season, and (D) different individuals sampled in the same breeding colony during the same vs. different breeding season. For OTU data, analyses were run on four dissimilarity index types (weighted and unweighted UniFrac, Bray–Curtis and Jaccard). Shown are observed dissimilarity difference values, 95% confidence intervals of the corresponding permutation-based null distribution, permutation-based probability values and effect size estimates (Cliff's d). Significant results are in boldface.

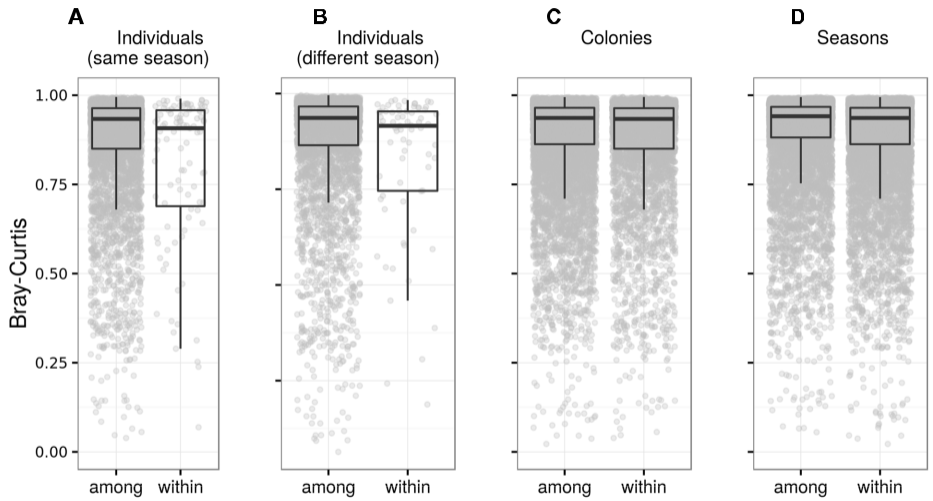

FIGURE 6 | Fecal microbiota differentiation in adult barn swallows. Box plots for Bray–Curtis dissimilarity among FM samples from the same vs. different individuals sampled during **(A)** the same breeding season and **(B)** different breeding seasons, **(C)** dissimilarities among FM samples from different individuals sampled during the same breeding season in the same vs. different colony, and **(D)** dissimilarities among FM samples from non-identical individuals sampled in the same breeding colony during the same vs. different breeding season. Comparisons based on other types of ecological dissimilarity are available in the **Data Sheet 1**.

TABLE 3 | Effect of individual identity, clutch identity, and breeding colony on FM divergence in juvenile barn swallows.

	Within vs. among individuals				Within vs. among nests			
	Observed diff.	95% CI range	*p*	Cliff's *d*	Observed diff.	95% CI range	*p*	Cliff's *d*
OTUs: weighted UniFrac	0.027	0.002 0.025	0.023	0.159	**0.022**	**0.007 0.012**	**0.001**	**0.142**
OTUs: unweighted UniFrac	**0.005**	**0.013 0.015**	**0.323**	**0.011**	**0.022**	**0.015 0.007**	**<0.001**	**0.144**
OTUs: Bray–Curtis	0.07	0.004 0.057	0.006	0.236	**0.064**	**0.015 0.02**	**<0.001**	**0.22**
OTUs: Jaccard	**0.003**	**0.008 0.011**	**0.37**	**0.018**	**0.03**	**0.008 0.006**	**<0.001**	**0.237**
KEGGs: Bray–Curtis	0.004	0.002 0.014	0.684	0.042	**0.016**	**0.003 0.009**	**0.001**	**0.184**
	Within vs. among localities							
	Observed diff.	96% CI range	*p*	Cliff's *d*				
OTUs: weighted UniFrac	0.002	0.004 0.003	0.101	0.017				
OTUs: unweighted UniFrac	**0.013**	**0.004 0.004**	**<0.001**	**0.083**				
OTUs: Bray–Curtis	**0.026**	**0.006 0.006**	**<0.001**	**0.084**				
OTUs: Jaccard	**0.009**	**0.002 0.003**	**<0.001**	**0.094**				
KEGGs: Bray–Curtis	0.003	0.002 0.002	0.977	0.028				

Results of permutation-based tests comparing dissimilarity among juvenile FM samples collected from (A) the same vs. different individuals in the same nest, (B) different individuals from the same vs. different nests located in the same breeding colony, and (C) individuals from different nests in the same vs. different breeding colony. For OTU data, analyses were run on four dissimilarity index types (weighted and unweighted UniFrac, Bray–Curtis and Jaccard). Only Bray–Curtis dissimilarity was used for predicted KEGG categories. Shown are observed dissimilarity difference values, 95% confidence intervals of permutation-based null distribution, permutation-based probability values and effect size estimates (Cliff's d). Significant results are in boldface.

experimental and comparative studies (Ley et al., 2008; David et al., 2014). We propose, however, that diet only has a limited effect on the differences in FM between adult and juvenile in barn swallow. While slight differences in adult vs. juvenile barn swallow diet have previously been reported, both these cohorts forage exclusively on taxonomically similar groups of flying insects (Turner, 1980). As a result, the potential effect of diet composition on FM is much more restricted in our study compared to research focused on omnivorous hosts. Moreover, our data on more than 50 passerine species with contrasting foraging specializations indicates that diet only has a negligible effect on interspecific variation in FM composition in this group (Kropáčková et al., unpublished data). Age-specific differences in gut physiology, morphology and diet digestion could also affect FM variation between adults vs. juveniles, especially as these undergo dramatic development after hatching in passerines and other bird taxa. Such changes include a gradual increase in relative gut mass and surface area, proliferation of enterocytes and mucous-secreting goblet cells (reviewed in Perry, 2006) and an increase in production of digestive enzymes (Caviedes-Vidal

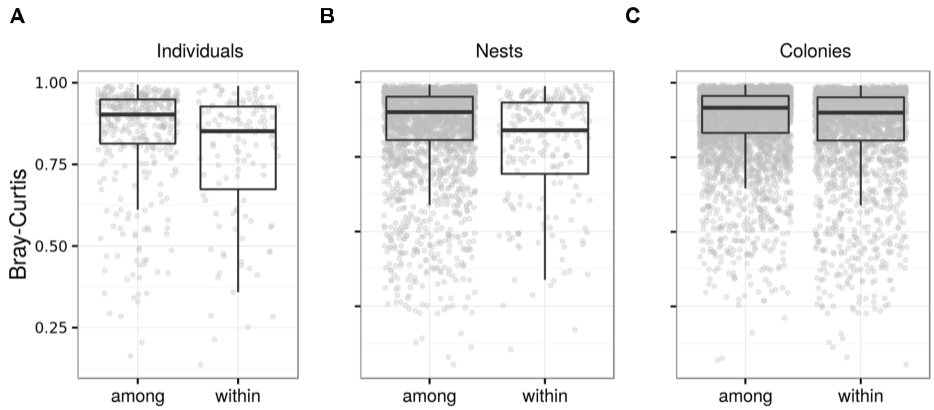

FIGURE 7 | Fecal microbiota differentiation in juvenile barn swallows. Box plots for Bray–Curtis dissimilarity among FM samples from **(A)** identical vs. different individuals from the same nest corresponding to different age-classes, **(B)** different individuals from identical vs. different nests placed in the same breeding colony and corresponding to the same age-classes, and **(C)** individuals from different nests that were placed in the same vs. different breeding colony. Comparisons based on other types of ecological dissimilarity are available in the **Data Sheet 1**.

TABLE 4 | Barn swallow parent vs. offspring similarity.

	Father vs. offspring similarity				Mother vs. offspring similarity			
	Observed diff.	95% CI range	*p*	Cliff's *d*	Observed diff.	95% CI range	*p*	Cliff's *d*
OTUs: weighted UniFrac	0.004	0.017 0.017	0.631	0.016	**0.026**	**0.022 0.022**	**0.031**	**0.148**
OTUs: unweighted UniFrac	0.01	0.016 0.016	0.188	0.074	0.005	0.024 0.024	0.616	0.038
OTUs: Bray–Curtis	0.005	0.016 0.018	0.639	0.022	**0.052**	**0.028 0.034**	**0.013**	**0.171**
OTUs: Jaccard	0.006	0.01 0.01	0.194	0.079	0.002	0.013 0.013	0.4	0.035
KEGGs: Bray–Curtis	0.005	0.019 0.018	0.328	0.003	0.007	0.017 0.017	0.724	0.104

Results of permutation-based tests comparing dissimilarity among parental vs. offspring FM composition. Analyses were run separately for offspring vs. mother and offspring vs. father pairs. For OTU data, analyses were run on four dissimilarity index types (weighted and unweighted UniFrac, Bray–Curtis and Jaccard). Only Bray– Curtis dissimilarity was used for predicted KEGG categories. Shown are observed value dissimilarity differences, 95% confidence intervals of permutation-based null distribution, permutation-based probability values and estimates of effect size (Cliff's d). Significant results are in boldface.

and Karasov, 2001), all of which increase digestion efficiency with host age (Karasov, 1990). Similarly, adaptive immunity and some components of innate immunity, are not fully developed in juveniles (Killpack et al., 2013), implying a reduced capability for management of associated microbiota in juveniles. Alternatively, FM variation between adults vs. juveniles could also be partly affected by age-dependent differences in the sources of bacteria invading the host's body. While the most likely source for juvenile FM are bacteria in the nest material or those acquired via parental transfer (González-Braojos et al., 2012a), adults are exposed to a much more diverse pool of environmental bacteria, both at the breeding ground and during the migration.

Taxonomic differences between adult and juvenile FM may provide a more mechanistic insight into those processes shaping FM differences between the two cohorts. Juvenile FM, for example, is characterized by an increase in the abundance of OTUs corresponding to lactic acid bacteria (LAB; genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Vagococcus*, *Weissella*, and *Olsenella*) and family Enterobacteriaceae (genera *Serratia*, *Lonsdalea*, and *Providencia*). LAB, and many Enterobacteriaceae, prefer energy-rich substrates and are capable of fermenting carbohydrates under anoxic conditions (Kandler, 1983). Their presence in juveniles, therefore, may reflect reduced production of digestive enzymes specific for this substrate (Caviedes-Vidal and Karasov, 2001). Presence of some LAB is believed to be generally beneficial as they stimulate the host's immune system and produce metabolites involved in the maintenance of gut microbiota homeostasis (Ljungh and Wadström, 2006). Further, some LAB species have been reported as contributing to the host's energy balance via improved feed conversion (Abe et al., 1995). On the other hand, while the specific effects of LAB and Enterobacteriaceae on passerine hosts are still poorly understood, it is thought that some may trigger negative consequences, including reduced growth rates and competition with the host for energy resources (González-Braojos et al., 2012b). Unlike juvenile FM, adult FM was enriched with bacteria utilizing relatively complex substrates, e.g., *Sedimentibacter*, *Clostridium* cluster XIVa, and *Proteiniclasticum* (family Clostridiaceae) or Flavobacterium and Chryseobacterium (family Flavobacteriaceae). In juveniles, we also observed an increase in the abundance of OTUs corresponding to taxa that may be associated with pathogenic or other detrimental effects on avian hosts. These include genera *Helicobacter* (Harbour and Sutton, 2008), *Campylobacter* (Benskin et al., 2015), *Rickettsia*

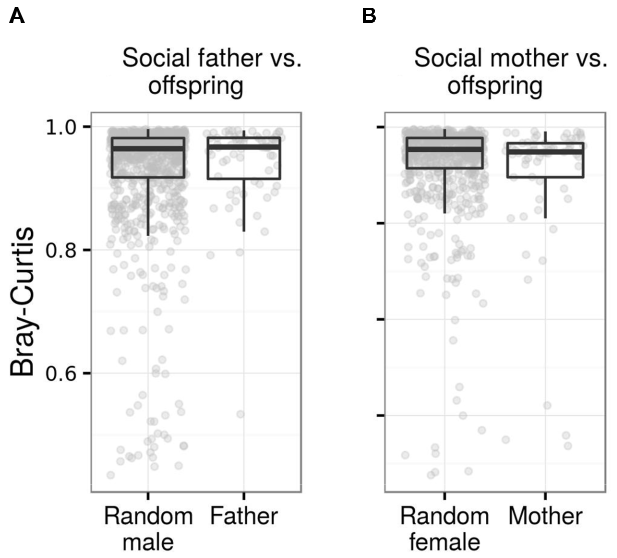

FIGURE 8 | Fecal microbiota similarity between young barn swallows and their social parents. Box plots for Bray–Curtis dissimilarity among juveniles and **(A)** their social fathers vs. non-social adult males and **(B)** social mothers vs. non-social adult females. Comparisons based on other types of ecological dissimilarity are available in the **Data Sheet 1**.

and *Diplorickettsia* (Ritchie et al., 1994), or *Suttonella* (Kirkwood et al., 2006), which may be associated with lowered capacity of the juvenile's immune system to cope with detrimental bacterial invaders (Killpack et al., 2013). Potential pathogens that were more abundant in adult FM included genera *Mycoplasma* and *Ureaplasma* (Sumithra et al., 2013). These bacteria often invade the urogenital tract of birds; hence, we speculate that their presence is associated with changes occurring in the urogenital tract during the breeding season or with sexual contact between colony members (Kreisinger et al., 2015b). We also detected an increase in OTUs of the family Xanthomonadaceae (genera *Wohlfahrtiimonas*, *Lysobacter*, *Luteimonas*, *Pseudoxanthomonas*, *Dokdonella*, and *Ignatzschineria*) in adult FM, as well as several Alphaproteobacterial OTUs (for example genera *Devosia*, *Hyphomicrobium*, *Porphyrobacter*, *Loktanella*) that are likely to be of environmental origin, suggesting a larger effect on adult FM of bacteria from environmental pools. However, as these taxa include both opportunistic pathogens and species harboring potentially important functions for vertebrate hosts, including defense against pathogens (Boutin et al., 2012), we cannot exclude their functional significance in barn swallow microbiota.

According to PICRUSt, the adult FM was enriched with a number of KEGGs that may be involved in interactions with the host's immunity system or with other FM members. Bacterial production of melanin, for example, can be associated with scavenging of superoxide radicals, which probably makes bacteria more resistant to the oxygen burst induced by the host's immune system (Plonka and Grabacka, 2006). Similarly, KEGG categories involved in the synthesis of metabolites that have putatively antibacterial and antifungal properties, such as sesquiterpenoid and macrolides, may promote competitive interactions with other FM members, thereby contributing to increased adult FM complexity, as discussed below. PICRUSt analysis also indicated that the adult FM may have a larger effect on host energy balance than that of juveniles as the abundance of those KEGGs associated with protein and carbohydrate digestion and absorption is increased. Bacterial fermentation of these substrates produces short-chain fatty acids that can be utilized as an energy source by the vertebrate host. Moreover, short-chain fatty acids are directly involved in the regulation of gut physiology and attenuate inflammatory responses (den Besten et al., 2013). The adult FM was also enriched with several KEGGs associated with ion balance (particularly: "Calcium signaling pathways" and "Endocrine and other factor-regulated calcium reabsorption") and pathways regulated by ion concentration (e.g., "Vasopressin-regulated water reabsorption" or "Gastric acid secretion"). Overall, FM predictions indicate higher interaction complexity between the host and gut microbiota and between individual members of the gut microbiota community in adults. On the other hand, several KEGGs found at higher levels in the adult FM are not directly associated with animal hosts (e.g., Isoflavim or Betalain biosynthesis), suggesting a greater importance of environmental bacteria in the adult FM.

Interestingly, differences in FM structure between adults and juveniles were observed not only in terms of richness and composition but also at the level of OTU co-occurrence pattern, with co-occurrence networks being more complex in adults. There was a significant correlation between co-occurrence coefficients calculated for adults and for different juvenile age-classes, with Alphaproteobacteria, Actinobacteria, and Clostridia being the main drivers of co-occurrence interaction, irrespective of host age. While this suggests that the overall shape of co-occurrence interactions remains invariant in relation to host age, co-occurrence strength increased with the end of the nestling period. Significant links in co-concurrence networks are often interpreted as direct interactive associations between two bacterial taxa (Faust et al., 2012). The abundance of two OTUs may be positively correlated, for example, if one utilizes the product of the other as a metabolic substrate (reviewed in Morris et al., 2013). On the other hand, secondary metabolites in certain species may suppress proliferation of other FM members, resulting in negatively correlated abundances (Ruiz et al., 2009). Alternatively, heterogeneity in environmental factors modulating FM composition may affect inter-individual variation in the FM community. Consequently, abundance correlations among OTUs may be driven by these extrinsic sources of FM variation rather than by direct mutual OTU interaction. In line with this explanation, inter-individual variation in adult FM was higher, implying an increased potential for detection of apparent interactive links between OTUs. At the same time, Alphaproteobacteria harboring OTUs of putatively environmental origin, were identified as one of the main drivers of co-occurrence interactions. On the other hand, Actinobacterial OTUs, which commonly produce secondary metabolites involved in interactions with other gut bacteria (Riley and Wertz, 2002), and Clostridia, which have been identified as important co-occurrence drivers in human gut microbiota (Faust et al., 2012),

were both involved in co-occurrence interactions in barn swallow FM more than expected by chance. This suggests that the patterns observed were at least partly driven by direct OTU vs. OTU interactions.

Temporal Stability of FM

Fecal microbiota is shaped by a wide variety of factors, some of which presumably have a stabilizing effect on the temporal consistency of its composition (Benson et al., 2010; McKnite et al., 2012) while others contribute to rapid turnover of FM members, introducing instability into the FM community over time (Amato et al., 2013; Wang et al., 2014). Our data on a free-living passerine bird population has extended current understanding of FM stability over time, with only one other study addressing this topic in this vertebrate group, however, using a captive population (Benskin et al., 2010). We show that, for both juveniles and adults, temporal consistency in taxonomic and functional profiles at the whole FM community level exhibited a rather low effect-size, despite its statistical significance. The most striking evidence for FM temporal stability has been provided for human populations, where individual-specific FM signatures persist for several years (Faith et al., 2013; Lim et al., 2014; Salonen et al., 2014, but see David et al., 2014). A high degree of temporal invariance in human FM may be maintained partly through long-term consistency in the social micro-culture environment and associated temporal stability of biotic and abiotic factors shaping FM (Mulder et al., 1998; Edstrom and Devine, 2001; Borland et al., 2007). In comparison, the few studies focused on FM temporal stability in other mammalian species typically show rapid within-individual FM composition fluctuations (Schloss et al., 2012; Becker et al., 2015; Hoy et al., 2015; but see Stevenson et al., 2014). Current knowledge on individual FM composition over time in wild populations is very limited (Waite et al., 2014; Baxter et al., 2015; Sun et al., 2016). Nevertheless, previous studies on free-living vertebrates have revealed pronounced population-wide changes in FM due to switches in diet composition, physiological state or health and infection status (Amato et al., 2013; Kreisinger et al., 2015a; Maurice et al., 2015; Sommer et al., 2016). Hence, the relatively low temporal stability in barn swallow FM detected in our study is in general agreement with the vast majority of current studies on vertebrates. As we controlled our analysis for systematic FM variation among breeding colonies, we can assume that the slight yet significant FM consistency observed was not caused by environmental heterogeneity within the populations sampled. In addition, given the small area of the breeding colonies (several hundreds of square meters), the high environmental homogeneity within colonies and low FM differentiation between colonies, it is unlikely that any environmental variation operating at the within-colony level could contribute to temporally consistent differences in FM between individuals.

It has recently been proposed that the temporal stability in microbial communities is maintained by a subset of "conditionally rare taxa" (Shade and Gilbert, 2015) that exhibit temporal persistence and typically occur at low abundance, though populations may exhibit an abrupt increase under certain circumstances. Our results, however, do not support any significant role of conditionally rare taxa as regards FM stability as signatures of FM consistency over time were consistently more pronounced in analyses utilizing OTU abundance compared with those based solely on OTU presence vs. absence. We conclude, therefore, that FM stability is driven by abundance invariance of relatively common OTUs and that a large proportion of OTUs that typically occur at low abundances are likely to persist for a limited period only in barn swallow FM. It could be argued that we were not able to detect any significant effect of conditionally rare taxa as these were below or at the detection threshold of our sequencing experiment. Although we cannot reject this possibility, we believe that any potential bias due to under-sequencing is unlikely to have affected the sensitivity of our analysis as high coverage index values indicate almost complete representation of FM diversity by sequencing data.

Temporal consistency effect sizes for juvenile FM sampled over several days were comparable with those observed in adults where the time-lag between samplings of the same individual was much longer, indicating a lower level of temporal FM invariance in young. This could theoretically be attributed to gradual FM succession associated with gradual changes in gut physiology and morphology and immune system during the early post-hatching period (Caviedes-Vidal and Karasov, 2001; Killpack et al., 2013). However, as our data do not provide evidence for successive changes in FM, we suggest that stochastic turnover of FM species is more rapid in juveniles. Knowledge regarding changes in FM stability in different ontogenetic stages remains relatively poor; however, our results are consistent with data from captive mice, where juveniles exhibited more rapid FM changes at the individual level compared with adults (Schloss et al., 2012). In addition, high stochasticity of microbial communities in early life stages is implied by numerous studies showing their dramatic changes during postnatal development (Schloss et al., 2012; Bäckhed et al., 2015; Mach et al., 2015).

Further evidence for increased stochasticity in the FM composition of juveniles was provided by our OTU-level analysis, which showed that only OTUs represented by a very low proportion of 16S rRNA reads, exhibited signs of temporal stability in juveniles, whereas both the number of OTUs with stable abundance over time and their relative representation in FM was much higher in the case of adults. Seventeen OTUs contributed significantly to FM stability in adults, both at the within- and between-season time-scale, and these represent potential candidates for involvement in long-term modulation of the host phenotype. Further correlative and experimental research, therefore, should focus on the relevance of these 17 OTUs as regards host traits involved in fitness pay-offs and mechanisms maintaining their temporal stability. At present, the role of individual FM species on host fitness in passerines is little understood (González-Braojos et al., 2012b; Benskin et al., 2015); nevertheless, the relevance of OTUs exhibiting consistent signs of temporal stability is indicated by numerous studies suggesting both beneficial and harmful effects in humans and other organisms. In barn swallow, several of these OTUs were Actinobacteria (genus *Dietzia, Corynebacterium* and *Tsukamurella*), i.e., they belong to a bacterial clade characterized by production of bacteriocins

and other secondary metabolites involved in interactions with other microbiota members (Riley and Wertz, 2002), implying their importance for FM structure. *Acinetobacter* genus (phylum Proteobacteria) are able to degrade chitin (Askarian et al., 2012), which may be of substantial importance for the barn swallow given its dependence on a chitin-rich diet. Genus *Mycoplasma* (phylum Tenericutes) include several potentially pathogenic species for birds (Sumithra et al., 2013). These bacteria support mechanisms enabling adherence to epithelial cells, implying a tight interaction with the host's immune system (Chu et al., 2003). Notably, the Lactobacillus OTU (corresponding to *L. reuteri* by blast search; 100% identity), known for its probiotic properties in poultry (Liu et al., 2007), exhibits a substantial level of temporal stability in barn swallow FM, as did *Streptococcus* OTU populations (corresponding to S. *thermophilus* by blast search; 100% identity), which also have a putative probiotic effect (Correa et al., 2005). Importantly, other abundant taxa of putatively environmental origin, such as Xantomonadaceae and Alphaproteobacteria, were not present in the OTU subset exhibiting signs of temporal stability, suggesting transient colonization of the gut by bacteria from the environmental pool as an important source of FM temporal variation at the within-individual level in barn-swallows. Although further research is needed to distinguish transient vs. resident members in passerine FM, a large proportion of putatively transient bacteria are probably associated with rapid passage of food through the digestive tract (Caviedes-Vidal et al., 2007; McWhorter et al., 2009), which may preclude their effective elimination or overgrowth by resident bacteria. Aside from the effect of transient bacteria, within-individual variation could theoretically be explained by a wide range of factors, including temporal changes in hormonal profiles or immune parameters (Koren et al., 2012; Org et al., 2016). Our own unpublished data suggest a tight association between cell-mediated immune response and FM composition in barn swallow, which is consistent with the latter explanation (Kreisinger et al., unpublished data). On the other hand, while there are contrasting differences in male and female hormonal profiles during the breeding season (Garamszegi et al., 2005), sex has been shown to have a negligible effect on FM composition in barn swallow (Kreisinger et al., 2015b). We therefore speculate that physiological changes modulated by variation in hormonal levels are unlikely to explain temporal variation in FM at the individual level.

FM Similarity between Offspring and Their Parents

Strict vertical transmission of FM from parents to progeny plays an important role in host vs. host-associated microbiota co-adaptations in several arthropod taxa, including the evolution of obligatory symbiotic interactions between host-associated microbiota members and their host. This results in a tight phylogenetic co-divergence between host-associated microbiota and their host (Janson et al., 2008), and perhaps in Dobzhansky–Muller type incompatibilities due to host-associated microbiota admixture in hybrids (Brucker and Bordenstein, 2012). In vertebrates, host-associated microbiota vs. host associations mediated by trans-generational transfer are probably not so tight; nevertheless, this mechanism still plays a significant role in genome evolution in some host-associated microbiota species (Falush et al., 2003). In mammals, initial inoculation of newborn young by vaginal microbiota during the delivery has a long-term effect on their FM composition (Salminen et al., 2004), while prebiotic compounds included in breast milk facilitate proliferation of beneficial microbes in the gut (Bode, 2012). The role of such parental effects on the FM of progeny has generally been poorly explored in non-viviparous vertebrate taxa (Lucas and Heeb, 2005). Where post-natal parental care exists in non-viviparous species, however, bacteria are likely to be transferred during food provisioning or other types of physical contact between parents and offspring (Lucas and Heeb, 2005). In barn swallow, we observed significantly higher similarity in FM composition between social mothers and their offspring but not in the case of FM comparisons between offspring and social fathers. The lower effect of social fathers on FM composition in progeny can be explained by the lower contribution of male barn swallows to parental care (Smith and Montgomerie, 1992; Møller, 1994). This results in a lower rate of social contact, which has previously been shown to shape microbial communities in birds (White et al., 2010; Kreisinger et al., 2015b). Although direct FM transfer during food provisioning is the most parsimonious explanation for mother vs. offspring similarity, we cannot exclude the possibility that it is mediated by *in ovo* maternal deposition of bioactive compounds that have the potential to modulate FM (Yurkovetskiy et al., 2013). On the other hand, we believe that mother vs. offspring similarity is unlikely to be caused by vertical inheritance of genes interacting with FM as a paternal effect on offspring FM was of a much lower effect-size and did not increase after expulsion of extra-pair (i.e., non-genetic) offspring, and mitochondria and heterogametic sex chromosomes are the only parts of the genome inherited exclusively maternally, and these are unlikely to have any considerable effect on FM structure (Benson et al., 2010; McKnite et al., 2012). Finally, there is some evidence for egg (and consequently embryo) inoculation by bacterial populations in the uterus (Funkhouser and Bordenstein, 2013). How often this occurs, however, and how this mechanism contributes to FM after hatching, remains unclear.

Conclusions

The aim of this study was to assess the strength of FM composition temporal consistency and the level of parental effect on juvenile FM composition. Both these factors are important from an ecological and evolutionary perspective as they help promote within individual and trans-generational consistency of phenotype traits linked with FM. Our data, however, revealed a limited role for these two factors.

At the whole community level, FM exhibited significant temporal consistency, both in adults and juveniles, though corresponding effect sizes were low. Nevertheless, we identified a subset of bacteria whose relative abundances exhibited pronounced levels of temporal consistency in adults, both at the within- and between-year time-scales. Consequently, these OTUs

may be involved in long-term modulation of the host phenotype. This possibility, along with identification of the mechanisms underlining stability of these OTUs over time, should be the subject of further empirical evaluation.

Our data also indicate a slight maternal, but not paternal, effect on FM composition in social offspring. This pattern may be explained by direct social transfer of FM, which has been proposed as a mechanism underlining gut microbiota heritability. Our data, however, are not fully consistent with this idea. The observed effect size of mother vs. offspring similarity was low and did not increase with offspring age. Consequently, pronounced differences between juvenile and adult microbiota are unlikely to be compensated for by a maternal effect during the nestling stage. Thus, the switch between juvenile-specific and adult-specific FM likely takes place after nest abandonment, when any parental effect is presumably to be limited. In addition, juvenile FM composition was highly variable during the nestling stage and, consequently, maternal effect on offspring FM is likely to persist for a limited period only.

DATA ACCESSIBILITY

Raw FASTQ files: http://www.ebi.ac.uk/ena/data/view/PRJEB 14586

AUTHOR CONTRIBUTIONS

Design of the study: JK and TA. Field sampling: TA, RM, AP, MA, OT, and JK. Laboratory analysis: LK, J-FM, and RM. Data analysis: JK. Financial funding: JK, TA, and LK. Manuscript drafting: JK, LK, and TA. All authors provided helpful comments and recommendations and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00050/full#supplementary-material

TABLE S1 | Detailed listing of barn swallow samples.

TABLE S2 | Taxonomic composition of barn swallow FM and its variation between adults and juveniles.

DATA SHEET 1 | Supplementary methods and analysis.

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3.5. Publikace E

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Podpis školitele

Variation in diet composition and its relation to gut microbiota in a passerine bird, the barn swallow

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ABSTRACT

Quality and quantity of food items consumed has a crucial effect on phenotypes. In addition to direct effects mediated by nutrient resources, an individual's diet can also affect the phenotype indirectly by altering its gut microbiota, a potent modulator of physiological, immunity and cognitive functions. However, little is still known about the effect of diet on gut microbiota in non-mammalian vertebrate hosts and in free-living populations. We developed a metabarcoding procedure based on cytochrome c oxidase I high-throughput amplicon sequencing and applied it to describe diet composition in a breeding colonies of an insectivorous bird, the barn swallow (*Hirundo rustica*). To identify putative diet-microbiota associations, we integrated the resulting diet profiles with an existing dataset for faecal microbiota in the same individual. Consistent with previous studies based on macroscopic analysis of diet composition, we found that Diptera, Hemiptera, Coleoptera and Hymenoptera were the dominant dietary components in our population. We revealed pronounced variation in diet consumed during the breeding season, along with significant differences between nearby breeding colonies. In addition, we found no difference in diet composition between adults and juveniles. Finally, our data revealed a correlation between diet and faecal microbiota composition, even after statistical control for environmental covariates affecting both diet and microbiota variation. While the strength of the diet-microbiota correlation was relatively low, our study suggests that variation in diet can induce significant microbiota changes in a non-mammalian host relying on a narrow spectrum of items consumed.

KEYWORDS: fecal microbiome, symbiosis, gastrointestinal tract, metabarcoding, insectivor

INTRODUCTION

Nutrient intake and energy metabolism are inherent properties of all cellular life forms. It is well established that nutrient composition and quantity obtained through diet has a decisive effect on traits tightly linked with fitness, including growth[1], reproduction[2], immunity[3] and various aspects of physiology[4]. At the same time, an animal's condition and/or health can have a causal effect on the composition of the diet consumed[5]. As an example, the preferred diet may vary with age due to differing nutritional demands related to specific ontogenetic stages[6].

In addition to the direct consequences of nutrient compounds on fitness-related traits, diet variation may also impose indirect effects by modulating populations of microbial symbionts hosted in the digestive tract of a given individual. This gut microbiota represents the largest fraction of microbial symbionts associated with animal hosts in terms of both cell count and encoded genes[7,8]. Notably, the gut microbiota is a potent modulator of host physiology and health status, with strong effects on the immune system, digestive tract morphology and digestion efficiency. At the same time, disruptions to the normal gut microbiota have been associated with a number of adverse consequences to host health[9,10].

Diet composition has been identified as one of the main drivers of gut microbiota variation in mammals. At larger phylogenetic scales, for example, repeated transitions between carnivory and herbivory were followed by consistent changes in gut microbiota content[11–13]. In omnivorous species, including humans, both long-term and short-term dietary habits impose gut microbiota changes that partly recapitulate transitions observed at the herbivore-carnivore continuum[14–16]. On the other hand, the effect of diet on gut microbiota has also been observed in species relying on a relatively narrow diet spectrum. For example, significant gut microbiota differences have been detected between folivore and frugivore lemurs[17], in bison fed on pasture or a grain diet[18] and even between strictly insectivorous bat species exploiting different prey spectra[19].

In non-mammalian taxa, the evidence for a dietary effect on gut microbiota is scarce and inconclusive. In comparison with mammals, bird diet appears to be a less important factor modulating gut microbiota[20]. Most studies undertaken on birds have found some support for the effect of diet on interspecific variation, particularly in passerines[21–24]. However, many of these studies had limitations related to the use of indirect dietary data and dietary data based on literature searches[25, but see 26,27]. Consequently, the rather high within-species variation in dietary items consumed in some cases could mean that an important source of variation was omitted from these analyses. Moreover, little is known about the effect of diet on intraspecific gut microbiota variation. There has been just one experimental study showing that diet intervention induced changes in gut microbiota composition in an omnivorous passerine bird[28]. To our knowledge, there has been no study exploring the effect of natural within-species diet variation on gut microbiota content. Alongside the effect of diet, most studies focused on avian gut microbiota in wild populations have

usually only identified a weak or moderate effect size of ecological gut microbiota predictors, with a large fraction of gut microbiota variation remaining unexplained[22,29,30]. As relatively small dietary changes can induce pronounced microbiota changes in mammals, it is tempting to speculate that a detailed knowledge of avian diet composition could help elucidate mechanisms driving their gut microbiota variation.

The use of traditional diet analysis methodologies for wild populations is challenging. Macroscopic examination of faecal samples or undigested food remains have commonly been applied[31,32]; however, this approach is time consuming and demanding as regards expertise. Moreover, there may be a non-negligible risk of limited taxonomic resolution or other specific biases[33,34]. DNA-based methodologies such as metabarcoding, which rely on deep sequencing of DNA markers bearing taxonomic information, represent a promising alternative that could partly overcome such challenges [35–37].

In this study, we developed procedures for metabarcoding-based diet profiling in insectivorous birds and applied this approach in studying dietary variation in breeding colonies of a migratory passerine bird, the barn swallow (*Hirundo rustica*). The barn swallow is a model free-living species widely used for research into reproductive biology and evolutionary ecology, including studies of sexual selection and sperm competition[38–40]. Importantly, the species' gut microbiota has recently been studied by a number of authors[41–45]. As such, this species represents a suitable non-mammalian model for research into interactions between diet and gut microbiota. Studies on diet-microbiota interactions in wild populations are still rare and, to our knowledge, our contribution represents the first attempt to integrate individual-based data on metabarcoding-based diet and gut microbiota composition in an insectivorous bird. Specifically, we used metabarcoding data to identify drivers of diet variation in the barn swallow and, in doing so, ask whether barn swallow diet varied temporally during the course of the breeding season or spatially between breeding colonies and whether adults and their nestlings rely on different spectrum of items consumed. As a further step, we combined existing data on barn swallow faecal microbiota[42] with diet profiles for the same individuals to test whether interindividual variation in diet was a predictor of gut microbiota composition.

MATERIAL AND METHODS

Sample collection

Collection of faecal samples from both adults and nestlings (6-12 days after hatching) was conducted from two populations (Šaloun farm, Lomnice nad Lužnicí [49° 4′ 7.762″ N, 14° 42′ 36.521″ E]; Hamr farm, Lužnice [49° 3′ 25.288″ N, 14° 46′ 10.82″ E]) in the Třeboňsko Protected Landscape Area (Czech Republic; distance between populations = 4.5 km) during the barn swallow breeding season from May to August 2014. For details on field procedures and faecal sample collection see Kreisinger *et al.*[42] and Petrželková *et al.*[39].

All field procedures were conducted in accordance with European Union Guidelines for Animal Care and Treatment and approved by the Animal Care and Use Committees at the Czech Academy of Sciences (041/2011) and Charles University in Prague (4789/2008-0).

Laboratory analysis

Metagenomic DNA from faecal samples was extracted using commercial PowerSoil kits (MoBio), with faecal microbiota subsequently profiled through high-throughput sequencing of 16S rRNA amplicons, as described in our previous studies[42,43]. In brief, the V3-V4 variable regions of 16S rRNA were amplified through a polymerase chain reaction (PCR) using universal primers. Next, sequencing libraries were prepared using TruSeq nano kits (Illumina) and sequenced on Illumina Miseq using the v3 kit (300bp paired-end reads) at Montpellier-SupAgro (France).

For the purpose of diet profiling, we used universal Cytochrome c oxidase subunit I (COI) primers (BF2-GCHCCHGAYATRGCHTTYCC and BR2-TCDGGRTGNCCRAARAAYCA) targeting a broad range of invertebrate taxa[46]. We selected these primers as *in vitro* and *in silico* tests indicate that they exhibit a lower level of PCR bias compared to existing alternatives.

To reduce problems associated with the formation of primer-dimers, sequencing libraries were prepared in three PCR steps:

1) COI pre-amplification by gene-specific primers, using a PCR mixture consisting of 5 µl of PCR mastermix, 0.6 µM of forward and reverse COI-specific primer and 3.8 µl of

metagenomic DNA. Our pilot PCR analysis revealed that the primers showed a strong affinity to the host DNA. To avoid amplification of host COI, 6 μM of a custom blocking primer containing C3 spacer modification on the 3' end and exhibiting a perfect match to the host COI (ACCGAAGAACCAGAATAGGTGTTGGTAAAGTAC) was added to the PCR reaction. To evaluate potential biases associated with this technique, a subset of samples (n = 23) was also amplified without the blocking primer. PCR cycling conditions consisted of an initial denaturation step (95°C, 5 minutes) followed by 22 cycles of denaturation (98°C, 20 seconds), blocking primer annealing (53°C, 15s), COI-specific primer annealing (47°C, 15s) and extension (72°C, 40s), followed by a final extension at 72°C for 5 min.

2) Amplification by primers including tails compatible with sequencing adaptors, using a PCR mixture comprising 5 μl of PCR mastermix, 2.8 μl of ddH_2O, 0.6 μM of forward and reverse COI primers flanked by tails complementary to Access Array sequencing adaptors (Fluidigm Corporation, USA) and 1 μl of PCR product from the 1st PCR round. PCR cycling conditions comprised an initial denaturation step (95°C, 5 minutes) followed by 15 cycles of denaturation (98°C, 20 seconds), primer annealing (50.5°C, 15s) and extension (72°C, 40s), followed by a final extension at 72°C for 5 min.

3) PCR-based ligation of sequencing adaptors, using a reaction mixture comprising Access Array sequencing adaptors (4 μl) along with PCR mastermix (10 μl), 4 μl of ddH_2O and 2μl of 25x diluted PCR product from the 2nd PCR round. PCR cycling consisted of an initial denaturation step (95°C, 5 minutes) followed by 16 cycles of denaturation (98°C, 20 seconds), primer annealing (55.5°C, 20s), and extension (72°C, 40s), followed by a final extension at 72°C for 5 min.

Kappa HIFI HotStart polymerase mastermix (Kapa Biosystems, USA) was used in all PCR reactions. Technical PCR duplicates were prepared for all samples. Products from the 3rd PCR round were quantified by GenoSoft software (VWR International, Belgium) based on band intensities after electrophoresis on 1.5% agarose gel and mixed at equimolar concentration. The final library was purified using Agencourt AmpureXP beads (Beckman Coulter Life Sciences). Products of the desired size were extracted by PipinPrep (Sage Science Inc., USA) and sequenced on Illumina Miseq (v3 kit, 300bp paired-end reads) at the Central European Institute of Technology (CEITEC, Masaryk University, Brno, Czech Republic).

Bioinformatic and statistical analysis

Bacterial 16S rRNA reads were processed using LOTUS pipeline[47] as detailed in Kreisinger *et al.*[42], the resulting operational taxonomic unit (OTU) table (i.e. abundance matrix for read counts of individuals in individual samples), OTU taxonomic annotations along with their representative sequences, and sample metadata were merged into a phyloseq database [48] for later analysis.

In the case of the COI dataset, regions corresponding to gene-specific primers were removed from fastq files using skewer[49]. Subsequently, the fastq files were quality-filtered (< 1 expected error per read) and denoised using R version 3.4.4[50], with the dada2 package[51] used to define reliable COI haplotypes. Technical duplicates showed significant consistency in Shannon diversities (Pearson correlation: $r = 0.982$, $p < 0.0001$) and composition of COI profiles (Procrustean analysis: $r = 0.996$, $p < 0.0001$). Consequently, we merged COI profiles for sample duplicates to obtain sample-specific COI profiles. To suppress any effect of PCR and sequencing artefacts, haplotypes that were not consistently present in both technical duplicates were eliminated from the dataset[e. g. ,52]. For a limited number of samples, we failed to sequence both duplicates (n = 3). In these cases, we eliminated all haplotypes whose presence was not confirmed in samples for which both duplicates were available.

For the purpose of taxonomic classification, 200 top blastn hits for each COI haplotype were downloaded from the NCBI nt database and used for the construction of a reference database. Dada2 implementation of RDP classifier[53] was subsequently applied for taxonomic assignment of COI haplotypes at an 80% posterior confidence threshold. Abundances matrix, representing read counts for individual haplotypes in each sample, along with sample metadata, taxonomic annotations and haplotype sequences were merged into a phyloseq database[48].

Krona pie-charts[54] were used to visualise the taxonomic content of the whole dataset. Next, all non-insect haplotypes (i.e. not corresponding to putative dietary items) were eliminated. Congruence in Shannon diversities (calculated after the exclusion of non-insect haplotypes; hereinafter termed dietary profile) between sample pairs amplified either with or without the blocking primer were assessed as intra-class correlations calculated using the rptR function in the R statistical environment[55]. We also evaluated

congruence in the composition of insect dietary profiles using Procrustean analysis, with Hellinger dissimilarity matrices scaled by Principal Coordinate Analysis (PCoA) used as inputs.

Analysis of covariance (ANCOVA) was used to test whether Shannon diversity (square-root-transformed) of dietary profiles was affected by locality, Julian date of sample collection, age class (i.e. adult vs. young) and by two-way interactions between these variables. Julian date was centred[56], both in this statistical model and all later analyses. We also checked whether diversity varied with sequencing depth (log-scaled). Significant predictors of dietary diversity were identified via step-wise backward elimination of nonsignificant variables from the initial full model (i.e. containing all the above-mentioned predictors). After visual exploration of divergence in dietary profile composition by Principal Coordinate Analysis (PCoA), variation in dietary composition due to the effect of locality, age class and Julian date of sample collection was analysed by distance-based redundancy analysis (db-RDA)[57] running on Hellinger and binary Jaccard dissimilarities among samples. We considered linear, quadratic and cubic effect of Julian date to account for potentially non-monotonic abundance changes in dietary items during the breeding season. Hellinger dissimilarities automatically account for the different number of sequences between samples. Jaccard dissimilarities were calculated after rarefaction of the abundance matrix (n = 536 sequences per sample, i.e. the minimum sequencing depth achieved). To demonstrate that rarefaction has a negligible effect on overall beta diversity, we calculated Jaccard dissimilarities for a subset of samples with > 5000 sequences (n = 47), which were rarefied to either 500 or 5000 seqs./sample. The resulting strength of correlation between these two distance matrices was high (Procrustean analysis: $r = 0.934$, $p = 0.0001$). The db-RDA model selection strategy was based on the forward step-wise approach implemented in the ordiR2step function (vegan package in R)[58]. The abundances of dietary taxa that varied due to the effects of predictors suggested by db-RDA were identified using generalised linear models with negative binomial distribution in the DESeq2 package[59]. Benjamini & Hochberg[60] false discovery rates were used for multiple testing corrections.

Procrustean analysis was applied to test for any correlation between interindividual divergence in diet and faecal microbiota profile. Furthermore, we employed db-RDA and variation partitioning analysis (varpart function in the R package vegan) to account for

direct and indirect effects of environmental factors, where the matrix of Hellinger or Jaccard dissimilarity in gut microbiota was considered as a response and matrices of explanatory variables (including linear, quadratic and cubic effects of Julian date, locality and age class) and divergence in diet composition (i.e. PCoA axis scores for Hellinger divergences in diet profiles) were included as explanatory and/or conditional variables. To prevent db-RDA model overfitting, we only considered PCoA axes for diet that exhibited a significant correlation with microbiota composition ($n = 10$ for Hellinger and $n = 11$ for Jaccard dissimilarities), selected using a forward selection approach (ordiR2step function from R package vegan). Finally, we applied the joint species distribution model (JSDM) from the boral package[61] to estimate pair-wise residual correlations between taxa abundances in the community matrix after accounting for the effects of environmental variables on taxa abundances and their mutual covariance. A similar approach was previously applied to search for cross-domain correlations between gut microbiota and the intestinal helminth community[62]. To run JSDM, we merged community matrices for insect genera and 16S rRNA OTUs, using a model offset corresponding to the log-transformed number of sequences for a given sample and given marker gene to account for uneven sequencing depth. Dietary genera and bacterial OTUs detected in < 10 samples were excluded. Locality, age class and Julian date of sample collection were considered as environmental covariates. We considered three JSDM versions, with the effect of Julian date modelled as either [i] a linear term only, [2] a linear and quadratic term, or [3] a linear, quadratic and cubic term. Later, we used the JDSM with Julian date modelled as a linear and quadratic effect only, as this exhibited the lowest deviance information criterion compared to the alternative fits ($\Delta DIC_{(quadratic\ vs.\ linear)} = 77.17$ and $\Delta DIC_{(quadratic\ vs.\ cubic)} = 186.3$). The model was fitted using default priors (described in boral documentation) and assuming negative binomial distribution of read counts for bacterial and dietary taxa. Diet vs. microbiota taxa correlations were estimated based on a Markov Chain Monte Carlo simulation consisting of 50 000 iterations. The thinning interval was set to 40 iterations, with the first 1000 iterations discharged as burn-in. Support for estimated parameters was assessed based on 95% posterior credible intervals.

RESULTS

Effect of blocking primers on COI amplification

While we collected 141 faecal samples, PCR amplification failed in 35 samples; hence, we only sequenced 106 samples for the COI profile. We obtained 2 369 181 high-quality reads that were grouped into 1 591 COI haplotypes. Median sequencing depth corresponded to 13 258 sequences per sample (range = 536 – 60 484). Later, we examined the sources of this relatively high frequency of PCR failure, as their non-random distribution could potentially affect interpretations of later analyses. We found that PCR failure was more common in adults than juveniles (Probability of success = 48.9% vs. 89.2%, chi-squared test: d.f = 1, χ^2 = 27.591, p < 0.0001). Based on electrophoresis gel band intensity, we observed lower PCR outputs for bacterial 16S rRNA amplicons in adults than juveniles (Welsh t test: d.f. = 124.44, t= -3.458, p = 0.001; amplicons prepared in parallel for the same samples). Hence, we suggest that the lower PCR success in adults was caused by an overall lower quantity and quality of DNA template. On the other hand, we did not detect any effect of sample location (chi-squared test: d.f. = 1, χ^2 = 0.037, p = 0.848) or Julian date (Welsh t test: d.f. = 45.882, t = 0.315, p = 0.755) on the probability of PCR failure. In addition, we found no difference in microbiota composition between samples that failed vs. those that passed the PCR step with diet primers (PERMANOVA for Hellinger distances: pseudo-$F_{(1,137)}$ = 1.001, R^2 = 0.06, p = 0.459) after statistical control for the effect of age and locality (i.e. predictors that impacted microbiota composition in our population)[42].

Insects representing putative dietary components represented the dominant fraction of COI profiles (47% of all reads, 961 haplotypes). Non-target taxa were represented by avian haplotypes (18% of reads), plants (10% of reads), fungi (namely Oomycetes, 3% of reads) and putative symbiotic Arachnida (Trombidiformes and Dermanyssidae, 6% reads; Supporting information Fig. A1). The relative abundance of avian haplotypes was significantly higher when blocking primer was not included in the first PCR reaction (62% of reads per sample vs. 1.3% of reads; Wilcoxon rank sum test: W = 11, p < 0.0001). After the exclusion of all non-target haplotypes, sample pairs that were amplified both with and without blocking primers (n = 23 pairs) exhibited high consistency in Shannon diversities (Intra-class correlation = 0.961, 95% bootstrap confidence intervals = 0.908 – 0.983, permutation-based p = 0.0001) and relative abundance of individual haplotypes

(Procrustean analysis: $r = 0.989$, $p < 0.0001$; Fig. 1). Consequently, diet profiles for sample duplicates generated with and without blocking primes were merged. We then excluded 24 samples (out of 106 sequenced samples) that included low numbers of target insect reads (< 500). Consequently, the final dataset included 82 samples (17 adults and 68 juveniles; Supporting information Table A1). Frequency of samples with a low vs. sufficient number of insect reads (i.e. < 500 insect sequences) did not vary between adults and juveniles (chi-squared test: d.f = 1, $\chi^2 = 0.199$, $p = 0.656$) or Julian date (Welsh t test: d.f. = 24.088, $t = -0.550$, $p = 0.588$). However, there was a higher percentage of samples with a low number of insect reads at Saloun farm (40%) than Hamr farm (4%; chi-squared test: d.f. = 1, $\chi^2 = 19.666$, $p < 0.0001$), which was paralleled by a higher fraction of no-target reads at Saloun farm (58.8%) than Hamr farm (31.3%) across all sequenced samples (Welsh t test: d.f. = 103.75, $t = 3.904$, $p = 0.0002$). This difference was mainly associated with increased percentage of symbiotic Arachnida (36.9% vs. 4.3% of reads) at the former location, suggesting that higher abundance of these nontarget taxa could compromise efficient amplification of insect DNA. Alternatively, there could have been higher incidence of insect taxa at Saloun farm locality that were poorly recovered by our wetlab protocol. Given the high dominance of insect genera within individual samples (Fig. 2), this should also result in different frequency of PCR failures between the two locations. However, as already mentioned above, we did not detect such an effect. Variation in hardly recoverable insect taxa between the two locations may lead to differences in PCR products concentrations. Nevertheless, contrary to this prediction, gel band intensities for diet amplicons after the second PCR step were the same for both localities (Welsh t test: d.f. = 103.57, $t = -0.446$, $p = 0.656$). We also did not detect any difference in microbiota composition between samples with low vs. sufficient number of insect reads, while accounting for locality-specific variation in microbiota content (PERMANOVA for Hellinger distances: pseudo-$F_{(1,103)} = 0.900$, $R2 = 0.009$, $p = 0.580$).

Diet profile variation

Haplotype-specific read counts were grouped into genus-level bins for all later analyses if not stated otherwise. We detected 171 insect genera or higher insect taxa (in the case of insufficient support for genus-level delimitation). In terms of reads counts, the most abundant insect order was Diptera (61% of reads per sample on average, dominated by the genera *Chironomus* and *Nephrotoma*). A considerable proportion of the dietary profile

comprised Hemiptera (17% of reads, dominated by the genus *Lygus*), Coleoptera (14% of reads, dominated by the genera *Aphodius* and *Psylliodes*) and Hymenoptera (6% of reads, dominated by ants of the genus *Lasius*; Supporting information Fig. A2). Other taxa were represented by < 1% reads per sample on average. The number of insect genera per sample ranged between one and 18 (median = 4). Individual samples were mostly dominated by a single insect genus (Fig. 2). Subsequently, rarefaction analysis for 1 – 5001 randomly selected reads per sample revealed that sequencing coverage corresponding to ~500 sequences per sample was sufficient to capture the majority of genus-level diversity (Supporting information Fig. A3).

Using ANCOVA, we found that the Shannon diversity of dietary profiles differed between localities ($F_{(1,79)} = 4.352$, $p = 0.040$). However, there was no difference in diversity between adults and juveniles ($F_{(1,80)} = 3.289$, $p = 0.074$, mean Shannon diversity [± S.E] = 0.428 ± 0.096 for adults and 0.667 ± 0.065 for juveniles) and we found no support for any other predictor of diet diversity ($p > 0.05$ in all cases).

Explorative insights provided by PCoA for Jaccard and Hellinger dissimilarities suggested an effect of both locality and Julian date on variation in dietary composition (Fig. 3). Specifically, scores for the second PCoA axis separated samples from different localities (Wilcoxon test: $W = 862$, $p < 0.0001$ for Jaccard and $W = 514$, $p = 0.005$ for Hellinger dissimilarities) and were correlated with Julian date of sample collection (Spearman correlation, rho = 0.248, $p = 0.025$ for Jaccard and rho = 0.413, $p = 0.0001$ for Hellinger dissimilarities). Constrained db-RDA models running on Hellinger and Jaccard dissimilarities provided comparable results (Table 1). However, neither PCoA nor db-RDA supported a difference in dietary composition between adults and juveniles. Inclusion of polynomial terms into the final db-RDA models suggested non-monotonic variation in dietary items during the breeding season. Subsequently, DESeq2 analysis aimed at identifying particular insect genera involved in this variation included both the effect of locality and Julian date. In the case of Julian date, we tested for the effect of cubic and quadratic polynomes via likelihood ratio tests. While no insect genera exhibited cubic association with Julian date of sample collection, the abundance of 14 insect genera exhibited quadratic correlation with sampling date (Supporting information Fig. A4). For example, flies from the genera *Pollenia* and *Hybomitra* and from the family Tabaninae, as well as crane flies (genus *Nephrotoma*) and ants (genus *Lasius*), were most commonly

detected in the middle of the breeding season. Conversely, beetles from the genus *Aphodius* were more common at the beginning of the breeding season, while mosquitoes from the genera *Culiseta* and *Ochlerotatus*, as well as Hemiptera from the genus *Lygus*, were more prevalent late in the breeding season. No insect genus exhibited significant variation between localities after statistical control for within-season variation (i.e. quadratic effect of sample collection date) and multiple testing corrections.

Association between diet variation and faecal microbiota composition

Bivariate Procrustean analysis suggested significant congruence between faecal microbiota and dietary profile composition (Sum of squared differences = 0.358, Procrustes correlation = 0.801, p = 0.001 for Jaccard and Sum of squared differences = 0.589, Procrustes correlation = 0.641, p = 0.001 for Hellinger dissimilarities; Supporting information Fig. A5). Furthermore, db-RDA and subsequent variation partition analyses indicated that diet had a low, though significant, effect on faecal microbiota, independent of environmental covariates ($R^2_{adjusted}$ = 0.058, $F_{(116,65)}$ = 1.468, p = 0.001 for Jaccard and $R^2_{adjusted}$ = 0.063, $F_{(10,66)}$ = 1.571, p = 0.001 for Hellinger dissimilarities). At the same time, faecal microbiota was also significantly affected by an independent effect of environmental covariates ($R^2_{adjusted}$ = 0.081, $F_{(5.65)}$ = 1.921, p = 0.001 for Jaccard and $R^2_{adjusted}$ = 0.064, $F_{(5.66)}$ =2.074, p = 0.001 for Hellinger dissimilarities). Finally, variation partitioning revealed a fraction of gut microbiota variation explained by both diet and environment ($R^2_{adjusted}$ = 0.037 for Jaccard $R^2_{adjusted}$ = 0.032 for Hellinger dissimilarities). JSDM indicated 85 highly supported (posterior confidence < 0.95) residual correlations between bacterial OTUs and insect genera present in the dietary profile (Fig. 4).

DISCUSSION

Variation in barn swallow diet

COI metabarcoding of faecal samples enabled us to recover a large number of insect taxa, corresponding to putative components of barn swallow diet. The taxonomic content of these dietary profiles was comparable with those from previous studies relying on undigested prey or faecal samples[32,63–67]. In particular, Diptera represented the largest fraction of reads in our study, followed by Coleoptera, Hemiptera and Hymenoptera. The dominance of individual insect groups tends to differ between previously published

studies, presumably because of dietary variation in space and throughout the breeding season[32,63,66,67]. We explicitly addressed this possibility by field sampling within two months of the breeding season at two ca. 4.5 km distant breeding colonies, and were able to show that both spatial variation and collection date affected diet spectra. This illustrates the high spatio-temporal variability of dietary items consumed and shows that the dietary patterns observed during short sampling periods cannot be easily generalised, even in aerial insectivores. In our study, potential prey availability was not quantified as we believe that obtaining such data can be problematic as regards aerial foragers, particularly as their hunting strategy, including intensity of hunting, height of hunting trips and their distance from the nest site, may vary dramatically with actual environmental conditions[63,68,69].

Offspring can be very demanding as regards the quality and quantity of nutrients required during the early post-hatching phases of development; hence, parents of many animal species supplement the offspring's diet with specific dietary items[70,71] or select microhabitats that satisfy their dietary requirements[72,73]. Furthermore, adults may switch their typical foraging preferences during the breeding season in order to provide their progeny with a high-quality diet. Previous studies on the barn swallow suggest that parents feed themselves with smaller dietary items than those they provide to nestlings[63,74]. In the present study, however, both dietary composition and diet alpha diversity failed to provide support for the idea that food composition differs between adults and juveniles. In fact, nestlings at our localities generally exhibited high fledging success and suffered no apparent signs of deprivation during the nestling period. This, together with the overall high quantity of flying insects at our field sites (located close (<1 km) to highly eutrophic human-made water reservoirs), indicates that food was not a limiting resource and that adults did not need to allocate the best food to nestlings.

Correlation between diet and faecal microbiota

While knowledge of gut microbiota in free-living vertebrates is gradually increasing, the extent to which their gut microbiota is affected by variation in diet is still not sufficiently understood. Most studies on wild vertebrate species have applied a comparative approach aimed at detection of microbiota variation between animal species[13,20,21,30] or populations[18,75]. However, to our knowledge, there have been just a few studies attempting to directly integrate metabarcoding data on dietary composition and

microbiota profiles on an individual basis[19,76–79].

In comparison with more widely studied mammals, passerine birds have a clearly distinct composition of host-associated microbial communities[11,22,30]. The microbiota in such communities is characterised by rapid temporal changes at the intra-individual level[42], with just a few bacteria exhibiting some level of stability over time. Based on current knowledge, interspecific differences in passerine gut microbiota composition appear to be rather low[22,30]. Furthermore, it has been shown that passerine gut microbiota structure can be affected by social contacts, age, sex, host immunity or blood concentrations of steroid hormones[41,43,45,80]. All the above-mentioned factors, however, usually explain just a limited fraction of total gastrointestinal microbiota variation. As such, we hypothesised whether the unexplained variation in gut microbiota may be related to actual diet composition. While bivariate Procrustean analysis indicated a significant correlation between gut microbiota and diet profile composition, use of this approach is problematic as bivariate approaches fail to distinguish direct links from indirect effects mediated by shared correlation of taxa abundance with environmental variables. To address this, we applied db-RDA modelling followed by variance partitioning, which indicated a significant, though relatively low, fraction of gut microbiota variation explained by variation in diet, independent of the effect of environmental covariates modulating gut microbiota and/or diet consumed. In addition, approximately 3.5% of gut microbiota variation was explained by the joint effect of diet and environmental covariates. Though we were unable to untangle causality in this particular case, we propose that variation in diet induced by spatio-temporal factors could be an important force behind the effect. JDSM identified 85 links between prey genera and bacterial OTUs and, in some cases, bacterial OTUs corresponded to putative insect symbionts (e.g. a positive link between *Lygus* and *Rikettsia* OTU 19), suggesting that gut microbiota can be at least partly affected by bacteria present in the diet. At the same time, however, variation in dietary items was also associated with abundance changes in several bacteria that are widespread residents of vertebrate guts (e.g. Lacobacillacea, Enterobacteriaceae, Clostidiales), suggesting that diet can also modulate proliferation of bacteria already residing in the barn swallow gut. Despite being significant, the overall effect of diet on the gut microbiota of barn swallows, and in birds in general, appears to be of lower importance than in mammals[20], providing further evidence for clear differences in host-microbiota interactions in these two vertebrate clades. Deducing mechanisms behind these

differences is rather challenging, given the current state of knowledge. Nevertheless, we speculate that the explanation involves differences in digestion physiology between the two groups. In particular, diet passage thought the gut is much faster in passerines than in mammals and, therefore, does not depend largely on bacterial fermentation[81,82]. Consequently, there would be a limited opportunity for bacterial populations within the gut to be affected by the diet consumed.

Methodical considerations

In our study, we used recently designed universal COI primers that are comparable with existing primers for ribosomal genes in terms of their capability to target a wide range of arthropod taxa[46]. The broad taxonomic coverage achieved by our protocol was also evident based on our sequencing data, where several plant and fungal taxa were effectively amplified alongside barn swallow COI, resulting in a large proportion of non-target sequences in our dataset. Consequently, researchers intending to adopt these primers should account for this and adjust target sequencing depth accordingly. Further, to uncovering potential biases in biological interpretations, researchers should also consider an in-depth missing values analysis, to identify sources of commonly occurring PCR failures and low numbers of target sequences.

To partly overcome the problem with non-target reads, primers blocking passerine COI amplification were added to the PCR reaction. Though this procedure is commonly used in metabarcoding-based diet analyses, it has been noted that blocking primers may systematically bias abundances of taxa in resulting profiles[83,84]. However, our data were unlikely to be affected as there was a high consistency in diversity and insect COI profile composition for sample duplicates that were prepared with and without blocking primers.

Conclusions

Using COI profiling of faecal samples, we described diet variation in a breeding barn swallow population and demonstrated that diet metabarcoding is a promising non-invasive alternative to traditional diet analysis approaches in insectivorous birds. We also showed that use of blocking primers does not bias the content of diet profiles, probably due to phylogenetic disparity between passerines and their insect prey. Barn swallow diet exhibited high between-sample variation, which was partly explained by differences between breeding colonies and abundance variation of prey taxa during the season.

Finally, our data provides correlative support for the effect of diet consumed on faecal microbiota composition, independent of environmental factors affecting both diet and faecal microbiota.

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AUTHOR CONTRIBUTIONS

JK, TA study design; TA, JK, OT, LS field sampling; LS, HP laboratory analysis; LS, JK data analysis; LS, TA, JK funding; LS, JK manuscript drafting; all authors provided helpful comments and recommendations and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA ACCESSIBILITY

Sequencing data are available at the European Nucleotide Archive under project accession number PRJEB 14586 for the 16S bacterial profile and PRJEB46476 for the COI profile. Accession numbers for each sample are provided in supporting information Table A1.

ETHICS DECLARATIONS

All field procedures were conducted in accordance with European Union Guidelines for Animal Care and Treatment and approved by the Animal Care and Use Committees at the Czech Academy of Sciences (041/2011) and Charles University in Prague (4789/2008-0).

FIGURE LEGENDS

Figure 1: **Consistence between diet profiles generated with or without blocking primer**, assessed based on A) Shannon diversity correlations and B) procrustean superimposition for Hellinger dissimilarities in diet profiles.

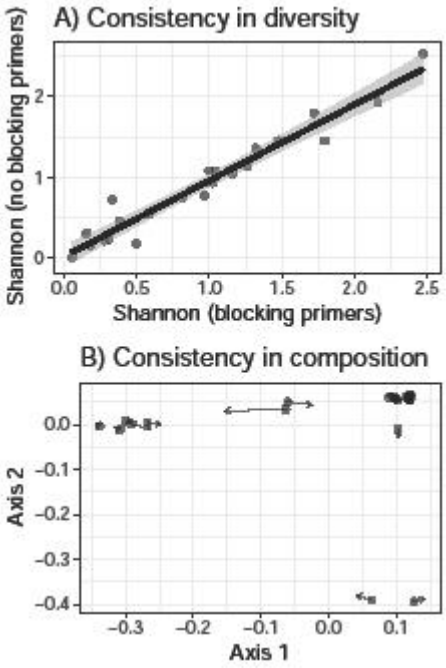


Figure 2: Insect genera detected by diet profiling of barn swallow faecal samples. The average proportion of reads is shown. Taxa present at low abundances (< 1% of all reads) are indicated as "others".

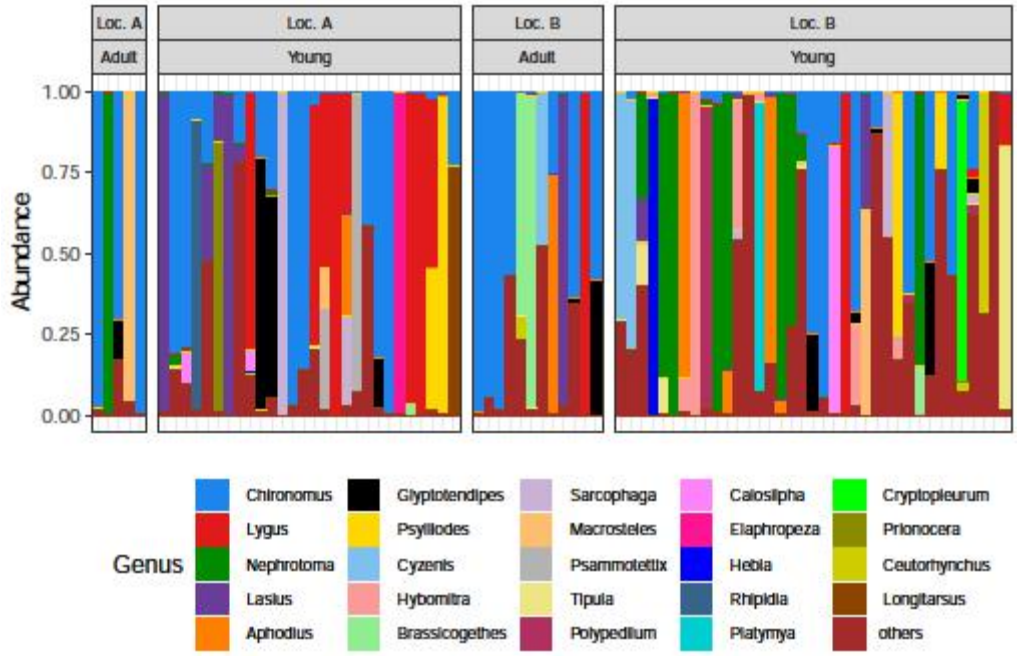


Figure 3: Variation in diet profile composition based on PCoA running on A) Hellinger and B) Jaccard dissimilarities. Samples from adult vs. young are indicated by different plotting characters. Samples taken during the breeding season are indicated with different shades of grey. Data for different localities are in different facets.

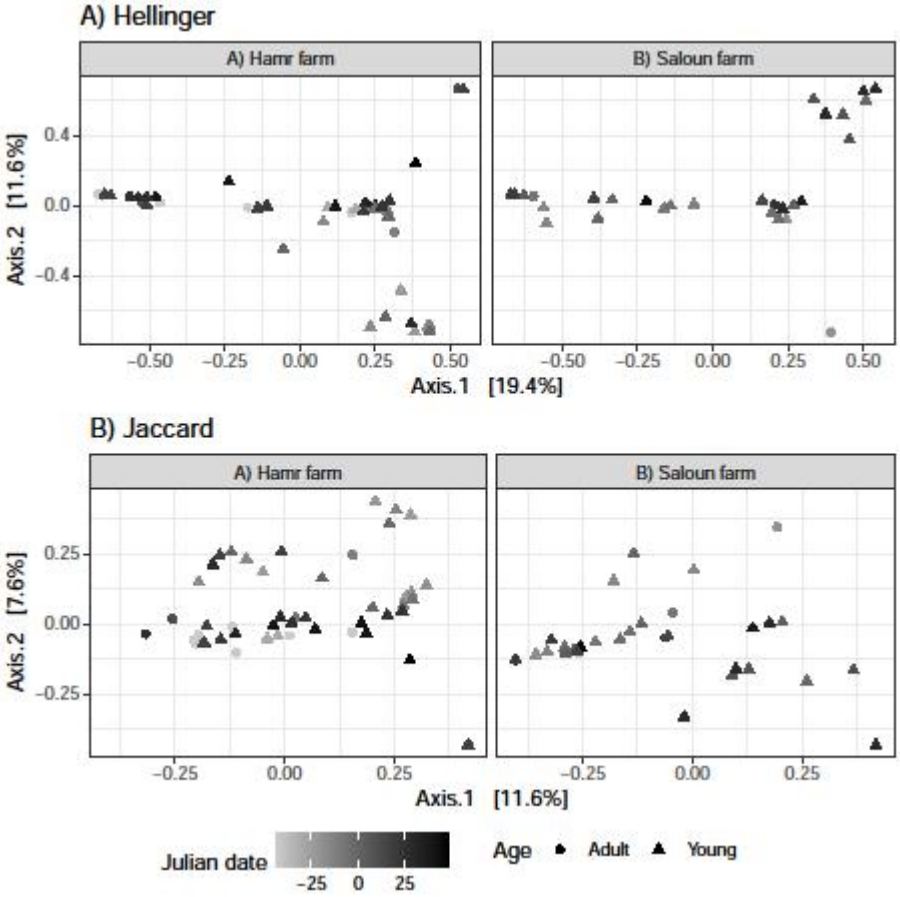
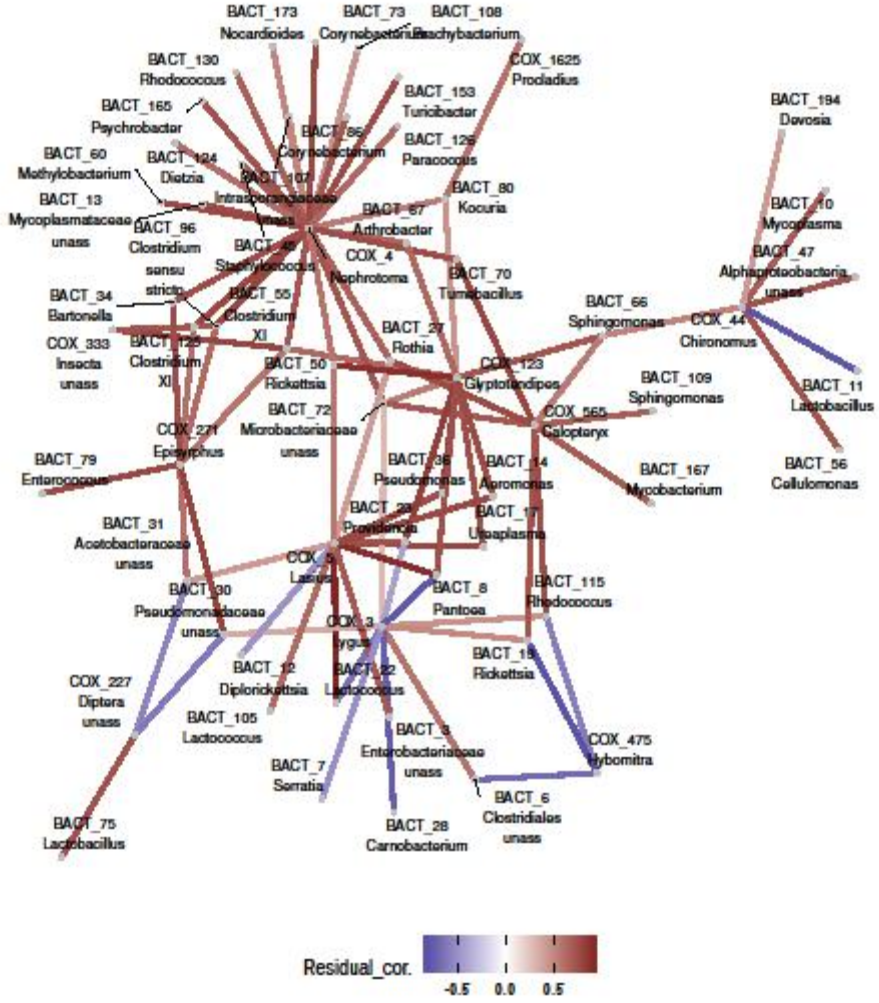


Figure 4: Residual correlations between bacterial OTUs and insect genera detected in faecal samples based on JSDM. Shown are correlations with posterior support > 0.95.



TABLES

Table 1: ANOVA table for db-RDA models testing the effect of Julian date, locality and age class on variation in the composition of insect profiles. The matrix of A) Hellinger or B) Jaccard dissimilarity in insect profile composition was used as a response. Models were constructed using the forward selection process (ordiR2step function from the R package vegan)

Dissimilarity	Predictor	Df	Variance	F	P
Hellinger	**(Julian date)^1**	1	0.018	1.853	0.028
	(Julian date)^2	1	0.020	2.029	0.010
	(Julian date)^3	1	0.033	3.297	0.002
	Locality	1	0.020	2.066	0.018
	Residual	77	0.763		
Jaccard	**(Julian date)^1**	1	0.012	2.527	0.001
	(Julian date)^2	1	0.010	2.083	0.003
	(Julian date)^3	1	0.010	2.037	0.001
	Locality	1	0.012	2.440	0.001
	Residual	77	0.375		

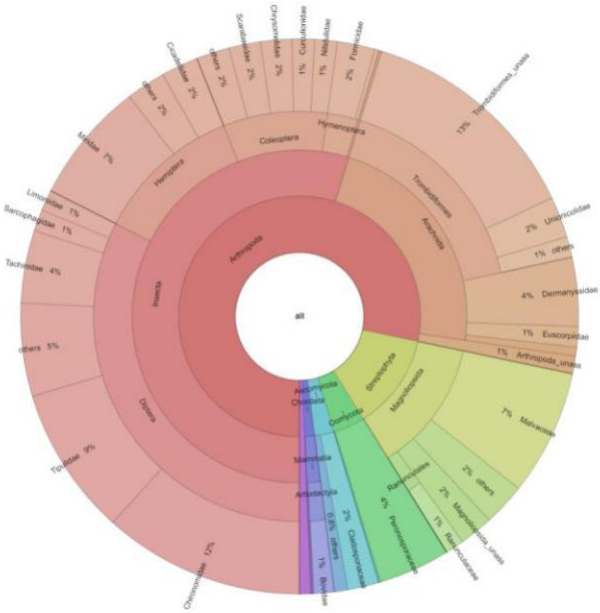
SUPPORTING INFORMATION

Table A1: Sample metadata and accession numbers for sequencing files. **Příloha 2**

Supporting information Appendix 1: Additional figures A1- A5.

Figure A1: Taxa detected in barn swallow COI profiles for samples amplified using A) both gene specific and blocking primers or B) gene specific primers only.

A) both gene specific and blocking primers



B) gene specific primers only

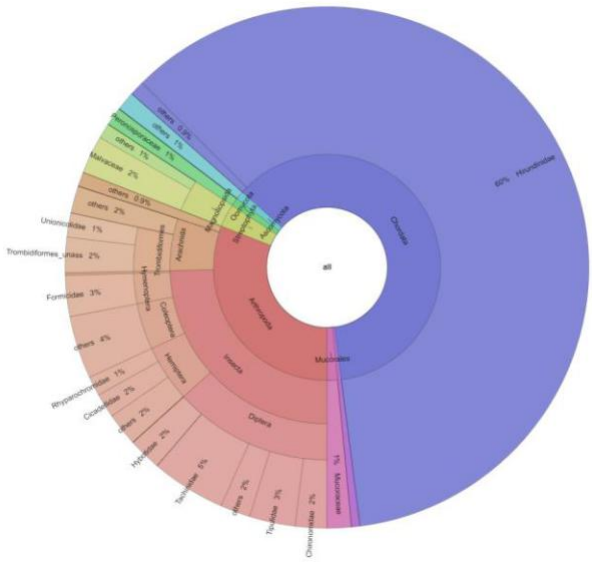


Figure A2: Insect taxa detected in barn swallow diet profiles.

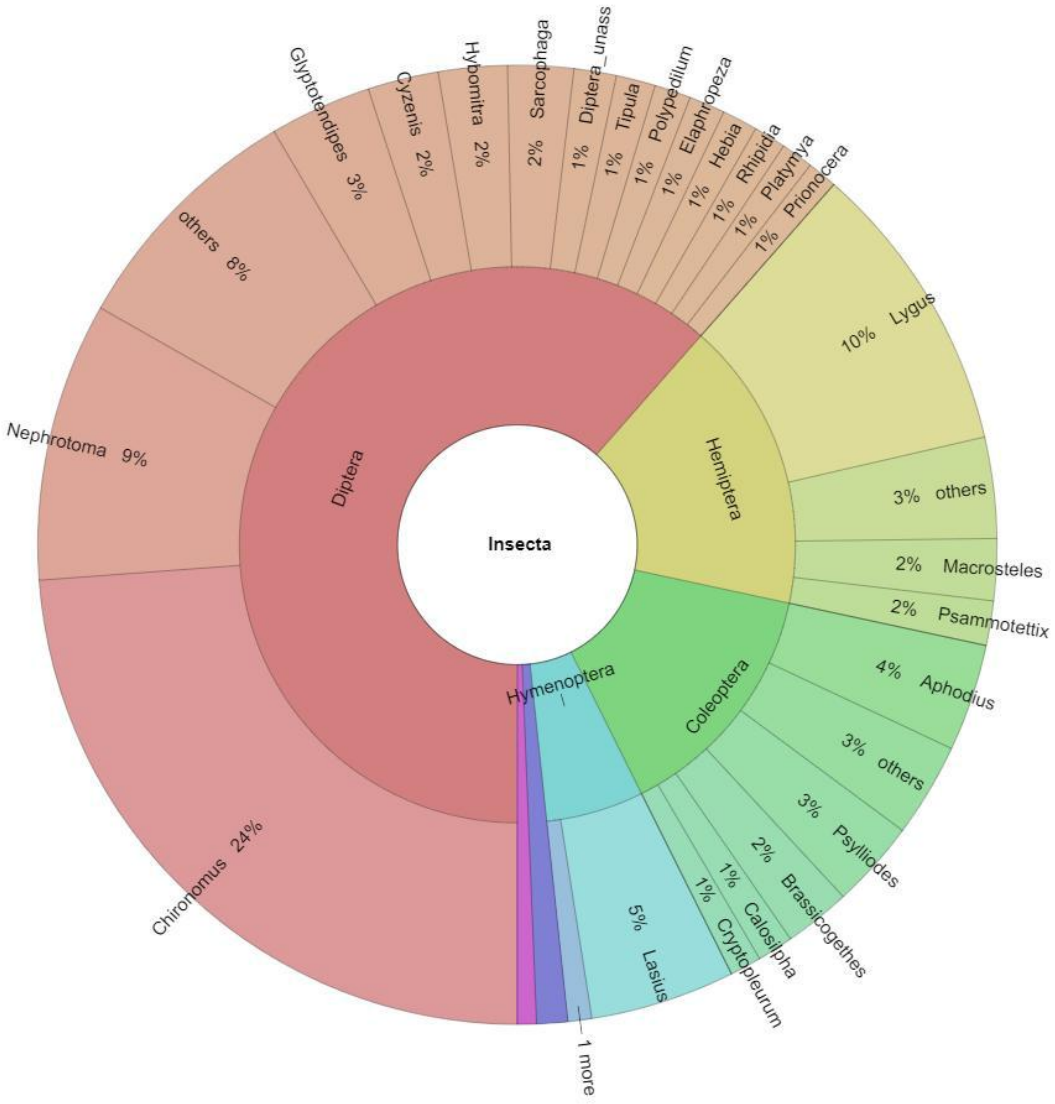


Figure A3: Rarefaction curves for number of observed insect genera within individual barn swallow samples. Curves were generated by 200 rounds of random subsampling of the origin community matrix. Lines indicate average values for given sequencing depths.

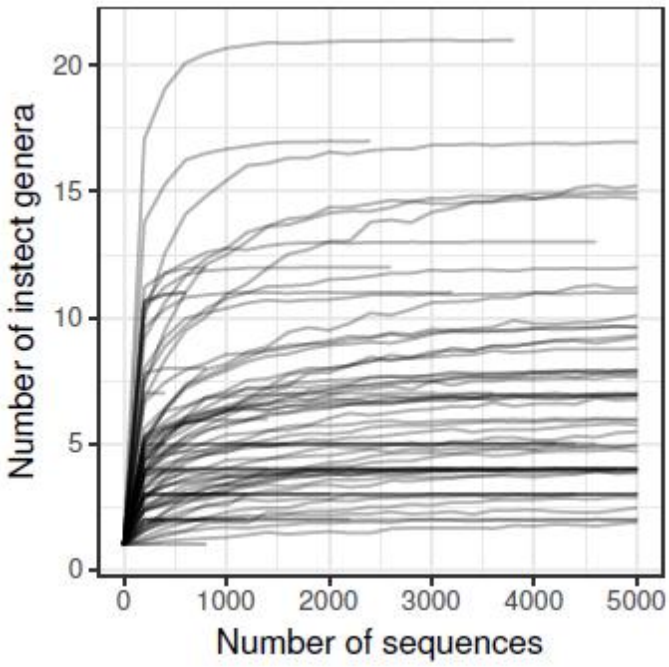


Figure A4: Temporal variation in the abundance of insect genera (expressed as read proportions). Shown are genera that exhibited significant abundance changes during the breeding season based on DESeq2 analysis. Regression curves correspond to local polynomial regression fits.

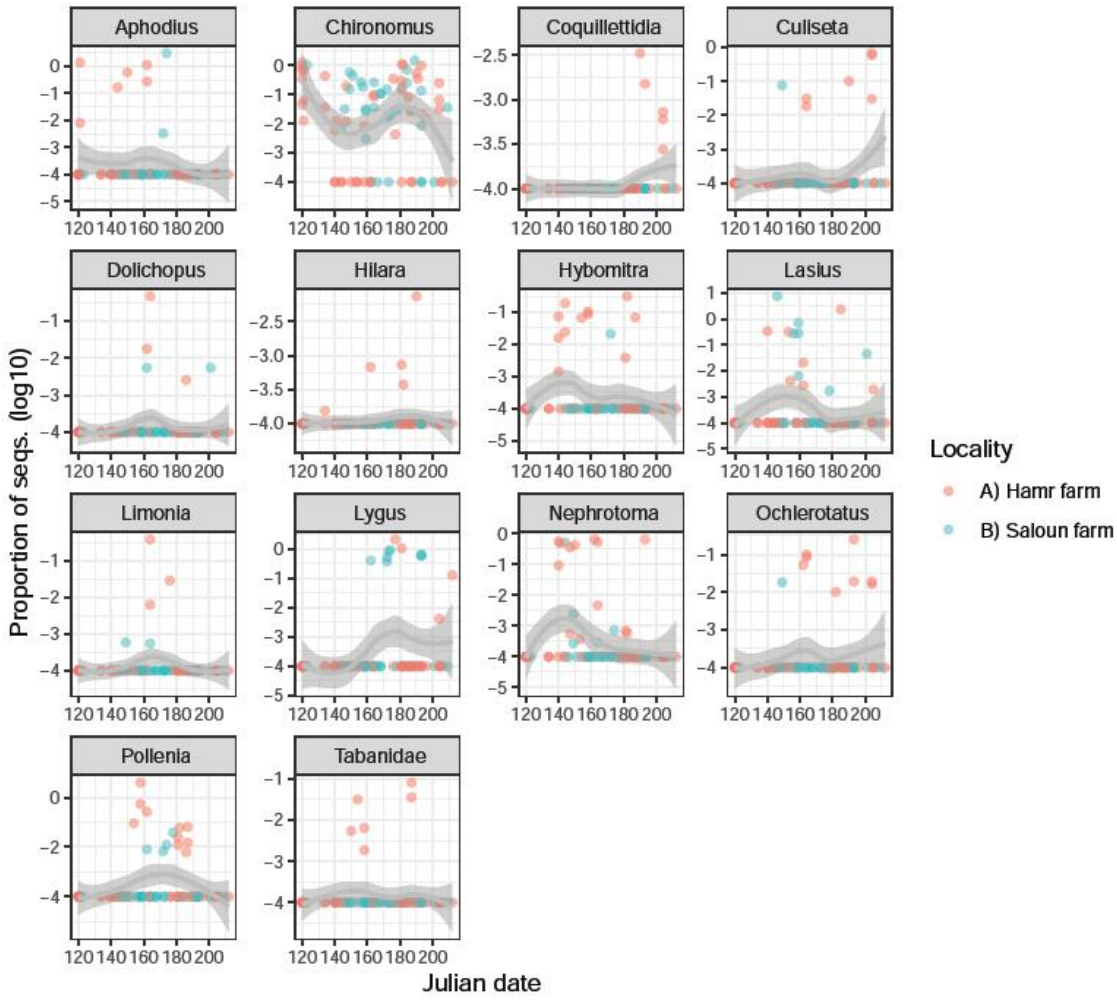
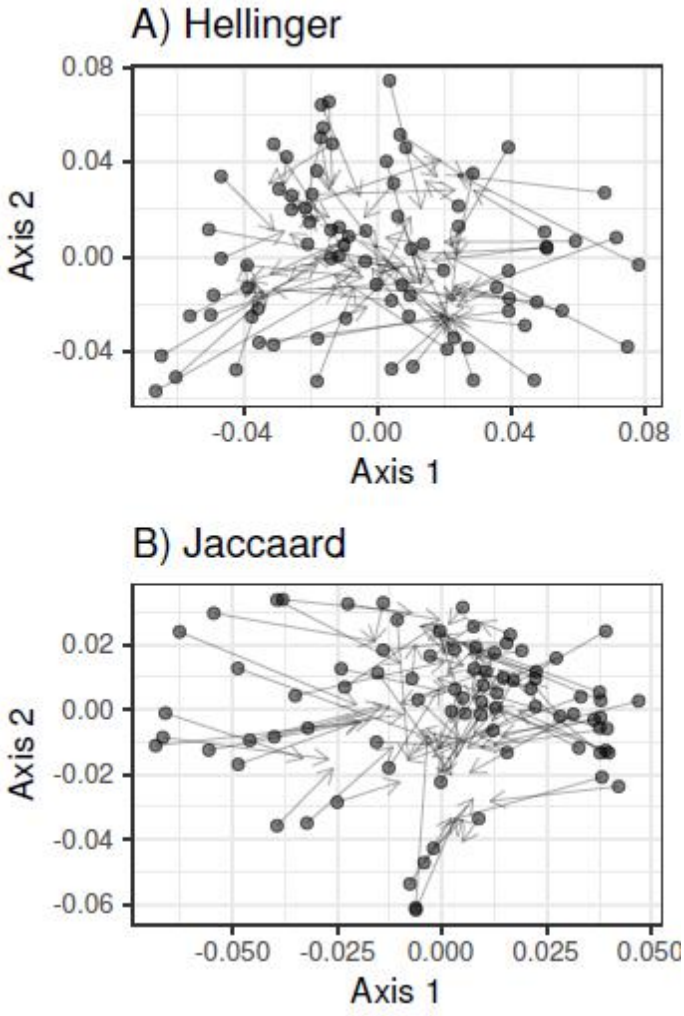


Figure A5: Procrustean superimposition for faecal microbiota vs. insect genera profiles.



3.6. Publikace F

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ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Fecal microbiota associated with phytohaemagglutinin--induced immune response in nestlings of a passerine bird

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Abstract

The vertebrate gastrointestinal tract is inhabited by a diverse community of bacteria, the so--called gut microbiota (GM). Research on captive mammalian models has re-vealed tight mutual interactions between immune functions and GM. However, our knowledge of GM versus immune system interactions in wild populations and non-mammalian species remains poor. Here, we focus on the association between GM community structure and immune response measured via the phytohaemagglutinin (PHA) skin swelling test in 12--day--old nestlings of a passerine bird, the barn swallow (*Hirundo rustica*). The PHA test, a widely used method in field ecoimmunology, as-sesses cell--mediated immunity. GM structure was inferred based on high--throughput 16S rRNA sequencing of microbial communities in fecal samples. We did not find any association between PHA response and GM diversity; however, our data revealed that the intensity of PHA response was correlated with differences in GM composi-tion at the whole--community level. Ten bacterial operational taxonomic units corre-sponding to both putative commensal and pathogens were identified as drivers of the compositional variation. In conclusion, our study suggests existence of GM -versus immune system interactions in a free--living nonmammalian species, which -corresponds with previous research on captive vertebrates.

KEYWORDS

fitness, immunity, inflammation, metabarcoding, microbiome, symbiosis

1 | INTRODUCTION

Vertebrates harbor diverse microbial communities in their guts (Ley et al., 2008; Qin et al., 2010) and these so--called gut microbiota (GM) are involved in many interactions with the host. In addition to its effect on gut function (Jumpertz et al., 2011; Sekirov, Russell, Antunes, & Finlay, 2010), interactions with the host's immune system have important consequences for the host's health and fitness. The different species comprising the GM regulate the host immune sys-tem contribute to its development during early ontogenetic stages (Belkaid & Hand, 2014; Kim, Park, & Kim, 2014; Sjögren et al., 2009; Wu & Wu, 2012) and affect the host's capacity to resist invading

pathogens (Ivanov et al., 2009). Simultaneously, the host supports a wide range of mechanisms, usually linked with the immune genes, that regulate GM content (Benson et al., 2010; Bolnick et al., 2014).

Most current research focussed on interactions between GM, and the host immune system has used captive-bred animals as a model. However, both taxonomic and functional composition varies considerably between wild and captive populations (Kreisinger, Čížková, Vohánka, & Piálek, 2014; McKenzie et al., 2017). Similarly, both immune parameters and their interindividual variation differ between wild and captive populations due to the altered genetic background of laboratory strains, a lower prevalence of parasites and pathogens and less variation in biotic and abiotic factors involved in immune trait modulation under captive conditions (Boysen, Eide, & Storset, 2011; Flies, Mansfield, Grant, Weldele, & Holekamp, 2015). Hence, results for GM versus immune system interactions obtained from captive populations do not necessarily reflect the selective forces that shape the host's immune system over GM-associated coevolutionary history (Maizels & Nussey, 2013). Moreover, our knowledge on host immune system versus GM interactions is largely based on mammalian species hosting different GM and having distinct immune system than other vertebrate taxa. Specifically, bacteria from the Firmicutes and Bacteroidetes phyla typically dominate in the mammalian GM (Ley et al., 2008), whereas nonmammalian vertebrate GM may comprise taxonomically more diverse bacterial consortia (Kropáčková, Těšický, et al., 2017; Sullam et al., 2012). Consequently, further studies dealing with free-living, nonmammalian species are essential for a deeper understanding of the evolutionary forces shaping interactions between GM and the host's immune system.

Here, we study the associations between GM structure and immune response in nestlings of a free-living passerine bird, the barn swallow (*Hirundo rustica*). The barn swallow is a migratory, insectivorous species with complex social system that breeds in colonies (Cramp & Perrins, 1993; Petrželková et al., 2015). The GM of barn swallows and other birds differs from that of conventional mammalian models (Hird, Carstens, Cardiff, Dittmann, & Brumfield, 2014; Kropáčková, Těšický, et al., 2017; Waite & Taylor, 2014), which makes birds a valuable model group for gaining a deeper insight into GM versus immune system interactions. Various aspects of immune system function have previously been studied in barn swallows and other free-living birds, predominantly related to reproductive behavior and sexual selection (Møller, 2001; Saino, Ambrosini, Martinelli, & Møller, 2002; Saino, Ferrari, Romano, Martinelli, & Møller, 2003). However, there have been few studies aimed at testing the association between immunity and associated microbial communities (Ruiz-Rodríguez et al., 2009).

We analyzed fecal microbiota profiles using high-throughput sequencing of 16S rRNA amplicons as a proxy for GM. Immune response was assessed via the phytohaemagglutinin (PHA) skin swelling test, which is the most widely used method for assessment of cell-mediated response in field ecoimmunology (Møller, 2001; Saino et al., 2002, 2003; Tella, Lemus, Carrete, & Blanco, 2008). The PHA assay is traditionally believed to reflect adaptive immune response mediated predominantly by T cells (Goto, Kodama, Okada, & Fujimoto, 1978; Tella et al., 2008). However, recent research suggests that immune mechanisms involved in PHA-induced swelling are more complex, comprising a strong component of innate immunity (Vinkler, Bainová, & Albrecht, 2010; Vinkler, Schnitzer, Munclinger, & Albrecht, 2012). A stronger PHA response is typically interpreted as beneficial due to its positive association with fitness- and condition-related traits (Bowers et al., 2014). Given the complex immunological background of PHA swelling, however, this may not hold universally. There are numerous examples showing no, or even a negative, relationship between PHA responsiveness and body condition, physiological stress, or health status (Møller & Petrie, 2002; Saks, Karu, Ots, & Hõrak, 2006; Vinkler et al., 2012). Despite these complexities, it is worth exploring the potential correlations between GM and PHA responsiveness as PHA-induced swelling is the most widely studied trait in ecoimmunological literature. In addition, previous research supports both positive (Saks et al., 2006) and negative (Merlo, Cutrera, & Zenuto, 2016) association between gut infection by eukaryotic parasites and PHA responsiveness, suggesting that extending such research on prokaryotic communities inhabiting the gut could be potentially fruitful. We therefore combine data on GM profiles with measures of PHA swelling in order to test whether there is any association between GM diversity and immune response in barn swallows. We also assess whether interindividual variation in PHA response is correlated with differences in GM composition and which specific bacterial taxa determine any such variation in PHA response.

2 | METHODS

2.1 | Field data acquisition

Data on fecal microbiota and PHA response were collected during 2014 (late April – late June) from barn swallow nestlings ($n = 58$) distributed in 32 clutches (Czech Republic, 49° 4′ 7.762″ N, 14° 42′ 36.521″ E, Supporting Information Table S1).

Tissue thickness of the left wing web (patagium) of 12-day-old barn swallow nestlings was measured using a standard thickness gauge (Mitutoyo, Japan). Subsequently, the PHA solution (0.10 mg of PHA-P dissolved in 20 μl of DPBS) was injected and the magnitude of the swelling reaction was measured after 24 hr. Both pre- and post-treatment tissue thickness measurements were performed three times by the same person (A.P., accuracy ~0.01 mm). Repeatability of these measurements was high (intraclass correlation coefficient = 0.973 and 0.967 for pre- and posttreatment measurements, respectively). Consequently, the average tissue thickness increment between pre- and posttreatment measurements was used as an index of PHA-induced swelling in subsequent analyses.

Fecal samples of 12-day-old barn swallow nestlings were collected prior PHA injection, placed in sterile cryotubes (Simport, Canada), and stored in liquid nitrogen during field works. After the field works, samples were preserved under −80°C until DNA extractions. Further details on fecal sample collection and storage,

together with a description of breeding site, are provided elsewhere (Kreisinger et al., 2017).

All field procedures were conducted in accordance with the Guidelines for Animal Care and Treatment of the European Union and approved by the Animal Care and Use Committees of the Czech Academy of Sciences (041/2011) and Charles University (4789/2008-0).

2.2 | Microbiome profiling and bioinformatic processing of 16S rRNA data

Metagenomic DNA was isolated from fecal samples using PowerSoil Mo Bio kits (Qiagen). The V3--V4 region of 16S rRNA was ampli-fied using S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) primers (Klindworth et al., 2013), tagged with 10 bp oligonucleotide indi-ces for demultiplexing. Technical PCR duplicates were prepared for all samples in order to check for microbial profile consistency. Sequencing libraries were prepared using TruSeq Nano Kits and se-quenced on Illumina Miseq using v3 chemistry.

The resulting 300 bp long paired--end reads were merged using Pear (Zhang, Kobert, Flouri, & Stamatakis, 2014) and demultiplexed using Mothur (Schloss et al., 2009). Lotus pipeline (Hildebrand, Tadeo, Voigt, Bork, & Raes, 2014) was used for quality filtering (elimination of sequences, if average $Q < 30$ and if average Q within 50 bp sliding dropped below 25) and elimination of chimeric se-quences. Subsequently, UPARSE algorithm (Edgar, 2013) imple-mented in Lotus was used for clustering of resulting high--quality reads at 97% similarity threshold to operational taxonomic units (OTUs). Taxonomic assignment of representative sequences for each OTU was performed using RDP classifier and Green Genes database (v. 13_5, DeSantis et al., 2006) as a reference. Representative se-quences were aligned using PyNAST (Caporaso et al., 2010) and a phylogenetic tree constructed using FastTree (Price, Dehal, & Arkin, 2010). The OTU table, sample metadata, taxonomic annotations, and phylogenetic tree were stored as a phyloseq object (McMurdie & Holmes, 2013) for further analysis. OTUs not assigned to phylum level, or those classified as chloroplasts (1% and 8.2% reads, respec-tively), were considered as sequencing artefacts and diet contam-inants, respectively, and eliminated from all downstream analyses. Details on laboratory procedures associated with microbiome profil-ing and bioinformatic processing of sequencing data were provided in a previous study on this species (Kreisinger et al., 2017).

2.3 | Statistical analysis

Barn swallow GM taxonomic content was visually summarized using Krona tools (Ondov, Bergman, & Phillippy, 2011). All the statistical

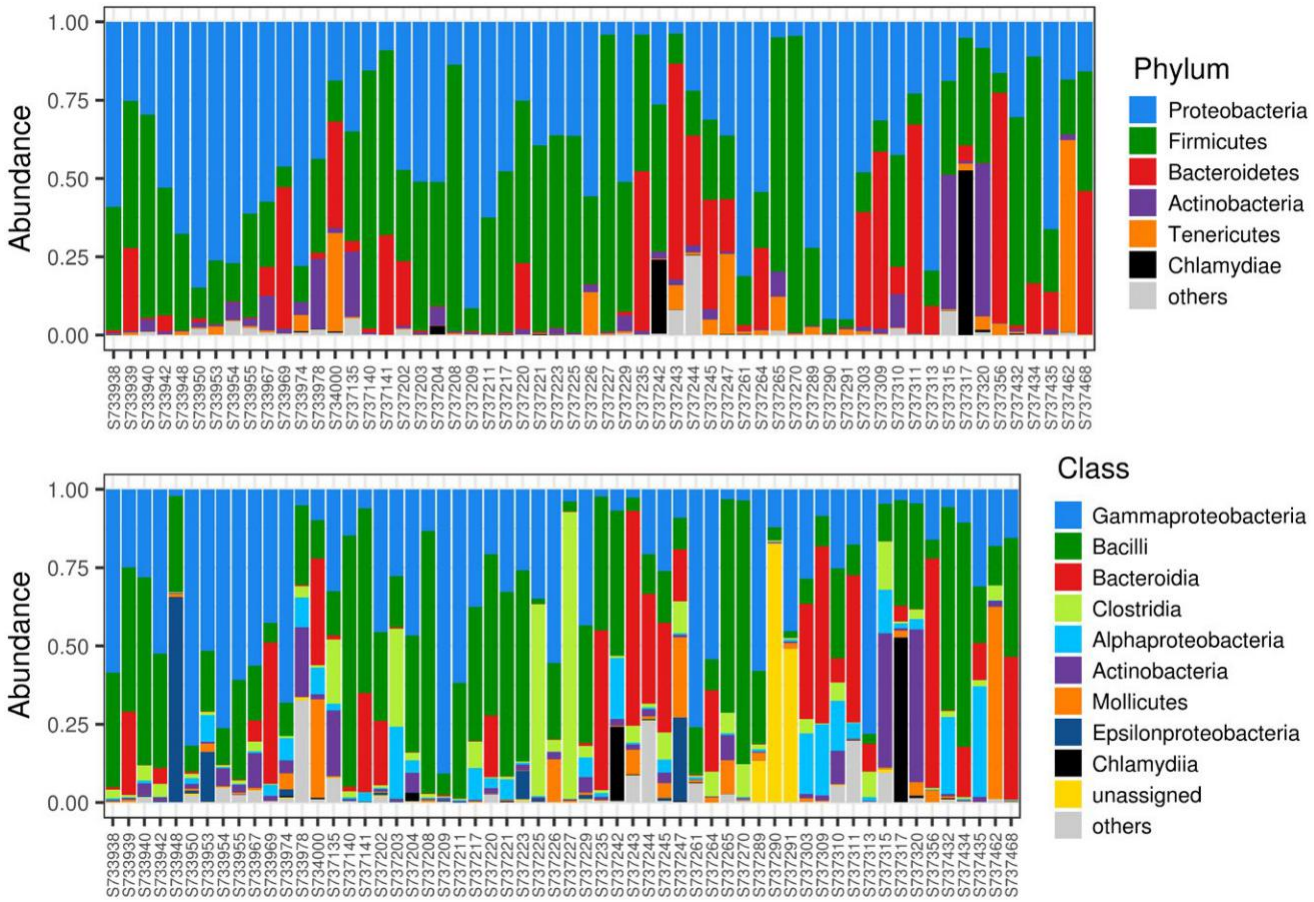

FIGURE 1 Bar plots indicating proportions of dominant bacterial phyla and classes in barn swallow fecal microbiota samples

analyses were conducted using packages running under R 3.4.3 soft-ware (R Core Team, 2016). As we detected significant correlation between PHA response and Julian date of fecal sample collection (Pearson $r = 0.324$, $p < 0.01$), we controlled all subsequent statistical analyses for effect of sampling date. Association between microbiota diversity (i.e., number of observed OTUs, Chao1 diversity estimates and Shannon diversities) and PHA response or Julian date of sample collection was tested using linear mixed--effect models (LME, R pack-age lme4; Bates, Mächler, Bolker, & Walker, 2015) with Gaussian dis-tribution of errors. Nest identity was included as a random intercept. Next, weighted UniFrac (Lozupone & Knight, 2005) and Bray–Curtis community dissimilarity between samples were calculated based on sample--specific OTU proportions. The effect of PHA--induced response and Julian date was assessed using distance--based redundancy analysis (db--RDA, Legendre & Anderson, 1999), with the matrix of between--sample dissimilarities included as a response. Permutation--based ANOVA (anova.cca function from R package vegan, Oksanen et al., 2013) was then used to test for significance of the constrained db--RDA axes. According to these analyses, only the first constrained db--RDA axis was significant ($p < 0.001$ for both UniFrac and Bray–Curtis dissimilarity), and the effect of the second constrained db--RDA axis was nonsignificant ($p > 0.3$ in both cases). We then extracted the scores for the first db--RDA axis and tested whether they were significantly associated with PHA response and/ or Julian date using LME. We argue that this analysis method is pref-erable to the default anova.cca, as this function cannot effectively

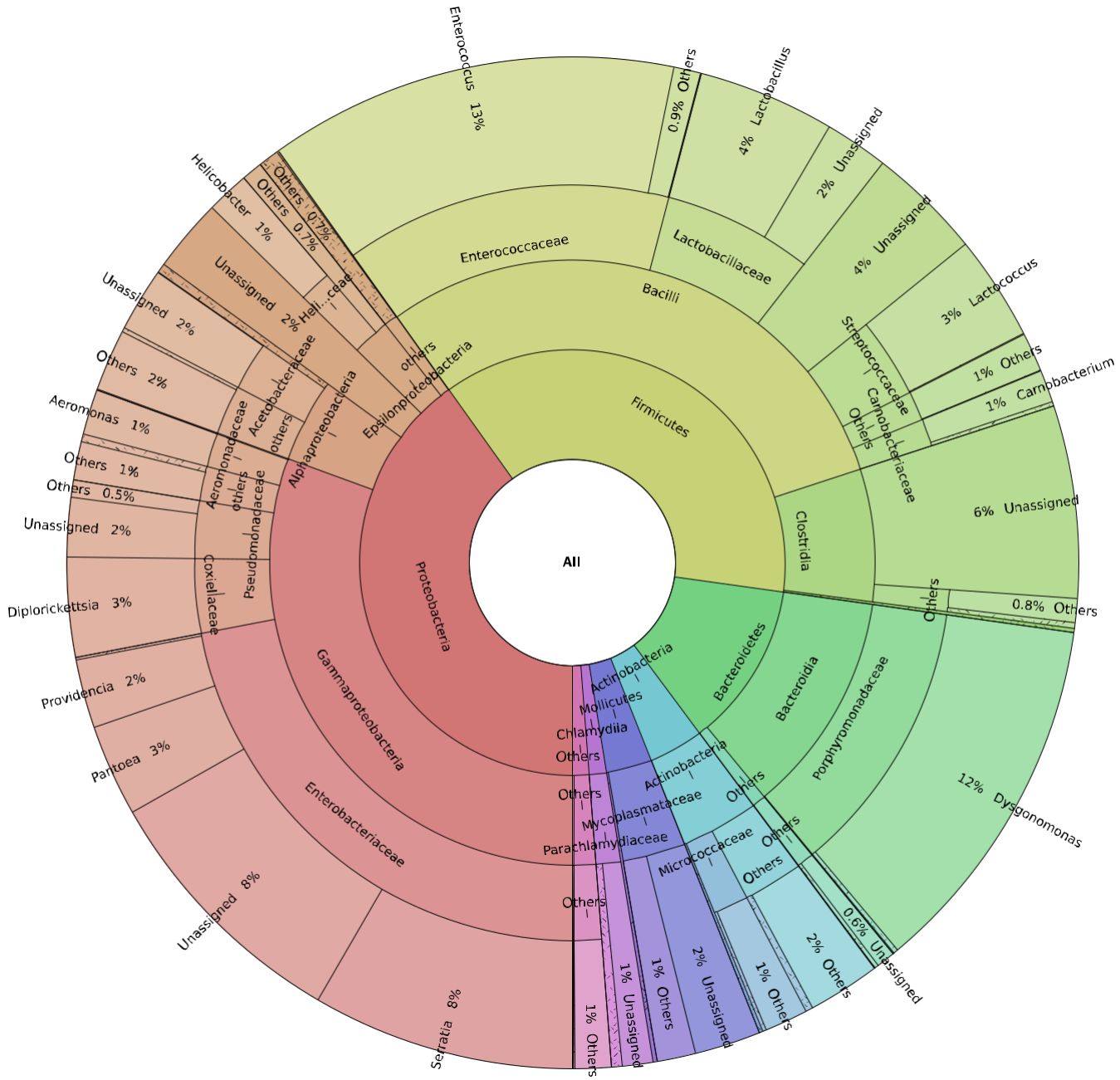

FIGURE 2 Summary of barn swallow GM taxonomic content. Rare taxa (represented by <2% reads) are labeled as "others"

account for pseudoreplications induced by sampling multiple individuals from the same nest.

Associations between abundance of OTUs and PHA response were tested using generalized LMEs from R package BhGLM for data with negative binomial distribution of errors (Zhang et al., 2017). OTU-specific read counts within individual samples were included as a response, while Julian date of sample collection and PHA response were included as explanatory variables. Log-transformed total number of reads per sample was specified as model offset (i.e., assuming number of reads per given OTU to be proportional to total number of reads per individual sample) and clutch identity as random effect. The qvalue method (Storey & Tibshirani, 2003) was subsequently used to account for false discoveries due to multiple testing. To optimize sensitivity of OTU-level analyses, we applied "independent filtering" procedure (Bourgon, Gentleman, & Huber, 2010) using DESeq2 R package (Love, Huber, & Anders, 2014) and considered only OTUs that passed this step (n = 196 OTUs, representing 96% of all high-quality reads). Procrustean analysis revealed high congruence between original and subsetted microbial profiles (Procrustean r = 0.9974, Procrustean sum of squares = 0.0052, p < 0.0001), suggesting that resulting OTU subset covered representative variation in the GM content.

3 | RESULTS AND DISCUSSION

After all filtering steps, we obtained 947,675 high-quality reads with a median sequencing depth per sample of 13,798 (range = 1,112–44,777) and an average number of 97% UPARSE OTUs per sample of 153.6 (range = 67–443). In line with our previous study on barn swallow nestlings from the same population (Kreisinger et al., 2017), the most abundant bacterial phyla were Proteobacteria (dominated by *Serratia*, *Pantoea*, *Providencia,* and *Diplorickettsia*) Firmicutes (dominated by genera *Enterococcus*, *Lactococcus*, *Lactobacillus,* and unassigned *Clostridia*), Bacteriodetes (dominated by the genus *Dysgonomonas*), and Actinobacteria. All Actinobacterial genera were represented by low percentage of reads (<1%), with *Rhodococcus* (0.58% of all read), *Rothia* (0.56% of reads), and *Corynebacterium* (0.55% of reads) being the most abundant (Figures 1 and 2).

We did not observe any association between PHA response and GM diversity (LME: p > 0.2 for all alpha diversity measures; Table 1). In theory, a correlation between immune functions and GM diversity might be expected as mutual interactions between immune gene allelic diversity (Bolnick et al., 2014), intensity of immune response (Hawley, Sydenstricker, Kollias, & Dhondt, 2005), parasite load (Kurtz et al., 2004; Madsen & Ujvari, 2006; Sommer, 2005), and overall GM diversity have repeatedly been reported in previous studies. However, consistent with our data, number of previous studies did not find any straightforward correlation between GM richness and immune phenotype (Chang, Hao, Offermanns, & Medzhitov, 2014; Jones et al., 2013; Vatanen et al., 2016).

Despite the lack of any relationship between GM diversity and PHA response, we observed significant correlation between magnitude of PHA swelling and variation in GM composition at the whole-community level, suggesting that individuals with similar GM composition had a similar PHA response. This association was specifically implied based on db-RDA ordination (Figure 3). In addition, LMEs running on scores for the first db-RDA axis (using both Bray–Curtis and weighted UniFrac distance) revealed a significant effect of PHA response after statistical control for Julian date of sample collection, while the effect of Julian date itself was significant only in the case of db-RDA for Bray–Curtis dissimilarity (Table 2).

According to OTU-centered negative binomial LMEs, ten OTUs represented by relatively low number of reads (~2.2% in total) exhibited significant association with the intensity of PHA response (Table 3 and Supporting Information File S1). Two of these OTUs, belonging to Lactic Acid Bacteria from genus *Enterococcus* and *Lactococcus,* were negatively related to PHA swelling. Intensity of PHA-induced swelling seems to strongly reflect general proinflammatory potential of given individual (Vinkler et al., 2010). Consequently, observed negative correlation between *Lactococcus* abundances and PHA response can be related to anti-inflammatory effect that was previously described for some probiotic species from this genus (Han, Lee, Park, & Paik, 2015; Luerce et al., 2014).

TABLE 1 Effect of PHA response and Julian date on GM alpha diversity (assessed as number of observed OTUs, Chao1 predictions of total GM diversity and Shannon index). Shown are LME estimates (Estimate) and corresponding standard errors (*SE*), deviance changes due to elimination of a given term from the model (χ^2) and associated degrees of freedom (*df*), and probability values (p)

Response	Explanatory var.	Estimate	*SE*	χ^2	*df*	*p*
Chao1	Intercept	1.897	0.079			
	Julian	−0.001	0.001	3.364	1	0.067
	PHA	0.028	0.024	1.388	1	0.239
Observed	Intercept	1.780	0.096			
	Julian	−0.001	0.001	0.933	1	0.334
	PHA	0.026	0.030	1.149	1	0.284
Shannon	Intercept	0.600	0.685			
	Julian	0.000	0.005	0.002	1	0.968
	PHA	0.152	0.208	0.645	1	0.422

Note. GM: gut microbiota; LME: linear mixed-effect model; OTUs: operational taxonomic units; PHA: phytohaemagglutinin.

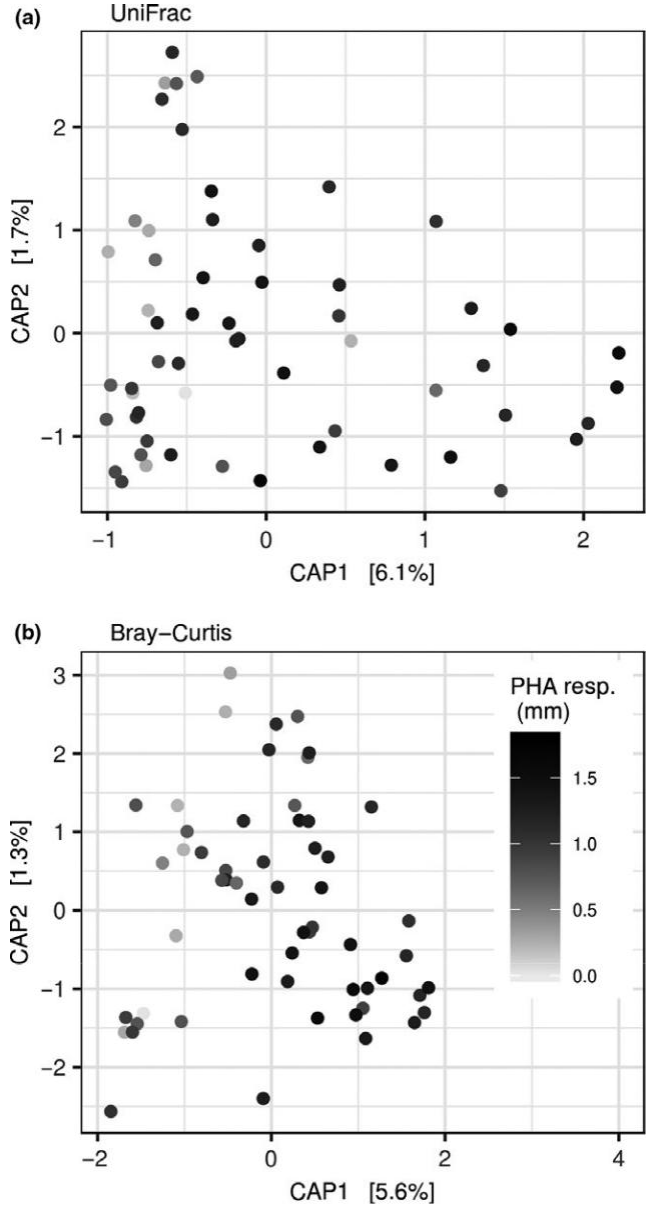

FIGURE 3 Db--RDA ordination of GM in barn swallow nestlings. Two dissimilarity types between samples were used as a response (Bray–Curtis and weighted UniFrac), while PHA swelling and Julian date of sampling were included as explanatory variables. Variation along the first two constrained axes is shown. Strength of PHA response (in millimeters) is indicated by color intensity of plotting characters

There are several plausible explanations for the negative correlation between abundances of *Enterococcus* OTU and PHA response. Similarly, as in the case of *Lactococcus*, some *Enterococcus* species exhibit probiotic properties. However, *Enterococcus* genus includes also several pathogenic strains, whose infection can directly affect host's immunity (Fisher & Phillips, 2009). Unfortunately, we are not able to distinguish between these two alternatives as 16S rRNA region used in our study does not allow reliable species--level assignment of this particular OTU. Interestingly, association of *Enterococcus* loads and phenotype was observed in another study on passerine juveniles (González--Braojos, Vela, Ruiz--de-- Castañeda, Briones, & Moreno, 2012). In particular, *Enterococcus* loads were negatively correlated with growth rates, that is, the phenotype trait that can covary with PHA response (Lifjeld, Dunn, & Whittingham, 2002) and other immune parameters as well (van der Most, de Jong, Parmentier, & Verhulst, 2011). Reduced PHA response was also associated higher abundances of OTU from genus *Rickettsia*, an insect--borne intracellular pathogen (Parola & Didier, 2001) commonly detected bird GM (Kropáčková, Těšický, et al., 2017).

On the contrary, intensity of PHA response tended to increase with increasing abundance of OTUs from genus *Bacillus, Staphylococcus* (putatively *Staphylococcus saprophyticus*; 100% identity according to blastn searches), *Dysgonomonas* and *Streptococcus*. Bacteria from genus *Bacillus* include many common gut symbionts of vertebrates. On the other hand, *S. saprophyticus* is opportunistic pathogen causing inflammatory diseases of urinary tract in humans (Hovelius & Mårdh, 1984) and *Dysgonomonas* can cause gut inflammation in immunocompromised human subjects (Bangsborg, Frederiksen, & Bruun, 1990). Both these OTUs were previously detected in bird GM (Kropáčková, Pechmanová, et al., 2017; Kropáčková, Těšický, et al., 2017; Xenoulis et al., 2010). However, their effect on physiology and health of avian are still unknown. Many *Streptococcus* species are vertebrate commensal, but some represent opportunistic pathogens of various vertebrate taxa including birds (Benskin, Wilson, Jones, & Hartley, 2009). Unfortunately, our data did not allow reliable species--level assignation of this particular OTU.

The contrasting effect of *Rickettsia* and *Staphylococcus* OTUs on PHA response suggests that putative bacterial pathogens can be associated both with attenuation and enhancement of PHA response. A similar contradictory pattern has been observed

Response	Explanatory var.	Estimate	*SE*	χ^2	*df*	*p*
Bray–Curtis	Intercept	−5.053	0.953			
	Julian	0.024	0.006	13.479	1	<0.001
	PHA	0.911	0.294	8.724	1	0.003
UniFrac	Intercept	−2.074	1.262			
	Julian	0.007	0.008	0.871	1	0.351
	PHA	0.769	0.366	4.523	1	0.033

Note. GM: gut microbiota; LME: linear mixed--effect model; PHA: phytohaemagglutinin.

TABLE 2 Effect of PHA response and Julian date on GM composition (corresponding to the first db--RDA axis for weighted UniFrac and Bray–Curtis dissimilarity). Shown are LME estimates (Estimate) and corresponding standard errors (*SE*), deviance changes due to elimination of a given term from the model (χ^2) and associated degrees of freedom (*df*), and probability values (*p*)

TABLE 3 OTUs associated with PHA response according to generalized LMEs for data with negative binomial errors. Shown are taxonomic assignation of individual OTUs, LME--based estimates, and corresponding standard errors (*SE*) for OTU abundance versus PHA response association, together with their probability (*p*) and *q* values

OTU name	Estimate	*SE*	*p* Value	*q* Value	Class	Family	Genus
OTU_105	−5.7648	1.0746	<0.001	0.0005	Lactobacillales	Streptococcaceae	*Lactococcus*
OTU_446	−5.5817	1.2421	0.0001	0.0035	Clostridiales	Unassigned	Unassigned
OTU_77	−3.774	0.9505	0.0005	0.0111	Lactobacillales	Enterococcaceae	*Enterococcus*
OTU_155	−3.5573	0.989	0.0013	0.0207	Pseudomonadales	Pseudomonadaceae	Unassigned
OTU_50	−2.6995	0.7293	0.001	0.0186	Rickettsiales	Rickettsiaceae	*Rickettsia*
OTU_280	2.7659	0.8043	0.002	0.0218	Lactobacillales	Streptococcaceae	*Streptococcus*
OTU_45	2.9869	0.8524	0.0017	0.0207	Bacillales	Staphylococcaceae	*Staphylococcus*
OTU_235	5.7617	1.6456	0.0017	0.0207	Bacillales	Bacillaceae	*Bacillus*
OTU_104	7.9446	1.4446	<0.001	0.0005	Flavobacteriales	Flavobacteriaceae	Unassigned
OTU_119	24.4801	0.2463	<0.001	<0.001	Bacteroidales	Porphyromonadaceae	*Dysgonomonas*

Note. LME: linear mixed--effect model; OTUs: operational taxonomic units; PHA: phytohaemagglutinin.

in the case of putative commensal or beneficial bacteria, with *Lactococcus* OTU abundance, in particular, being negatively re-lated to PHA swelling and *Bacillus* sp. being positively related. We propose that these seemingly contrasting results are related to both interaction complexity between bacteria and the verte-brate immune system and to the complex immunological back-ground of the PHA swelling response. Further studies targeting specific components of the bird immune system are required, therefore, in order to obtain a better understanding of how (a) host immune system interacts with GM and (b) how the overall pattern of such interactions differ from well--established mam-malian models.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

TA and JK designed the study. AP, MA, TA, RM, and OT carried out field sampling. LS, J--FM, and RM performed laboratory analysis. JK and LS performed data analysis. TA, LS, and JK secured fund-ing. JK drafted the manuscript. All authors provided helpful com-ments and recommendations and approved the final version of the manuscript.

DATA ACCESSIBILITY

FASTQ files for individual samples are available at European Nucleotide Archive (project accession number: PRJEB27618, http://www.ebi.ac.uk/ena/data/view/PRJEB27618).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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RESEARCH ARTICLE

Cloacal Microbiome Structure in a Long-Distance Migratory Bird Assessed Using Deep 16sRNA Pyrosequencing

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Abstract

Effects of vertebrate-associated microbiota on physiology and health are of significant inter-est in current biological research. Most previous studies have focused on host-microbiota interactions in captive-bred mammalian models. These interactions and their outcomes are still relatively understudied, however, in wild populations and non-mammalian taxa. Using deep pyrosequencing, we described the cloacal microbiome (CM) composition in free living barn swallows Hirundo rustica, a long-distance migratory passerine bird. Barn swallow CM was dominated by bacteria of the Actinobacteria, Proteobacteria and Firmicutes phyla. Bacteroidetes, which represent an important proportion of the digestive tract microbiome in many vertebrate species, was relatively rare in barn swallow CM (< 5%). CM composition did not differ between males and females. A significant correlation of CM within breeding pair members is consistent with the hypothesis that cloacal contact during within-pair copu-lation may promote transfer of bacterial assemblages. This effect on CM composition had a relatively low effect size, however, possibly due to the species' high level of sexual promiscuity.

Introduction

Vertebrate digestive tracts are inhabited by a taxonomically and functionally diverse commu-nity of bacteria, usually dominated by obligatory anaerobes [1,2]. Indeed, the cell and active gene count of this community may exceed that of the host genome by at least one order of mag-nitude [3]. Hence, it is no surprise that gastrointestinal tract microbiota (GTM) interact with a broad range of host physiological systems and provide ecosystemservices of considerable value. In particular, GTM affect metabolism efficiency [4,5], modulate the host's immune sys-tem [6], play a significant role in defence against pathogens [7,8] and enable synthesis of sub-stances that cannot be synthesised by enzymes encoded by the host's genome [9,10]. GTM dysbiosis is often associated with metabolic [11,12], autoimmune [13] and neurological disor-ders [10,14] and can also increase the risk of pathogen invasion [7,8].

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Recent advances in parallel high-throughput sequencing have enabled detailed insights into the complex interplay between GTM and vertebrate physiological status [5,11]. To date, most of this research has been focused on biomedical aspects of host/GTM interactions in humans and captive-bred mammalian model species [3,4,11,15]. The effect of GTM on host physiology also has relevance to ecological and evolutionary studies of wild populations. GTM composition has been shown to be associated with mate choice [16], for example, including propensity for within- and extra-pair copulations [17,18]. There is also evidence that social contact mediates horizontal transfer of GTM from parents to progeny [19–21], between sexual partners [22,23] or between members of a social community [7,24]. This transfer can have a long-lasting effect on fitness-related traits such as metabolism efficiency or pathogenesis susceptibility. Despite its potential importance, the current low knowledge level on GTM composition in free-living non-mammalian vertebrates and on factors shaping intra- and inter-specific variation, but see [21,25,26] precludes any general conclusions.

In this study, we focus on cloacal microbiome (CM) composition in a free-living population of barn swallows (Hirundo rustica), an insectivorous long-distance migratory passerine bird. The barn swallow is a traditional model species for research into reproductive biology and evo-lutionary ecology, and especially for studies of sexual selection and sperm competition [27–29]. To date, there has been no attempt to extend this research by including information on GTM composition, despite it having particular relevance in these fields.

Biogeographically, e.g. [1], CM is a subset of GTM colonising the distal part of the gut com-municating directly with the urogenital tract and the external environment. Factors associated with inter-individual CM variation in wild bird populations have already received some atten-tion, particularly as regards to horizontal transfer of CM from parents to progeny [21] or between sexual partners during copulation [25,30,31]. Many of these studies used cultivation based methods that only capture a low proportion of total CM, e.g. [32]. A few studies have used culture independent methods, such as Automated rRNA Intergenic Spacer Analysis (ARISA), Denaturing Gradient Gel Electrophoresis (DGGE) or cloning and clone sequencing of 16s rRNA amplicons [25,33] however, these approaches are also likely to suffer from com-promised CM coverage and taxonomic resolution.

In order to analyse barn swallow CM composition, we applied 454 pyrosequencing of 16s rRNA amplicons. The resulting data were used to assess whether sex, breeding pair identity or colony identity influenced inter-individual variation in microbiota composition during the breeding season.

Methods

Field sampling

We sampled CM from seven barn swallow breeding pairs (i.e. seven males and seven females) from two colonies, each around 4.5 km from the village of Lužnice in the Czech Republic (49° 3'56.90"N, 14°45'20.38"E). Both colonies (ca. 40 breeding pairs at each locality, hereafter"-Kotrbů" and "Šaloun") were located in small livestock farms. The composition of livestock differed between these two localities. Cattle and pigs dominated in Kotrbů, whereas sheep and goats were more common in Šaloun. CM sampling was performed during the nestling period (second breeding attempt, late June). We assume that the last within-pair copulations occurred approx. 2–3 weeks before the data collection—given that within-pair copulations occur mostly during or before egg laying and the length of incubation period is 12–18 days in this species [34]. The CM was collected using sterile DNA-free microbiological nylon swabs (minitip FLOQSwabs, Copan, Italy) inserted ca. 10 mm inside the cloaca for approx. 20 seconds and gently twisted by approx. 360 degrees. These samples were then stored in 2 ml DNA-free

microcentrifuge tubes (Simport, Canada) at -80°C until sample processing, which was performed within one month of sample collection. The samples were collected over three consecu-tive days in order to minimize the probability that observed inter-individual variation was biased by temporal fluctuations in CM composition.

All field procedures were approved by the Animal Care and Use Committees at the Czech Academy of Sciences (041/2011), and Charles University in Prague (4789/2008-30). Owners of farms, where we collected samples, gave us the permission to conduct this work.

Pyrosequencing

DNA was extracted in a sterile laminar flow cabinet using the Qiagen Stool kit (Qiagen, Germany). Bacterial barcoding was performed using the universal primers MPRK341F (CCTAYGGGRBGCASCAG) and MPRK806R (GGACTACNNGGGTATCTAAT) that amplify the ~466 bp fragment, including the V3 and V4 regions of Escherichia coli 16S rDNA [35]. Sequences of these primers were included in fusion primers used to perform polymerase chain reactions (PCR). Forward fusion primers, represented by adaptor B sequence (Lib A), the unique tag sequence from the Roche MID library and the MPRK341F primer sequence, dif-fered between individuals sequenced. The reverse fusion primer consisted of the Titanium adaptor A sequence (Lib A) and the MPRK806R primer sequence.

PCR was performed using a 30 μl solution consisting of 1x Qiagen Multiplex PCR Master Mix (Qiagen, Germany), forward and reverse fusion primers at final concentration 0.5 μM, and 8 μl of DNA solution. PCR conditions were as follows: initial denaturation at 95°C for 15 min; followed by 35 cycles of 94°C (30 sec), 56°C (90 sec), 72°C (60 sec); and a final extension at 72°C (20 min). PCR products were incubated at 70°C for three minutes and then stored on ice. The samples were then run on 1% agarose gel and bands of appropriate size were excised from the gel and purified using the QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions using 30 μl of buffer in the elution step. Concentration of the purified PCR product was measured using a Qubit fluorometer (Invitrogen, USA) and nor-malised. Pyrosequencing was performed via a single run on a GS Junior sequencer (ROCHE, Switzerland) using Titanium chemistry according to the manufacturer's instructions. Demulti-plexed sff files have been deposited in the European Nucleotide Archive: http://www.ebi.ac.uk/ ena/data/view/PRJEB7057.

Analysis of 454 data

Sequences with low quality scores (average quality score < 0.25), that included more than three ambiguously determined nucleotides, that were shorter than 200bp, or that did not perfectly match forward primer sequences or tags were excluded from further analysis. Mid- and primer regions were trimmed using QIIME 1.8.0 [36] and the resulting fasta file was denoised using the Acacia software [37], while chimeric sequences were identified and filtered out using USEARCH [38]. As recommended by May et al. [39], the TBC algorithm [40] was used to clus-ter the resulting high quality sequences into operational taxonomic units (OTUs) with a 97% similarity threshold. TBC output was subsequently parsed using custom R [41] and UNIX scripts to produce a QIIME formatted OTU table (presenting the sequence count for OTUs in individual samples).

Taxonomy of representative sequence for OTUs was assigned using RDP classifier [42], with a posterior confidence level of > 0.80. Representative sequences were aligned using PyNast and Greengenes Core Set Alignment [43] and a minimum evolution phylogenetic tree was constructed based on the procedure implemented in FastTree [44]. Hellinger distances between samples were calculated based on OTU abundance data. In addition, the phylogenetic

tree, together with data on OTU abundances, was used to calculate both unweighted and weighted UniFrac distances [45] between samples. To avoid potential bias associated with unequal sequencing depth, distances were calculated based on a random subsample corresponding to 1600 reads (i.e. approximate minimum achieved sequencing depth) per individual.

The Chao1 index [46], phylogenetic diversity index (computed as total branch length), and number of OTUs detected in individual samples were calculated to provide further information on CM alpha diversity. In addition, total OTU richness for individual samples was estimated based on best-fitting parametric model implemented in CatchAll [47]. Coverage of CM diversity by our sequencing data was assessed based on rarefaction analysis and Goods coverage index [48]. Paired t-tests were used to test whether alpha diversity differed between males and females. Distances between samples were visualised using non-metric multidimensional scaling (NMDS) and distance-based redundancy analysis (db-RDA), implemented in the vegan package [49], was used to test whether CM composition differed systematically between males and females and between breeding colonies. The betadisper function (analogous to Levene's test of equality of variance), was used as a next step to assess whether inter-individual variation in Hellinger and UniFrac distance differed between males and females. Finally, we applied the Wilcoxon signed rank test to detect differences in proportional composition of individual bacterial phyla and families between males and females. The same approach was used to compare the proportion of individual OTUs (i.e. number of reads for a given OTU in a given sample divided by total number of reads for a given sample) that were represented by < 0.1% reads (number of OTUs = 123, see S2 Table for more detail). The q-value method was applied to account for false discoveries when using multiple comparisons [50] (q-value threshold was set to 0.05). In addition, corrected moment estimates of k parameter of the negative binomial distribution was calculated for these OTUs. This index is widely used in parasitology to quantify the level of parasite aggregation among hosts. Low values of this index imply highly aggregated distribution, whereas high values (k > 20) indicate near-Poisson distribution of infection intensities [51].

Two analytical approaches were applied to test whether individuals from the same breeding pair exhibited a higher level of similarity than expected by chance. First, we compared the observed mean of within-pair distances (Hellinger and both weighted and unweighted UniFrac) with the null distribution of mean distances for randomly paired males and females (n = 1000 randomly generated pairs). This individual-centred approach is highly conservative due to the relatively low sample size of our study. Second, an OTU-centred resampling approach was used to assess whether relative abundances of individual OTUs were non-randomly correlated between males and females within individual breeding pairs. This analysis was run on a subset of 153 OTUs occurring in 4 individuals and including 77% of the original high quality reads. Within-pair correlation of each OTU proportion was assessed using Spearman's correlation coefficient (Spearman's r); the Fisher's z-transformed mean being used as the within-pair similarity index. In the next step, randomized matrices (n = 1000) were constructed by reshuffling the individual identity in the original matrix of OTU proportions for individual samples which at the same time accounted for sex identity (i.e. randomly selected males was paired with randomly selected females). Mean Fisher's z-transformed Spearman's r was computed for each randomised matrix, as described above, and the resulting null distribution was used to assess statistical significance of within-pair community correlation. The outcome of these analyses were also expressed as community-specific standardised effect sizes (SES) using the formula (CORor–mean CORsim)/sdCORsim [52], where CORor is the mean of Fisher's z transformed correlations within actual pairs, mean CORsim is the mean Fisher's z transformed Spearman's r for randomised matrices, and sdCORsim is its standard deviation. We tested this approach using different OTU filtering criteria, Pearson correlations and raw

instead of Fisher's z-transformation correlation coefficients, and found that the results of these analyses remained unchanged.

All statistical analyses were performed in R 3.1.0 [53], the statistical significance for all tests being two-tailed. The 'phyloseq' package in R [41] was used for filtering and manipulating com-munity data.

Results

We analysed 71,100 sequences that passed quality filtering and were not chimeric, with the number of high quality sequences ranging between 1,656 and 8,110 (mean = 5,078) per sample. Sequences were clustered in 981 OTUs (754 non-singleton; details in S1 Table). The Goods coverage index ranged between 0.975 and 0.998 (mean = 0.992). This, along with the results of rarefaction analysis (presented in S1 Fig), suggest that the sequencing depth in our study was sufficient to capture the majority of CM alpha diversity. Based on taxonomic assignation, bac-teria from the phyla Proteobacteria, Firmucutes and Actinobacteria dominated the CM. We further recorded members of 17 other bacterial phyla and two archaebacterial phyla (Crenarch-aeota in one OTU and Euryarchaeota in two OTUs) at low frequencies (Fig 1; see S2 Fig for more details on taxonomic classification). The level of inter-individual variation in CM compo-sition was pronounced as just four OTUs were detected in all samples and only 52 OTUs in more than 50% of individuals.

The mean OTU number per sample, as predicted using the Chao1 index, was 179 (range = 107–424). CatchAll predictions of OTU richness were comparable with Chao1 estimates (range = 112–570 OTUs per sample, see S1 Table). The number of observed OTUs showed no variation between males vs. females (Paired t-test: $t_{(d.f.=6)} = 0.375$, $p = 0.721$). Non-significant difference between males and females was revealed also based on other alpha-

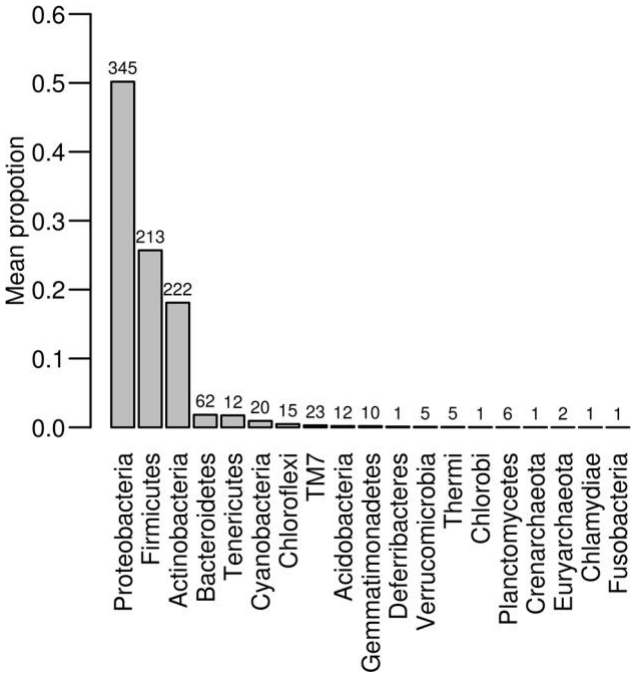

Fig 1. Taxonomic composition of barn sw allow cloacal microbiome. Bar heights correspond to the proportion of sequences assigned to individual bacterial phyla. Numbers above the bars indicate number of 97% TBC OTUs corresponding to a given phylum.

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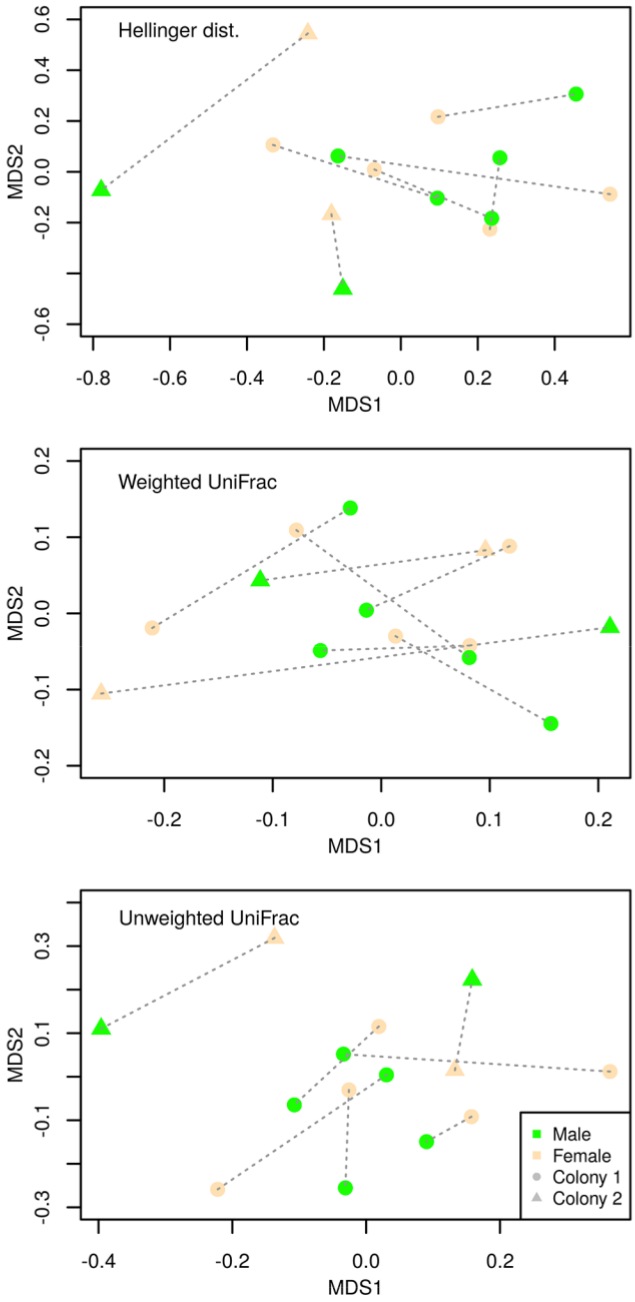

Fig 2. Betadiversity of barn swallow cloacal microbiome. Non-metric multidimensional scaling, based on Hellinger, unweighted and weighted UniFrac distances for barn swallow cloacal microbiota. Green and brown symbols indicate males and females, respectively. Circles and triangles correspond to the two localities sampled (Kotrbů and Šaloun, respectively). Individuals belonging to the same breeding pair are indicated by the same plotting character and connected by a dashed line.

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diversity indexes ($p > 0.2$ in all cases). Db-RDA suggested no difference in CM composition between males and females (Hellinger distances: $F_{(d.f = 1,12)} = 0.769$, $R^2 = 0.055$, $p = 0.906$, weighted UniFrac: $F_{(d.f = 1,12)} = 0.671$, $R^2 = < 0.01$, $p = 0.672$ and unweighted UniFrac: $F_{(d.f = 1,12)} = 0.977$, $R^2 < 0.01$, $p = 0.520$; Fig 2). Similarly, betadisper provided no support for differences in inter-individual CM variation between males and females (Hellinger: $F_{(d.f = 1,12)} = 0.017$, $p = 0.8977$,

weighted UniFrac: $F_{(d.f. = 1,12)} = 0.176$, $p = 0.682$ and unweighted UNIFRAC: $F_{(d.f. = 1,12)} = 0.219$, $p = 0.648$, respectively). We found no difference in proportion of individual bacterial phyla and families between males and females (Wilcoxon signed rank test: $p > 0.1$ and $q > 0.1$ in all cases). Furthermore, out of 123 OTUs with at least 0.1% high quality reads (see S2 Table), none showed any variation in relative abundance between males and females (Wilcoxon signed rank $p > 0.03$ and $q > 0.05$ in all cases). These OTUs exhibited highly aggregated distribution among sampled individuals (median value of k parameter = 0.172, inter-quartile range = 0.057–0.337, S2 Table). Db-RDAs models, based on unweighted UniFrac and Hellinger distances, suggested that individuals from different breeding colonies tended to be colonised by different bacterial OTUs ($F_{(d.f = 1,12)} = 1.357$, $R^2 = 0.102$, $p = 0.010$ and $F_{(d.f = 1,12)} = 1.618$, $R^2 = 0.131$, $p = 0.019$, respectively); however, this was largely influenced by individuals from a single breeding pair. When performing the same analysis using weighted UniFrac distances, between colony differences were not significant ($F_{(d.f = 1,12)} = 1.009$, $R^2 = 0.080$, $p = 0.380$; Fig 2).

Sample-centred permutations did not suggest a higher within-pair correlation than that expected by chance (Hellinger distances: $p = 0.108$, weighted UniFrac distances: $p = 838$ and unweighted UniFrac $p = 0.220$). An OTU-centred permutation model, however, indicated higher relative OTU abundance correlations between individuals in the same breeding pair than expected by chance ($p = 0.002$; Fig 3; standardised effect size = 2.910; untransformed mean Spearman's $r = 0.101$). The result remained significant after exclusion of Cyanobacteria OTUs and OTUs most likely corresponding with arthropod-associated bacteria (see Discus-sion); i.e. not an integral part of Barn Swallow microbiota ($p = 0.004$, SES = 2.490, untrans-formed mean Spearman's $r = 0.089$).

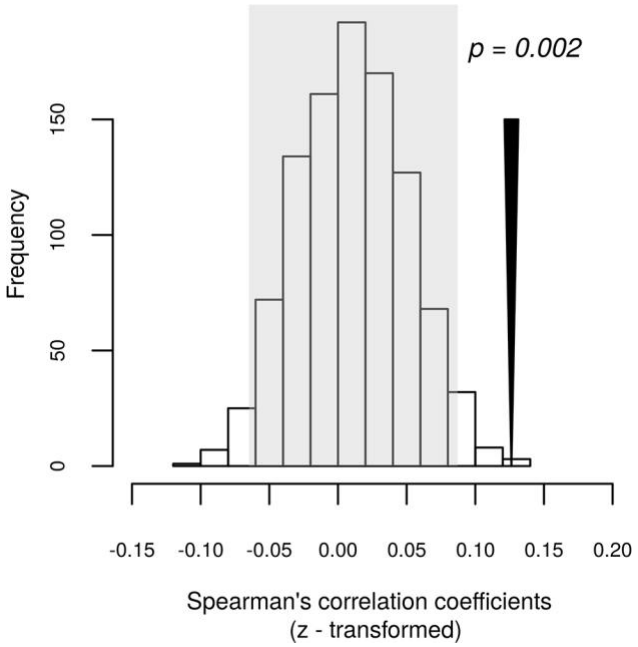

Fig 3. Correlation of OTU abundance between males and females within individual breeding pairs. Histogram showing the distribution of simulated means of Fisher's z transformed Spearman's correlation coefficient computed based on the correlation of relative abundance of individual OTUs between males and females belonging to the same breeding pair. The grey area indicates the 95% confidence interval for the simulated means. The black arrow corresponds to the mean Fisher's z transformed Spearman's correlation coefficient calculated based on the original community table. Permutation-based significance is indicated above the arrow.

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Discussion

Barn swallow CM was dominated by species of the phyla Proteobacteria, Firmucutes and Acti-nobacteria, with a further 17 bacterial and two archaebacterial phyla recorded at low relative abundances (< 5% of high quality reads). Despite such high CM phylogenetic diversity, the community exhibited only moderate diversity at the OTU level. We detected less than 1,000 OTUs (754 non-singleton), with the number of OTUs per individual predicted using the Chao1 index ranging between 107 and 424.

To date, most research on animal-associated microbiomes has been dedicated to bacteria colonising mammalian hosts [3–5,11,15,54]. Compared to the typical GTM of most mamma-lian species studied thus far, barn swallow CM taxonomic composition appears to be rather distinct. The phylum Bacteriodetes (along with Firmicutes), for example, usually dominates the GTM of most mammalian species [2,11,15,55,56], but was represented by less than 5% of high quality reads in the barn swallow. On the other hand, the Phyla Proteobacteria and Actinobac-teria, which were abundant in barn swallow CM, are usually under-represented in the GTM of mammalian species [55], but see [54]. Differences between mammalian GTM and barn swal-low CM could conceivably be due, at least in part, to the distal position of the cloaca in the digestive tract and its intermittent connection with the urogenital tract and the external envi-ronment. Our recent data, however, have shown no pronounced difference between CM and GTM community structure in passerine birds (Kropackova, unpublished results). Furthermore, a number of recent high-throughput sequencing studies have also shown bird GTM to be dom-inated by Proteobacteria, Actinobacteria and Firmicutes [57–61].

At a lower taxonomic level, many genera dominating in CM, such as Enterobacter, Strepto-coccus, Enterococcus, Clostridium, Lactobacillus, Lactococcus, Turicibacter and members of the Ruminococcaceae family, are facultative symbionts or commensals inhabiting the digestive tracts of many vertebrate species [2,56,62]. We also detected several OTUs, such as Staphylo-coccus, Janthinobacterium, Corynebacterium, Aerococcus and Brevibacterium, that commonly colonise the skin's surface [63] and OTUs associated with other parts of the animal's body, such as Rothia, Porphyromonas Enhydrobacter and Actinobacillus, see for example [64]. Finally, barn swallow CM composition may also partly reflect the bird's diet, which is com-posed of flying insects and other arthropods present in aerial plankton. Several abundant OTUs, including Hamiltonella, Rickettsiella and Wohlfahrtiimonas, correspond to symbiotic or pathogenic bacteria of arthropods [65–67]. Their widespread presence in barn swallow CM, therefore, is most probably a consequence of its foraging specialisation.

High inter-individual variation appears to be a general feature of the core mammalian GTM microbiome [12,19,68,69], but see [70]. This also appears to be true for barn swallow CM, with most OTUs detected in a single individual only and a relatively low proportion detected in more than 50% of individuals. Rarefaction analysis suggests this level of inter-individual het-erogeneity is unlikely to be an artefact caused by insufficient sequencing depth. High interindi-vidual variation in OTU presence vs. absence was further underscored by low values of k parameter, indicating highly aggregated OTU distribution among sampled hosts. This is com-parable with the aggregation pattern observed in vertebrate macroparasites [51]. The barn swal-low is a trans-Saharan migrant spending more than half-a-year outside its breeding locality [71]; hence we speculate that high inter-individual CM variation may be shaped, to some extent, by the heterogeneity of biotic and abiotic factors over the migration and wintering peri-ods. If tso, the CM could be viewed as a 'carry-over' effect that might contribute to variation in reproductive output over the breeding season [72]. Interestingly, NMDS and db-RDA indi-cated no pronounced difference in CM in individuals from different breeding colonies. Although a larger sample size would be desirable for a more robust conclusion, this result

suggests that variation in environmental conditions operating at small spatial scales during the breeding season has a limited effect on CM composition.

It has previously been shown that animal-associated microbiome composition is correlated with physiological stress [73], hormonal status [74], reproduction [75] and metabolic rate [4]. In barn swallows, there is a pronounced sexual difference in parental care investment [76], along with overall physiological and hormonal status [77,78], over the breeding season. Never-theless, our data suggest that these aspects are not associated with systematic differences in CM between males and females, with neither dominant OTU abundance nor CM taxonomic com-position exhibiting any apparent sex-dependent variation. Furthermore, both CM alpha (i.e. OTU richness) and beta diversity (i.e. level of inter-individual variation) were comparable between males and females, which is consistent with recent work on New World vultures [79]

Previous experimental and correlative studies have demonstrated cloacal contact during within-pair copulation to be an important factor shaping CM composition and contributing to CM similarity between individuals of the same breeding pair [25,30,31]. At the same time, CM composition has been suggested to have an important influence on within- and extra-pair mate choice and propensity to copulation in general, as both beneficial and potentially pathogenic bacteria may be transmitted during copulation [17,18,22,23,31]. Indeed, permutation-based analysis of barn swallow CM suggests that OTU abundance is correlated between individuals of the same breeding pair. The effect-size of this pattern is rather small, however, which is con-sistent with NMDS ordination and, more explicitly, with resampling tests based on between-sample distances, which show that CM similarities within breeding pairs were not lower than expected by chance. It is possible that within-pair similarities in CM may be, at least partly, jammed by CM transfer during extra-pair copulations, which occur frequently in the study populations [29]. In addition, it is worth mentioning that samples were collected approx. 2–3 weeks after egg fetilization. Recent manipulative study of White et al. [25] showed that similar-ity of CM community between social partners in kittiwakes (Rissa tridactyla) decrease rapidly after experimental prevention of copulations. Consistent with this observation, our data indi-cate that the potential for sexually transmitted bacteria to result in a major long-term CM shift in barn swallow is rather low.

Supporting Information

S1 Fig. Rarefaction analysis. Rarefaction curves for the number of 97% OTUs detected in indi-vidual samples according to sequencing depth. Calculations were based on 10 sub-sampled data-sets for each sequencing depth (0–3000 randomly selected sequences). Colours correspond to individual breeding pairs. Males and females are indicated by triangles and circles, respectively. (JPG)

S2 Fig. Taxonomic classification of barn swallow cloacal microbiota. Barplots showing taxo-nomic assignment (based on RDP classifier; 80% confidence threshold) of 454 sequences to A) Phylum and B) Class level for sequences corresponding to the five most abundant phyla (repre-sented by Proteobacteria, Firmicutes, Actinobacteria, Tenericutes and Bacteroidetes). This sub-set accounts for ca. 87% of high quality sequences generated during this study. Facets (A-H) correspond to individual breeding pairs. Samples within facets are sorted according to sexual identity (F = females, M = males). Detailed taxonomic classification of the dominant OTUs is provided in S2 Table.
(JPG)

S1 Table. Details on samples used in this study.
(XLS)

S2 Table. List of dominant OTUs detected in the barn swallow cloacal microbiome. Shown are OTUs represented by > 0.1% sequences and detected in at least two individuals. The Table includes information on taxonomic classification to genus level, proportion of high quality reads represented by a given OTU (Prop. Seqs.), the proportion of individuals for which a given OTU was detected (Prop. Individual) and the corrected moment estimate of k of the negative binomial distribution (k param.).
(XLS)

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Author Contributions

Conceived and designed the experiments: JK TA. Performed the experiments: JK DC TA. Analyzed the data: JK LK. Contributed reagents/materials/analysis tools: JK TA. Wrote the paper: JK. Provided comments and recommendations that improved the manuscript: JK DC LK TA. Approved the final version of the manuscript: JK DC LK TA.

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RESEARCH ARTICLE

Gut microbiota in a host–brood parasite system: insights from common cuckoos raised by two warbler species

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One sentence summary: This paper shows that fecal microbiota varied between cuckoo and warbler nestlings when accounting for the effect of foster/parent species, highlighting the importance of host-intrinsic regulatory mechanisms in comparison with environmental factors.

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ABSTRACT

An animal's gut microbiota (GM) is shaped by a range of environmental factors affecting the bacterial sources invading the host. At the same time, animal hosts are equipped with intrinsic mechanisms enabling regulation of GM. However, there is limited knowledge on the relative importance of these forces. To assess the significance of host-intrinsic vs environmental factors, we studied GM in nestlings of an obligate brood parasite, the common cuckoo (*Cuculus canorus*), raised by two foster species, great reed warblers (*Acrocephalus arundinaceus*) and Eurasian reed warblers (*A. scirpaceus*), and compared these with GM of the fosterers' own nestlings. We show that fecal GM varied between cuckoo and warbler nestlings when accounting for the effect of foster/parent species, highlighting the importance of host-intrinsic regulatory mechanisms. In addition to feces, cuckoos also expel a deterrent secretion, which provides protection against olfactory predators. We observed an increased abundance of bacterial genera capable of producing repulsive volatile molecules in the deterrent secretion. Consequently, our results support the hypothesis that microbiota play a role in this antipredator mechanism. Interestingly, fosterer/parent identity affected only cuckoo deterrent secretion and warbler feces microbiota, but not that of cuckoo feces, suggesting a strong selection of bacterial strains in the GM by cuckoo nestlings.

Keywords: metabarcoding; bacteria; feces; deterrent secretion; great reed warbler; Eurasian reed warbler

INTRODUCTION

Vertebrate gastrointestinal tracts are colonized by taxonom-ically and functionally diverse bacterial communities that interact with a broad range of host physiological processes and provide benefits to the host via increased digestion and vitamin synthesis efficiency, protection against pathogens and stimulation of the immune and nervous systems (Jumpertz *et al.* 2011; Koch and Schmid-Hempel 2011; Cryan and Dinan

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2012). Gut microbiota (GM) exhibits pronounced variation at the interindividual and interspecific levels (Baxter *et al.* 2015; Yuan *et al.* 2015; Lewis, Moore and Wang 2016; Kreisinger *et al.* 2017; Kropa´ckov˘a´ *et al.* 2017), which can modulate host phenotype (Claus *et al.* 2008; Han *et al.* 2016). Understanding the factors behind GM variation is considered a crucial endeavor in current ecological research as it improves our understanding of the mechanisms involved in three-way interactions between GM, host and its environment.

There are two basic sources of variation affecting GM com-munities, i.e. host-intrinsic and environmental factors. Host-intrinsic factors may be triggered by dozens of genes, being related, for example, to immune functions and facilitating selec-tion of appropriate microbes from the environment or regulation of microbial populations already present in the host's gut (Ben-son *et al.* 2010; McKnite *et al.* 2012; Bolnick *et al.* 2014; Kropa´ckov˘a´ *et al.* 2017). Environmental factors include a range of biotic and abiotic agents that affect the composition and spatiotempo-ral variation of environmental bacterial sources colonizing the gut. Physical contact with conspecifics and associated intraspe-cific microbiota transfer are other important sources of envi-ronmental variation shaping symbiotic bacterial communities (Lucas and Heeb 2005; Kreisinger *et al.* 2017; Ambrosini *et al.* 2019). There are also a plethora of factors linked to the exter-nal environment, including diet composition (Bodawatta *et al.* 2018; Loo *et al.* 2019b; Teyssier *et al.* 2020) and eukaryotic gut parasites that can directly affect the within-gut environment, thereby shaping associated microbial communities (Kreisinger *et al.* 2015; Newbold *et al.* 2017; Aivelo and Norberg 2018). Last but not the least, effects induced by environmental factors may vary depending on host-intrinsic regulatory mechanisms. For exam-ple, pronounced variation in GM changes has been observed following the introduction of wild-living species into captivity (McKenzie *et al.* 2017). Host-specific GM changes have also been recorded following infection by intestinal helminths, which can be explained by variation in host genetic factors interacting with both GM and the parasite (Reynolds *et al.* 2014). While the relative effect of environmental vs host-intrinsic factors on GM variation has been intensively studied in mammals (Benson *et al.* 2010; Campbell *et al.* 2012; Nelson *et al.* 2013; Menke *et al.* 2017), it has been somewhat neglected in non-mammalian vertebrate taxa, which can harbor strikingly distinct GM (Lucas and Heeb 2005; Ruiz-Rodrıguez´ *et al.* 2009a; Hird *et al.* 2014; Kreisinger *et al.* 2017; Loo *et al.* 2019b).

Obligate avian brood parasitism represents a reproductive strategy whereby species lay eggs in the nests of other species that then foster the parasitic offspring (Davies 2000). Brood parasites utilizing multiple foster species represent a unique type of natural experiment allowing the role of host-intrinsic vs environmental factors to be disentangled, with parasitic progeny from the same genetic background being exposed to different environmental conditions, i.e. foster species. Surpris-ingly, GM variation in brood parasites has received relatively little attention. To our knowledge, there have been only four studies focused on GM variation in avian brood parasites and/or their fosterers (Ruiz-Rodrıguez´ *et al.* 2009a,b, 2018; Hird *et al.* 2014), three of which compared GM between parasitic chicks and the fosterers' own chicks (Ruiz-Rodrıguez´ *et al.* 2009a,b, 2018). As these studies always comprised only one foster species of brood parasite system, they say little about the relative effects of host-intrinsic vs environmental factors on GM. In our study, we address for the first time the role of environmental vs host-intrinsic factors on GM of brood parasites raised by two different foster species and compare it with the GM of the fosterers' genetic offspring.

We focus on GM variation in an obligate brood parasite, the common cuckoo (*Cuculus canorus*), which exploits a range of passerine bird species as potential fosterers (Moksnes and Røskaft 1995; Stokke *et al.* 2018). Approximately 40 h after hatch-ing, a common cuckoo chick evicts its nest mates, thereby reduc-ing all potential bacterial interactions with the fosterer's off-spring (Honza, Voslajerov˘a´ and Moskat´ 2007). We analyzed the GM of cuckoo chicks raised by two foster species, the great reed warbler (*Acrocephalus arundinaceus*) and the Eurasian reed war-bler (*A. scirpaceus*), and contrasted it with the GM of the genetic progeny of both foster parent species. This allows us to test whether host-intrinsic mechanisms shape GM structure. Great reed warblers are around three times larger than Eurasian reed warblers (Cramp 1992) and, consequently, cuckoos raised by the former species grow faster and have higher body mass at fledg-ing than cuckoos raised by the latter species (Kleven *et al.* 1999). While the breeding habitats of the two fosterer species are com-parable, with minor differences in microhabitat structure (Dyrcz 1981; Leisler 1981; Saino 1989), the two species differ in the diet provided to the parasitic and own chicks (Dyrcz 1979; Grim and Honza 1996, 1997), which ensures sufficient variation in envi-ronmental component of so-called nidobiome, i.e. a complex of processes shaping microbial colonization in neonates (Campos-Cerda and Bohannan 2020). Consequently, we asked if such a sort of environmental variation affects GM of warbler's own and adoptive offspring. As in many previous studies (Hird *et al.* 2015; Lewis, Moore and Wang 2016; Kropa´ckov˘a´ *et al.* 2017; Grond *et al.* 2019; Loo *et al.* 2019a), we used fecal microbiota analyzed by high-throughput sequencing of 16S rRNA amplicons as a GM proxy for both cuckoo and warbler offspring. Furthermore, by adopting the metabarcoding approach, we were able to replicate previous research on warbler and cuckoo nestling diet (Grim and Honza 1997), i.e. one of the main sources of vertebrate GM vari-ation (Ley *et al.* 2008; Zhu *et al.* 2017; Youngblut *et al.* 2019). The main aim of diet profiling was to confirm that (i) nestling diet differs according to foster/parent species and (ii) individual fos-ter/parent species provisions their genetic progeny and cuckoo nestlings with a comparable diet. In addition to fecal samples, we also analyzed the microbial content of a dark secretion of putative cecal origin (Roder¨ *et al.* 2014) produced by cuckoos but not warbler nestlings. This secretion repels nest predators owing to its high concentration of volatile compounds, many of which are putative by-products of bacterial metabolism (e.g. butyric acid, acetic acid and indoles; Roder¨ *et al.* 2016). It has been suggested that symbiotic microbes also contribute to the secre-tion's repulsive properties (Roder¨ *et al.* 2016). Consequently, the secretion may represent a further example of microbiota facili-tating chemical communication in vertebrates (Theis *et al.* 2013; Lam *et al.* 2018), thus extending the host's phenotype beyond a capacity inherent to its genome. Surprisingly, there are no stud-ies aimed at profiling the secretion microbial community.

METHODS

Field sampling

Samples were obtained from a fishpond system situated between Hodonın´ (48° 51 N, 17° 07 E) and Mutenice˘ (48° 54 N, 17° 02 E) in South Moravia, Czech Republic. All samples were col-lected during the breeding season between 30 May and 10 July 2015. Around 46% of great reed warbler nests were parasitized by

common cuckoos in the population, while the cuckoo parasitism rate in Eurasian reed warbler nests was around 10% (Jelınek´ *et al.* 2016). Warbler nestlings were sampled 6–10 days after hatch-ing and cuckoos 7–17 days after hatching. We collected fecal samples from 20 common cuckoo, 16 great reed warbler and 9 Eurasian reed warbler nestlings, along with 15 samples of common cuckoo nestlings' deterrent secretions (Table S1, Support-ing Information). Only one nestling was sampled from each fos-ter brood. Day of year of sampling did not differ between sam-ples collected from great reed warbler and Eurasian reed warbler nests (*t*-test: $t = 0.351$, $P = 0.727$) or between cuckoo and warbler samples (*t*-test: $t = 0.265$, $P = 0.792$).

Nestlings were temporarily removed from the nest and both fecal (in warblers and cuckoos) and deterrent secretion (in cuck-oos) samples were collected directly into sterile DNA/RNA free cryotubes (Simport, Beloeil, Canada) filled with a self-made DNA/RNA-stabilizing buffer based on RNAlater (protocol avail-able upon request) and kept at −20° C until the end of the field work. Then, they were transferred into the laboratory and kept at −80° C.

All field procedures were approved by the ethical committee of the Czech Academy of Sciences (Animal Care Protocol num-bers 173/2008 and 128/2010) and by the relevant conservation authorities (permits JMK20189/2010 and MUHO 2680/2014 OZP˘) .

Microbiota profiling and bioinformatic processing of 16S rRNA data

We isolated metagenomic DNA using PowerSoil kits (Mo Bio Laboratories Inc., Carlsbad, USA) and subsequently ampli-fied the V3–V4 region of 16S rRNA using S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GAC-TACHVGGGTATCTAATCC) primers (Klindworth *et al.* 2013) and tagged both of them with 10 bp oligonucleotides for multi-plexing. Technical PCR duplicates were prepared for all sam-ples in order to check the consistency of microbial profiles. PCR yields were low in the case of two fecal samples. Thus, we did not use them for preparation of sequencing libraries. Sequencing libraries were prepared using TruSeq nano kits and sequenced on Illumina MiSeq using v3 chemistry (300 bp paired-end reads). Further details on laboratory procedures associated with microbiota profiling and subsequent bioinformatic pro-cessing of sequencing data are provided by Kreisinger *et al.* (2017).

Diet metabarcoding

To gain an insight into the diet of cuckoo and warbler nestlings, metabarcoding analysis was applied using the same fecal metagenomic DNA samples as for bacterial 16S rRNA profil-ing. Previously published universal cytochrome c oxidase sub-unit I (COI) primers targeting a broad range of invertebrate taxa (Elbrecht and Leese 2017) were employed for this purpose. Details on laboratory procedures and data processing associated with the metabarcoding experiment are available in Supporting Information A1.

Bioinformatic and statistical analysis

Fastq files were demultiplexed and primers trimmed using skewer (Jiang *et al.* 2014). Next, we trimmed low-quality 3 ends (250 base pairs from forward and 220 base pairs from reverse reads being retained), eliminated low-quality sequences (maximum expected error per sequence < 1) and denoised quality-filtered files using DADA2 (Callahan *et al.* 2016). After the denoising, forward and reverse files were merged using DADA2 and chimeric 16S rRNA variants were identified and eliminated using UCHIME (Edgar *et al.* 2011) and the GOLD database (Mukherjee *et al.* 2017).

The taxonomy of the resulting unclustered 16S rRNA variants (hereafter operational taxonomic units, i.e. OTUs) was assigned using RDP classifier and the Greengenes reference database (v. 13.8.; DeSantis *et al.* 2006). PyNAST (Caporaso *et al.* 2010) was employed for sequence alignment and an OTU phylogenic tree was subsequently constructed using FastTree (Price, Dehal and Arkin 2010). The resulting OTU table, sample metadata and phy-logenetic tree were merged into a phyloseq database (McMurdie and Holmes 2013) for the purposes of further statistical analysis.

In our study, we provide separate analyses regarding GM between (i) cuckoo and warbler fecal samples and (ii) between cuckoo deterrent secretion and cuckoo feces. Shannon diver-sity indices were calculated for alpha diversity analysis using rarefaction-based normalized OTU tables (random subsetting of read counts per sample corresponding to 1268 sequences, i.e. minimal sequencing depth). Shannon diversity was sub-sequently used as a response variable in analysis of vari-ance (ANOVA) to test whether microbial diversity varied due to explanatory variables that included the effect of foster/parent species (Eurasian reed warbler vs great reed warbler) and the effect of sample type (cuckoo vs warbler nestling feces or cuckoo feces vs cuckoo deterrent secretion). Variation in microbial com-position between samples was assessed using two types of ecological dissimilarity, each capturing different aspects of GM divergence, i.e. Bray–Curtis, which accounts for OTU abundance (Bray and Curtis 1957), and a binary version of Jaccard dissim-ilarity, which accounts for OTU absence or presence only (Jac-card 1901). Furthermore, to check if bacterial phylogeny affects GM variation pattern, UniFrac dissimilarities were calculated (Lozupone and Knight 2005; Supporting Information A1). In order to account for uneven sequencing depth between samples, we rarefied the OTU table to achieve the same sequence coverage per sample prior to calculation ($n = 1268$ sequences per sample, i.e. minimal sequencing depth).

Between-sample variation in GM composition was visualized using non-metric multidimensional scaling (NMDS) considering two axes. As stress values were relatively high (~0.2 in some cases, specified below), we also provided NMDS solutions for three axes (Supporting Information A1). Using PERMANOVA (adonis function from the vegan package in R; Oksanen *et al.* 2013), we tested whether there was any divergence in microbial composition between cuckoo and warbler nestling feces and whether composition varied according to foster/parent species identity. Next, we tested using PERMANOVA whether there was any difference in diversity and composition between cuckoo feces and deterrent secretion in the microbial profiles. Finally, generalized linear models with negative binomial error distribu-tion, obtained from the DESeq2 package (Love, Huber and Anders 2014), were employed for differential abundance analyses. Read counts for individual bacterial genera were used as response, and explanatory variables included the effect of foster/parent species and the effect of sample type (cuckoo vs warbler nestling feces or cuckoo feces vs cuckoo deterrent secretion). Significance of the explanatory variables was tested using like-lihood ratio tests. Model fitting and statistical testing included all default steps implemented in the DESeq2 pipeline. All statis-tical analyses and their visualizations were conducted using R v.3.4.4 (R Core Team 2018) and the R-based packages mentioned above.

RESULTS

Characteristics of cuckoo and warbler microbial profiles

After all filtering steps, our final dataset comprised 475 604 sequences assigned to 1651 OTUs (mean number of sequences per sample = 8200). According to phylum-level classification, Fir-micutes bacteria were the dominant component of both warbler and cuckoo microbiota (average proportion of reads 55% for war-bler feces, 51% for cuckoo secretion and 56% for cuckoo feces). Substantial variations in abundance patterns were observed in other bacterial phyla. Proteobacteria, for example, tended to be more abundant in warbler feces (26% of reads) compared to cuckoo secretion samples (15% of reads) and cuckoo feces (11% of reads), and while Bacteroidetes abundance was relatively high in some cuckoo samples (25% of reads in secretion and 8% in feces), it was low in warbler feces (2% of reads). Tenericutes, Actinobacteria, Fusobacteria and Chlamydia all occurred at relatively high levels in some samples (Fig. 1); however, their uneven distribution prevented us from drawing any general conclusions as to systematic variation due to the effect of offspring or fos-ter/parent species identity. A more detailed overview of the taxa in our dataset is provided in Figure S1 and Table S2 (Supporting Information).

Variation in fecal microbiota with respect to nestling and foster/parent species

Foster/parent species had no effect on Shannon diversity esti-mates (ANOVA, $F_{(1,41)} = 0.004$, $P = 0.953$, Fig. 2); however, fecal microbiota exhibited higher richness in cuckoo nestlings com-pared with warbler nestlings (ANOVA, $F_{(1,41)} = 5.867$, $P = 0.020$, Fig. 2). We found significant differences between cuckoo and warbler nestling fecal microbiota composition, and a signifi-cant effect of foster/parent species for all GM dissimilarities but weighted UniFrac (Table 1; Fig. 3; Supporting Information A1). DESeq2 analysis further identified six bacterial genera that were more abundant in warbler feces (*Rhodobacter*, *Sodalis*, '*Candida-tus* Arthromitus' and *Lactobacillus*, along with Legionellaceae and Mycoplasmataceae, which were not classified to genus level; Table S3, Supporting Information), and five genera that were more abundant in cuckoo fecal samples (*Escherichia*, *Methylobac-terium*, *Ruminococcus*, *Cetobacterium* and *Clostridium*; Table S3, Supporting Information).

Separate PERMANOVA analyses found no effect of foster species on cuckoo fecal microbiota composition (Table 2). On the other hand, separate PERMANOVA of warbler samples revealed a significant difference in composition between the two species (Table 2). Furthermore, DESeq2 identified four bacterial gen-era whose relative abundances varied between the two war-bler nestling species (*Sodalis*, *Marinomonas*, *Carnobacterium* and an unclassified Mycoplasmataceae; Table S3, Supporting Infor-mation).

Differences in GM between cuckoo feces and deterrent secretion

We collected both feces and deterrent secretions for 10 cuckoos (five raised by great reed warblers and five by Eurasian reed warblers). Interindividual differences between fecal and secre-tion microbiota profiles were not intercorrelated (Mantel test, $P > 0.2$, range of correlation coefficients: 0.145 to −0.168 for all dissimilarity types). Consequently, we did not account for within-individual covariation in microbiota structure between fecal and deterrent secretions in subsequent analyses.

While we observed a marginally non-significantly higher bacterial diversity in cuckoo deterrent secretion compared with cuckoo feces (ANOVA, $F_{(1,33)} = 4.087$, $P = 0.051$; Fig. 2), PER-MANOVA revealed pronounced differences in the composition of cuckoo fecal microbiota vs deterrent secretion (PERMANOVA, $p < 0.002$ for all GM dissimilarity types but weighted UniFrac; Table 3; Supporting Information A1). In addition, deterrent secretion microbial profiles were also distinct from those of both war-bler and cuckoo nestling fecal microbiota according to NMDS (Fig. 3). DESeq2 identified 43 bacterial genera differentiating cuckoo secretion profiles from those of cuckoo fecal micro-biota (e.g. *Methylobacterium*, *Marinomonas*, *Streptococcus*, *Sedimini-bacterium*, *Anaerofilum*, *Fusobacterium*; Table S3, Supporting Infor-mation). As in the case of the cuckoo fecal microbiota, cuckoo deterrent secretion microbial diversity was unaffected by fos-ter species (ANOVA, $F_{(1,33)} = 0.713$, $P = 0.405$; Fig. 2). Unlike fecal microbiota, however, deterrent secretion composition var-ied significantly between cuckoos raised by different warbler species (Table 2). Despite the significant difference, the effect size of deterrent secretion composition variation due to foster species was low, DESeq2 only identified the genus *Clostridium* as more abundant in the deterrent secretions of cuckoos raised by great reed warbler compared with cuckoos raised by Eurasian reed warbler (log2fold change = −8.224, adjusted $P < 0.001$; Table S3, Supporting Information).

Diet variation

Using COI metabarcoding, we were only able to retrieve useful data on diet composition for 49% of fecal samples. This was mainly due to poor PCR amplification of the COI and a high rep-resentation of reads corresponding to taxa that could not be con-sidered as diet components within the resulting COI profiles. As such, just 21 samples yielded diet profiles useful for quan-titative analyses. Consistent with the basic assumptions of our study, statistical analysis of this subset revealed that parents of the same species provided a comparable diet to both genetic progeny and cuckoo parasites, though the diet fed by the two parent species was different. Specifically, Eurasian reed warbler brought a more diverse diet that included a higher percentage of true bugs, while beetles tended to predominate in the diet pro-visioned by great reed warbler parents. For more details on the diet profile analyses, see Supporting Information A1.

DISCUSSION

We asked to what extent environmental and host-intrinsic fac-tors affect the GM structure during early stages of the post-natal development. To achieve this goal, we studied brood parasite's GM exposed to two distinct environmental contexts represented by two different foster species and compared it with GM of fos-terer's genetic progeny. Despite the two environmental contexts that were characterized by distinct diet composition, one of the main forces shaping GM in vertebrates (Ley *et al.* 2008; Zhu *et al.* 2017; Youngblut *et al.* 2019), we showed in our study system that host-intrinsic factors dominated over environmental fac-tors. At the same time, however, variation explained by both host-intrinsic and environmental effects was rather low. This is consistent with most studies on free-living birds, where GM exhibit pronounced interindividual variation and rapid tempo-ral changes (Kreisinger *et al.* 2017; Escallon *et al.* 2019; Grond *et al.* 2019). Consequently, variables predicting systematic avian GM

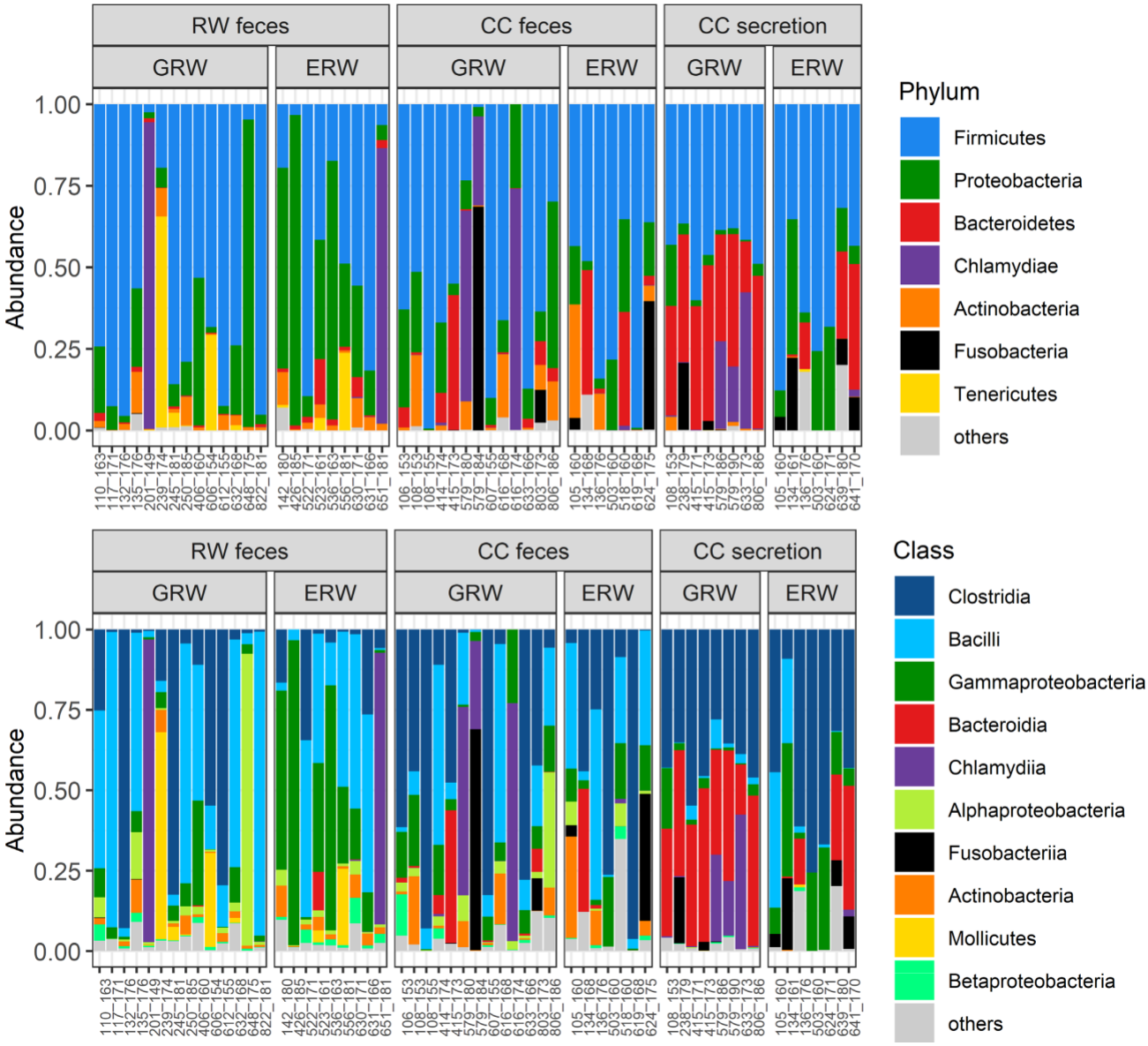

Figure 1. Proportion of dominant bacterial phyla and classes with relative abundance > 1% in feces and deterrent secretion of common cuckoo (CC feces and CC secretion) and feces of warbler nestlings (RW feces) raised by great reed warblers (GRW) or Eurasian reed warblers (ERW).

Table 1. Compositional differences in fecal microbiota between cuckoo and warbler nestlings (offspring) raised by two foster/parent species, Eurasian and great reed warblers. Calculations were based on PERMANOVA and two types of community dissimilarity. Significant predictors are in bold.

Dissimilarities	Variables	Df	*F*	R^2	*P*
Bray–Curtis	Foster/parent species	**1, 40**	**1.752**	**0.040**	**0.001**
	Offspring	**1, 40**	**1.679**	**0.039**	**0.001**
Jaccard	Foster/parent species	**1, 40**	**1.493**	**0.034**	**0.003**
	Offspring	**1, 40**	**2.427**	**0.055**	**0.001**

changes at both interpsecific and interindividual levels are typ-ically of low effect size.

Our conclusion that host-intrinsic factors have more deci-sive effect on GM than environmental factors is based on sys-tematic differences in the composition and diversity of fecal microbiota between cuckoo and warbler nestlings, irrespective of foster/parent species. GM differences between brood parasite young and genetic progeny were already reported by previous studies on the great spotted cuckoo (*Clamator glandarius*) fos-tered by magpies (*Pica pica*; Ruiz-Rodrıguez´ *et al*. 2009a, 2018). In addition, Ruiz-Rodrıguez´ *et al.* (2018) noted that the GM of great spotted cuckoo nestlings fostered by magpies was a mix-ture of GM from magpie nestlings and the great spotted cuckoo adults. However, these results do not allow direct comparison of

Table 2. Separate PERMANOVA analyses testing for the effect of foster/parent species (Eurasian or great reed warbler) on microbiota composition in (A) warbler nestling feces, (B) cuckoo nestling feces and (C) cuckoo nestling deterrent secretion. Calculations were based on two types of community dissimilarity. Significant predictors are in bold.

		A: Warbler feces			
Dissimilarities	Variables	Df	*F*	R^2	*P*
Bray–Curtis	Parent species	**1,21**	**1.978**	**0.086**	**0.001**
Jaccard	Parent species	**1,21**	**1.590**	**0.070**	**0.002**
		B: Cuckoo feces			
Dissimilarities	Variables	Df	*F*	R^2	*P*
Bray–Curtis	Foster species	1,18	1.129	0.059	0.241
Jaccard	Foster species	1,18	0.891	0.047	0.703
		C: Cuckoo deterrent secretion			
Dissimilarities	Variables	Df	*F*	R^2	*P*
Bray–Curtis	Foster species	**1,13**	**1.607**	**0.110**	**0.023**
Jaccard	Foster species	**1,13**	**1.924**	**0.129**	**0.003**

Table 3. Composition of cuckoo fecal vs cuckoo deterrent secretion microbiota (sample type) between individuals raised by two foster species, Eurasian and great reed warblers. Calculations were based on PERMANOVA and two types of community dissimilarity. Significant predictors are in bold.

Dissimilarities	Variables	Df	*F*	R^2	*P*
Bray–Curtis	Foster species	**1,32**	**1.624**	**0.046**	**0.003**
	Sample type	**1,32**	**1.667**	**0.047**	**0.002**
Jaccard	Foster species	**1,32**	**1.655**	**0.045**	**0.012**
	Sample type	**1,32**	**2.998**	**0.082**	**0.001**

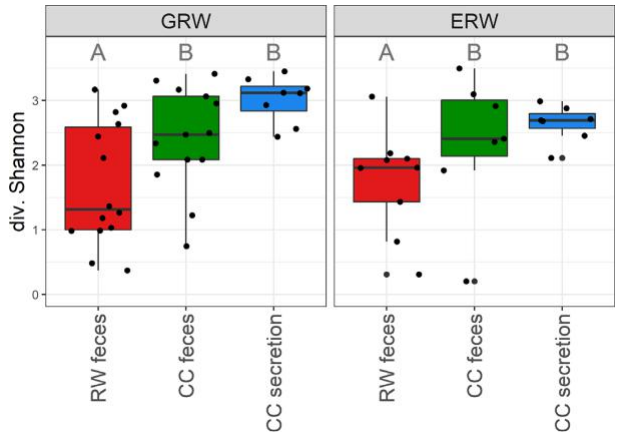

Figure 2. Boxplots for Shannon diversity of microbiota associated with feces and deterrent secretion of cuckoo nestlings (CC feces and CC secretion) and feces of warbler nestlings (RW feces) raised by great reed warblers (GRW) or Eurasian reed warblers (ERW). Groups A and B are significantly different ($P = 0.020$).

environmental vs host-intrinsic factors as they were based on uniform environmental context represented by a single foster species. In addition, unlike the common cuckoo, the great spot-ted cuckoo does not evict its nest mates, which increases the complexity of this model system due to social GM transmission among individuals sharing the same nest (see also Kreisinger *et al.* 2017; Ambrosini *et al.* 2019). Interestingly, all dissimilar-ity measures, with the exception of weighted UniFrac, provided congruent support for microbiota divergence between cuckoo and warbler nestlings. We assume that lack of host-specific sig-nal in the case of weighted UniFrac was caused by relatively low phylogenetic divergence of bacterial taxa that, according to DESeq2 analyses, varied between cuckoo and warbler nestlings.

Unfortunately, our data cannot provide direct insights into the mechanisms causing the differentiation in fecal microbiota between warbler and cuckoo nestlings. Nevertheless, variation in gut anatomy and function between cuckoos and passerines is the most likely source of the observed divergence. The most striking difference in the lower digestive tract of these two avian groups is probably the considerable reduction in passerine ceca, which are otherwise well developed in cuckoos (Clench and Mathias 1995; Ruiz-Rodríguez *et al.* 2009a) and typically host an abundant bacterial community involved in food decompo-sition (White 2005; Skadhauge 2012). Based on our data, we propose that migration of bacteria from cecal content to fecal material may have contributed to compositional differences in fecal microbiota between cuckoo and warbler nestlings. Consis-tent with this possibility, deterrent secretion composition (puta-tively produced in ceca; Roder¨ *et al.* 2016) was more dissimi-lar to warbler fecal microbial samples than to those of cuck-oos. In addition, most bacterial genera that were more abundant in cuckoo feces (e.g. *Ruminococcus*, *Clostridium* and *Cetobacterium*) corresponded to obligatory anaerobes predisposed to coloniza-tion of cecal content (Julliand *et al.* 1999; Zhu *et al.* 2002; Tsuchiya, Sakata and Sugita 2008; Suzuki and Nachman 2016). At the same time, however, we cannot exclude contributions of other factors, e.g. differences between passerine and cuckoo immune systems.

The deterrent secretion produced by the cuckoo chick is a rare example of a chemical anti-predation defense in birds (Canestrari *et al.* 2014; Roder¨ *et al.* 2014; Trnka *et al.* 2016). The

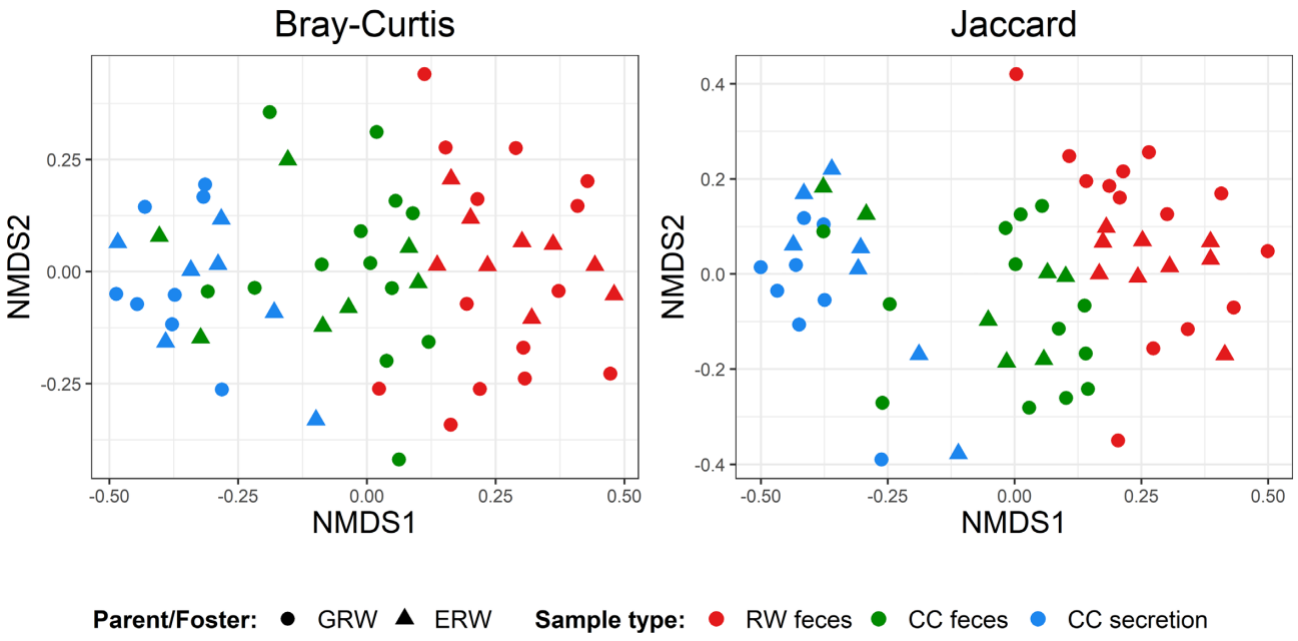

Figure 3. NMDS considering two axes for fecal and deterrent secretion microbiota of cuckoo (CC feces and CC secretion) and fecal microbiota of warbler nestlings (RW feces). Species identity of fosterers/parents (great reed warbler (GRW) or Eurasian reed warbler (ERW)) is indicated by different plotting characters as detailed in the figure legend. Two types of dissimilarity were used for NMDS ordination: Bray–Curtis (stress = 0.22) and Jaccard (stress = 0.20).

secretion is believed to be of cecal origin (Roder¨ *et al.* 2016). Though this view requires direct verification, a similar type of secretion, so-called 'cecal feces', is known to be produced in ceca of various avian lineages (Clarke 1979; Villanua´ *et al.* 2006). More-over, there is also indication that the cecal feces, similarly to cuckoo's deterrent secretion, provide protection against preda-tors (Swennen 1968). The cuckoo's secretion contains high con-centrations of volatile molecules directly linked with its repul-sive properties. Several of the bacterial genera over-represented in the cuckoo deterrent secretion (compared to cuckoo feces), i.e. *Ruminococcus*, *Bacteroides* and *Parabacteroides*, are known to pro-duce short-chain fatty acids and other volatile molecules, pre-viously detected in the deterrent secretion at high concentra-tions (Stack, Hungate and Opsahl 1983; Koh *et al.* 2016). Further-more, the deterrent secretion was also enriched by the genus *Fusobacterium*, whose volatiles cause oral malodor in humans (Sterer and Rosenberg 2011) and form a component of scent gland microbiota, important for chemical signaling, in mam-malian carnivores (Theis *et al.* 2013). As such, our data sug-gest that symbiotic microbiota contributes to the expression of antipredatory chemical signaling in the common cuckoo. Nev-ertheless, further research should employ functional metage-nomic and proteomic tools in order to link GM changes observed at the taxonomic level with functional pathways responsible for production of deterrent volatiles.

Compared to the deterrent secretion, cuckoo fecal microbiota was enriched with several genera corresponding to lactic acid bacteria (i.e. *Carnobacterium*, *Vagococcus*, *Lactobacillus*, *Lactococcus* and *Streptococcus*), which are common inhabitants of avian GM (Kropa´ckov˘a´ *et al.* 2017; Bodawatta *et al.* 2018; Grond *et al.* 2019). Members of this bacterial clade rely on anoxic decomposition of monosaccharides, prefer energy-rich substrates and exhibit tolerance to high acidity levels (Hijum *et al.* 2006; Nazef *et al.* 2008). Consequently, we speculate that a higher proportion of lactic acid bacteria in feces may reflect avian GM functional variation in different gut compartments and spatial variation of biotic and abiotic conditions within the gut. Fecal microbiota is also more likely to be affected by bacteria from external envi-ronmental pools than secretion microbiota. This is supported by the fact that plenty of aerobic bacteria, and/or bacteria that are regularly detected in environmental sources (e.g. *Sphingomonas*, *Photobacterium*, *Marinomonas*, *Sediminibacterium* and *Phycicoccus*), were more abundant in cuckoo feces than in cuckoo deterrent secretion.

Our data provide ambiguous support for the effect of fos-ter/parent species on GM structure in nestlings. While we observed significant differences in fecal microbiota composition between great reed warbler and Eurasian reed warbler juveniles, we were unable to distinguish the extent to which this variation was affected by environmental or host-intrinsic factors, given the correlative nature of the data used. Nevertheless, consistent with the findings of the previous studies, nest environment has already been shown to be an important predictor of GM at the intraspecific level in passerines (Kreisinger *et al.* 2017; Teyssier *et al.* 2018; Ambrosini *et al.* 2019). Moreover, a study by Grond *et al.* (2017) reported that shorebird hatchling's GM exhibited com-parable composition with microbiota from environmental sam-ples, which supports the effect of environmental bacterial pools on avian GM. In the case of cuckoo GM samples, the effect of nest environment and diet was confirmed for microbiota associated with the deterrent secretion, but not for fecal samples. How-ever, just one bacterial genus (*Clostridium*) at moderate abun-dance was associated with these changes, suggesting a limited effect of rearing conditions on cuckoo GM content.

We conclude that interspecific variation in host-intrinsic reg-ulatory factors and gut compartment variation were the most probable sources of the observed changes in GM, the effect of environmental variation apparently being of lower importance in our model system. Moreover, our results also imply that symbiotic GM may have played an important role in the evo-lution of the cuckoo nestling's unique chemical antipredatory defense.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

DATA ACCESSIBILITY

Sequencing data are available at European Nucleotide Archive under project accession numbers PRJEB38922 and PRJEB38897. Accession numbers for each sample are provided in Table S1 (Supporting Information).

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AUTHOR CONTRIBUTIONS

Study design: JK, PP; field sampling: MP, PP, MH; laboratory analysis: LS, JFM; data analysis: LS, JK; funding: JK, LS, MH; manuscript drafting: LS, JK. All authors provided helpful com-ments and recommendations and approved the final version of the manuscript.

Conflicts of interest. None declared.

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3.9. Publikace I

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Podpis školitele

PLOS ONE

RESEARCH ARTICLE

Variation between the oral and faecal microbiota in a free-living passerine bird, the great tit (*Parus major*)

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Abstract

The gastrointestinal tract of vertebrates is inhabited by diverse bacterial communities that induce marked effects on the host physiology and health status. The composition of the gastrointestinal microbiota is characterized by pronounced taxonomic and functional variability among different regions of the vertebrate gastrointestinal tract. Despite the relatively solid knowledge on the among-region variations of the gastrointestinal microbiota in model mammalian species, there are only a few studies concerning among-region variations of the gastrointestinal microbiota in free-living non-mammalian vertebrate taxa. We used Illumina MiSeq sequencing of bacterial 16S rRNA amplicons to compare the diversity as well as taxo-nomic composition of bacterial communities in proximal vs. distal parts of the gastrointestinal tract (represented by oral swabs and faecal samples, respectively) in a wild passerine bird, the great tit (*Parus major*). The diversity of the oral microbiota was significantly higher com-pared to the faecal microbiota, whereas interindividual variation was higher in faecal than in oral samples. We also observed a pronounced difference in taxonomic content between the oral and faecal microbiota. Bacteria belonging to the phyla Proteobacteria, Firmicutes and Actinobacteria typically dominated in both oral and faecal samples. A high abundance of bacteria belonging to Tenericutes was observed only in faecal samples. Surprisingly, we found only a slight correlation between the faecal and oral microbiota at the within-individual level, suggesting that the microbial composition in these body sites is shaped by independent regulatory processes. Given the independence of these two communities at the individual level, we propose that simultaneous sampling of the faecal and oral microbiota will extend our understanding of host vs. microbiota interactions in wild populations.

Introduction

Animal bodies are inhabited by taxonomically and functionally diverse communities of bacteria [1–3] that modulate their host's physiology and health status [4,5]. The modulations mediated by microbial communities are believed to be beneficial in most cases. However, adverse

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effects can be elicited by obligatory pathogenic bacterial species invading host bodies or facul-tative pathogens dwelling in immunocompromised hosts [6–8].

The host-associated microbiota exhibits pronounced spatial variation among different parts of the animal body [1,9–11]. Out of this complex system of host-associated microbial consor-tia, the microbiota of the gastrointestinal tract (hereafter GIT) has attracted considerable research attention during the past two decades. The GIT microbiota provides important bene-fits to the host, including increased efficiency of food digestion [12–14], stimulation of the immune system [15,16], defence against pathogens [17,18] and beneficial effects on the devel-opment and functioning of the gut and central nervous system [19,20]. At the same time, GIT microbiota dysbiosis is associated with metabolic, autoimmune and neurological disorders [21–24]. Last but not least, many bacterial pathogens invade animal bodies through the GIT [25–27], providing a further argument for the importance of the GIT microbiota on animal fitness.

Most current studies focusing on the GIT microbiota, use the microbiota of faecal samples, putatively representing the GIT microbiota of the lower intestine, as a proxy [3,28–30]. How-ever, there is compelling evidence that microbial composition exhibits marked variation between different compartments of the GIT [9,31–33]. Such variation occurs due to spatial changes in biotic and abiotic factors among different GIT regions, including acidity levels, concentrations of oxygen, cholic acids and nutrients as well as changes in the host's immunity [34–36]. As the relative contributions of these mechanisms to microbial structure may be in-dependent both at the between-GIT region level and the interindividual level, the resulting within-individual correlation of the microbial content among different GIT regions can be of rather low effect size (Kreisinger et al., in prep, [37]). At the same time, the microbiota in dif-ferent GIT regions can be associated with distinct effects on the hosts' phenotype [33,38]. Con-sequently, studies relying on samples from single GIT regions, or those based solely on faecal samples, can provide only a limited view on the outcome of interactions between the GIT microbiota and the host.

A few studies have analysed host vs. microbiota interactions using microbiota samples from multiple GIT regions [9,33,37]. However, as acquiring the corresponding samples was typically performed in a destructive way, this approach does not enable assessments of tempo-ral variation of the GIT microbiota based on longitudinal sampling of the same individual. Furthermore, destructive sampling might be ethically questionable in the case of research on protected wild species. Although biopsies taken from different GIT regions offer a non-destructive alternative [39–41], such an approach is methodically challenging, which limits its broader applicability. Therefore, an application of alternative sampling protocols that are non-destructive but allow sampling of multiple GIT regions is desirable.

Moreover, most current knowledge on microbial variation along the GIT relies on data from mammalian captive-bred models and humans [40–42]. Patterns of spatial variation of the microbiota along the GIT in wild populations and in non-mammalian taxa are still under-studied, however [9,31,32,43]. This sort of knowledge is crucial for understanding host vs. microbiota interactions, since previous studies, mostly based on samples from single GIT regions, have revealed that the taxonomic and functional content of the GIT microbiota varies considerably between captive-bred and wild populations of the same species [44–47], as well as between mammals vs. other vertebrates [28,30,48,49]. Consequently, approaches based on sampling of multiple GIT regions are essential for understanding host vs. microbiota interac-tions in wild populations, including resulting effects on the host's fitness.

Here we applied high-throughput amplicon sequencing of bacterial 16S rRNA to study the variation in microbiota among samples of proximal and distal parts of the GIT (repre-sented by oral swabs and faecal samples) noninvasively collected in a free-living passerine bird.

According to research on mammals, microbial communities of both the proximal and distal GIT are shaped to a large extent by host-intrinsic regulatory mechanisms, while the effect of environmental bacteria on the composition of these communities is usually limited [43,50]. At the same time, however, host-specific factors affecting microbial populations differ between the proximal and distal GIT in mammals. Diet composition, infection by intestinal parasites and genetic factors are crucial factors leading to lower GIT microbiota variation [51–53]. On the other hand, specific properties of the saliva and gingival crevicular fluid, and to a lesser extent diet, have important effects on the proximal GIT microbiota [54]. Importantly, how-ever, distinct mechanisms seem to drive the variation of host-associated microbiota in mam-mals vs. non-mammalian vertebrates [28,30,48,55].

In this study, great tit (*Parus major*) was selected as a model species. The great tit is an emi-nent model species for the functionally and evolutionary oriented branches of ecological research [56,57]. Despite the current interest in the emerging topic of host vs. GIT microbiota interactions [58–61], there is still rather limited knowledge on these interactions in the great tit [62]. Importantly, no previous studies on the great tit have used culture-independent high-throughput sequencing to characterize the microbial communities associated with this host. In addition, to our knowledge, variation in the microbiota colonizing different parts of body of this species as well as other passerines has not yet been addressed. We compared the diversity, interindividual variation and taxonomic composition of the microbiota from the proximal vs. distal parts of the GIT. Finally, we tested if there was any correlation between the proximal and distal GIT microbiota at the within-individual level.

Materials and methods

Field sampling

Faecal and oral samples used in this study were collected from putatively unrelated adult individuals (n = 29) of the great tit population breeding in artificial nest boxes in the Dablicky haj forest (50˚08'12.4"N, 14˚27'57.2"E, Prague, Czech Republic). The sampling locality is covered by secondary deciduous forest with a minor admixture of coniferous trees. All samples were obtained within one week in mid-May 2014.

Collection of microbial samples was performed as follows: adult individuals were captured in mist nets and placed in clean paper bags for approx. 15–20 minutes. Samples of faeces were subsequently collected from the paper surface. The oral microbiota was sampled using sterile microbiological nylon swabs (minitip FLOQSwabs, Copan, Italy) by wiping the oral cavity and upper side of the beak. Both faecal and oral samples were immediately placed in sterile DNA/ RNA free cryotubes (Simport, Canada) filled with a self-made DNA/RNA-stabilising buffer on the basis of RNA later (protocol available upon request) and transferred to -80˚C within two days. The sex of sampled individuals was determined by external phenotypic traits (e.g. [63]). Data on the body mass and tarsus length were used for calculation of the scaled body mass index following Peig and Green [64]. Birds were then individually marked using aluminium rings following the regulations of the Czech Bird Ringing Centre and released.

All field procedures were approved by the ethical committee of the Czech Academy of Sci-ences (107/2009).

Microbial genotyping

Metagenomic DNA from faecal and oral samples was extracted in a laminar flow cabinet using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., USA). To optimise the efficiency of DNA isolation, samples were homogenised using a MagnaLyzer (Roche, Switzerland) for 30s at 6000rpm and extracted DNA was eluted in 50 μl of elution buffer. From 29 sampled

individuals, DNA isolated from oral and faecal samples was not of sufficient quantity in 9 and 12 cases, respectively, and therefore these samples were not included in further analyses. The final dataset thus included 17 faecal and 20 oral samples (S1 Table).

Following the recommendations of Klindworth et al., [65], primers covering the V3-V4 variable region of bacterial 16S rRNA (i.e. S-D-Bact-0341-b-S-17 [CCTACGGGNGGCWGCAG] and S-D-Bact-0785-a-A-21 [GACTACHVGGGTATCTAATCC]) were used during the PCR step. Both forward and reverse primers were tagged with 10bp barcodes designed by TagGD software [66]. For the polymerase chain reaction (PCR) we used 8 μl of KAPA HIFI Hot Start Ready Mix (Kapa Biosystems, USA), 0.37 μM of each primer and 7 μl of DNA template. PCR conditions were as follows: initial denaturation at 95˚C for 5 min, followed by 35 cycles each of 98˚C (20 sec), 61˚C (15 sec) and 72˚C (40 sec), and a final extension at 72˚C (5 min). For individual samples, we prepared technical PCR duplicates. The PCR products, together with negative controls (PCR products of blank DNA isolates), were run on a 1.5% agarose gel and concentration of the PCR product was assessed based on gel band intensity using GenoSoft software (VWR International, Belgium). Samples were subsequently pooled at equimolar concentration. As we did not observe any visible PCR products in negative controls, therefore this type of samples was not included into the final pool. The pooled samples then were run on another 1.5% agarose gel, with bands of appropriate size excised from the gel and purified using the High Pure PCR product Purification Kit (Roche, Switzerland) according to the manufacturer's instructions. Sequencing adaptors were ligated using TruSeq nano DNA library preparation kits (Illumina, USA) and the resulting amplicon libraries sequenced on a single Miseq run (Illumina, USA) using v3 chemistry and 2 × 300 bp paired-end reads. Raw sequencing data are avialable at http://www.ebi.ac.uk/ena/data/view/PRJEB19204 and sample metadata in S1 Table.

Bioinformatic processing of 16S rRNA data

Paired-end Illumina reads were merged using PEAR [67], and de-mutiplexed using mothur [68] and custom R/Bioconductor scripts (available from the authors on request). We then used the Lotus pipeline [69] for quality filtering of the FASTQ files. Sequences were excluded if the average quality score was lower than 30 or if the average quality score within a 50 bp sliding window decreased below 25. UCHIME (implemented in the Lotus pipeline) [70] was used alongside the gold.fna database (available at http://sourceforge.net/projects/microbiomeutil/files) for the detection and elimination of chimeric sequences. The resulting 16S rRNA sequences were clustered at a 97% similarity threshold using UPARSE [71] in order to define Operational Taxonomic Units (OTU). Taxonomic assignation of representative sequences for each OTU was performed using the RDP classifier [72] and the GreenGenes reference database, (version gg_13_5) [73]. Representative sequences were further aligned using PyNAST [74], the maximum likelihood tree being constructed using FastTree [75]. We observed a considerable excess of chloroplast sequences in our dataset (17.7%). Chloroplast OTUs together with OTUs that were not assigned to any bacterial phylum were considered as diet contaminants or sequencing artefacts, respectively, and we excluded them from all downstream analyses. The resulting OTU tables, sample metadata, OTU tree and taxonomic annotations for individual OTUs were merged into a phyloseq object [76] for statistical analysis in R version 3.2.3 [77].

Statistical analyses

In order to account for uneven sequencing depth among samples, statistical analyses were calculated based on the rarefied OTU table unless otherwise stated. The number of observed OTUs, Shannon diversity and Chao1 based predictions of total microbial diversity for

individual samples were calculated using phyloseq [76]. Linear Mixed Effect models (LME, package lme4) [78] were used to test differences in diversity between faecal vs. oral microbiota. To account for statistical nonindependence, the effects of an individual were included as a ran-dom intercept. In addition, analysis of variance (ANOVA), running separately on samples from each GIT region, was applied to test differences in microbial alpha diversity between males vs. females and due to scaled body mass index.

We further used Principal Coordinate Analysis (PCoA) based on Bray-Curtis, Jaccard, weighted and unweighted UniFrac [79] distances between samples to visualize the contrast in the composition between faecal and oral microbiota. Adonis (i.e. analysis of variance based on distance matrices) was applied to assess the statistical significance and proportion of variance explained by the contrast in microbial composition between faecal and oral samples. Individ-ual identity was included as a constraint for permutations (i.e. "strata") in adonis models to account for data nonindependence. Betadisper was further applied to test for the difference in interindividual variation of microbial composition between the two GIT regions. The effects of sex and scaled body mass index were assessed via adonis analyses running separately on fae-cal and oral samples. For individuals where both oral and faecal microbiota were analysed

(n = 8), we used Pearson correlations to assess if there was any interrelationship in microbial alpha diversity between the two GIT regions. Next, within-individual correlations of the microbial composition between oral vs. faecal samples was assessed via Mantel's test. Finally, using Spearman's correlations, we tested if relative abundances of OTUs were correlated between the two GIT regions. This analysis was run on a subset of 216 OTUs that were present both in the oral and faecal microbiota of those individuals for which both these samples were available.

The LME-based approach was further used to identify OTUs whose abundances differed between oral and faecal samples. These analyses were performed on a subset of 240 OTUs (comprising 90.5% of all high-quality reads) that were detected in at least five samples. For each OTU-specific LME, Box-Cox transformed read counts were used as a response, whereas the effect of GIT regions and individual identity were included as the explanatory variable and random intercept, respectively. In addition, Box-Cox transformed total number of reads per individual samples was included as an offset in LMEs (i.e. assuming its direct relationship with the number of reads per tested OTUs in individual samples). To account for deviance from a Gaussian error distribution, the significance of the GIT region effect was assessed based on permutations. In particular, observed deviance changes due to the elimination of the GIT re-gion effect for the initial model were compared with the null distribution of deviance changes extracted from LMEs, where both the number of total and OTU-specific read counts were ran-domly resampled (10 000 permutations). The Qvalue method [80] was used to account for false discoveries due to multiple testing. The effect of a given OTU was considered to be signif-icant if the permutation-based p value and associated qvalue were lower than 0.05. The abun-dance pattern of OTUs that were overrepresented in the oral cavity or faecal samples was visualized using a heatmap (function aheatmap from R package NMF).

Results

Our dataset included 207 497 high-quality reads that were clustered into 1127 non-chloroplast OTUs. There was a significant decrease of alpha diversity in faecal compared to oral micro-biota according to the observed number of OTUs and Shannon index, as well as according to Chao1 (Fig 1, Table 1). Only 384 (34%) OTUs were detected in both the oral and faecal micro-biota, whereas 541 (48%) and 202 (18%) OTUs were detected exclusively in oral and faecal samples, respectively. Clear differences in the composition of oral vs. faecal microbiota were

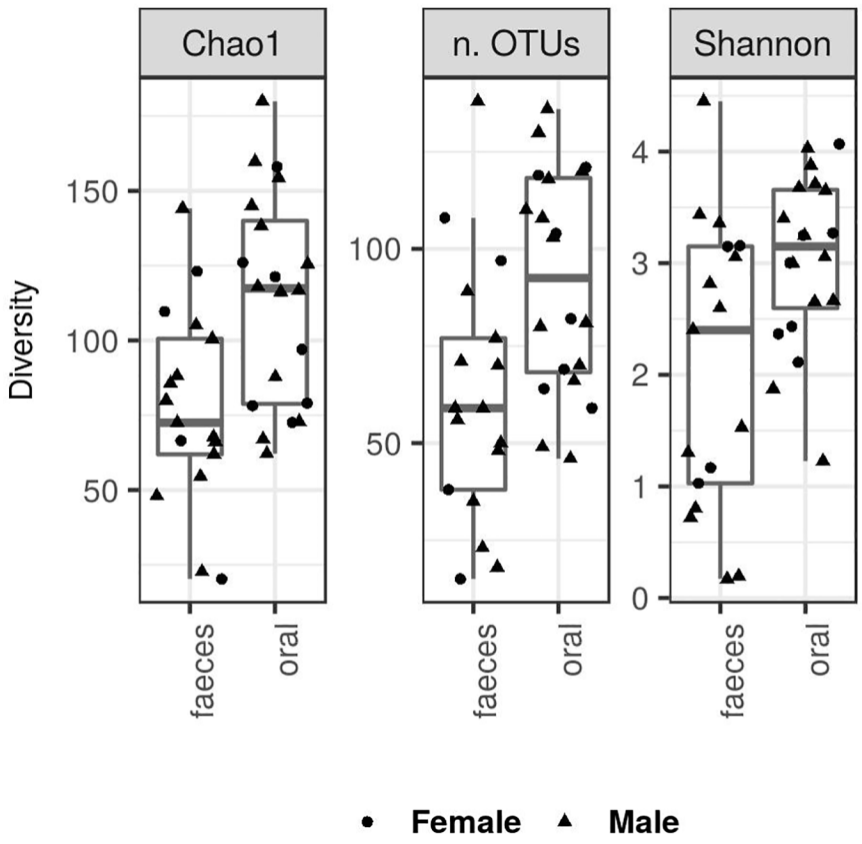

Fig 1. Diversity of the faecal and oral microbiota of the great tit. Alpha diversity was measured as Chao1, number of observed OTUs and Shannon diversity. To account for uneven sequencing depths, alpha diversities w ere calculated based on rarefied OTU tables.

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also revealed based on PCoA (Fig 2) and the associated adonis analyses (Table 2). In addition, the interindividual variation in the microbial composition, as assessed by betadisper analyses, w as low er in oral compared to faecal samples for all types of community dissimilarities, but no significant difference w as found in the unw eighted UniFrac (Table 2). In line w ith these results, plots visualising the taxonomic composition on the Phylum and Class levels indicated differentiation in the microbial composition betw een faecal and oral samples as w ell as a higher interindividual variation of faecal microbiota (Fig 3, S2 Table). Gammaproteobacteria (genera *Diplorickettsia*, *Pseudomonas*, *Erwinia*, *Escherichia/Shigella*, *Serratia* and *Acinetobac-ter*), Alphaproteobacteria (genera *Methylobacterium*, *Rickettsia* and *Sphingomonas*) and Actinobacteria (genera *Corynebacterium* and *Pseudonocardia*) w ere the dominating bacterial

Table 1. Diversity of the faecal and oral microbiota of the great tit.

	Oral mean ± SE	Fecal mean ± SE	χ2	P
Chao1	119.0186 ± 10.0396	78.4456 ± 9.1118	8.21050	0.00416
Observed	92.2500 ± 6.6153	62.2353 ± 8.1274	7.93768	0.00484
Shannon	3.0168 ± 0.1724	2.0839 ± 0.3136	7.05950	0.00788

Alpha diversity w as measured as Chao1, number of observed OTUs and Shannon diversity. Significance w as assessed based on LME. Mean ± SE for individual sample groups, LME based likelihood-ratio statistic associated probability values are shown.

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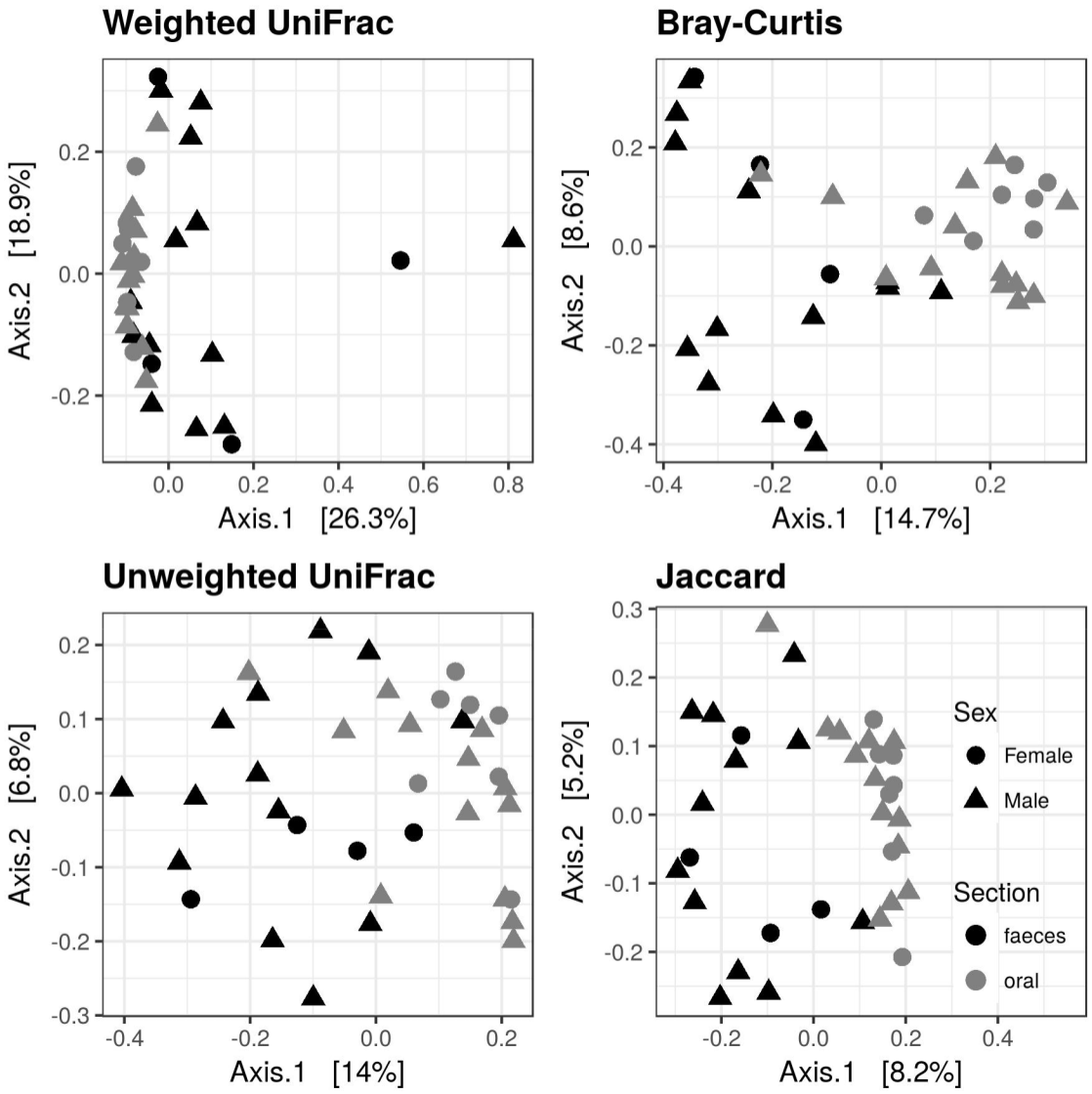

Fig 2. Differences in the composition between the oral and faecal microbiota of the great tit. PCoA w as performed for four dissimilarity indexes. Sex is indicated by different plotting symbols.

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classes of both the oral and faecal microbiota. How ever, the abundance of Bacilli (represented by genera *Staphylococcus* and *Lactobacillus*) and Betaproteobacteria (represented by genera *Methylobacillus*, *Comamonas* and *Herbaspirillum*) w as increased in oral compared to faecal samples. At the same time, several faecal samples exhibited high abundances of Mollicutes (represented by genera *Ureaplasma* and *Mycoplasma*), Clostridia and Chlamydia, i.e. bacterial classes that w ere detected in very low abundances in oral samples.

OTU-level analyses identified 33 OTUs (represented by 35.8% reads) w hose relative abundances differed betw een oral and faecal samples (Fig 4). OTUs corresponding to the genera *Ureaplasma* (phylum Tenericutes), *Delftia* (phylum Proteobacteria), *Carnobacterium* (phylum Firmicutes), *Deinococcus* (phylum Deinococcus-Thermus), *Chryseobacterium* and *Elizabeth-kingia* (both phylum Bacteroidetes) w ere more abundant in faecal samples. On the other hand, OTUs w here the most striking relative abundance increase in oral compared to faecal samples

Table 2. Differences in the composition between the oral and faecal microbiota of the great tit.

			composition				interindividual variation		
		Df	Mean Sum Sq.	F	R^2	p	Mean Sum Sq.	F	p
Weighted UniFrac	GIT region	1	0.47575	4.09496	0.10474	0.00781	0.24614	20.64496	0.00006
	Residuals	35	0.11618				0.01192		
Unweighed UniFrac	GIT region	1	0.78810	3.58368	0.09288	0.00781	0.00204	0.92960	0.34158
	Residuals1	35	0.21991				0.00220		
Bray Curtis	GIT region	1	1.51171	4.31907	0.10985	0.00391	0.09235	14.12805	0.00062
	Residuals2	35	0.35001				0.00654		
Jaccard	GIT region	1	0.92409	2.56352	0.06825	0.00391	0.00884	9.14144	0.00465
	Residuals3	35	0.36048				0.00097		

Differences in composition w ere analysed using adonis, w hereas differences in interindividual variation w ere assessed using betadisper. Both analyses w ere performed on four types of dissimilarity indexes.

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was observed belonged to the genera *Pseudomonas*, *Acinetobacter*, *Methylobacillus*, *Herbaspiril-lum* (all from phylum Proteobacteria), *Catellicoccus*, *Staphylococcus*, *Tumebacillus* (phylum Firmicutes) and to *Arthrobacter*, *Brevibacterium* and the families Intrasporangiaceae and Nocardioidaceae (phylum Actinobacteria).

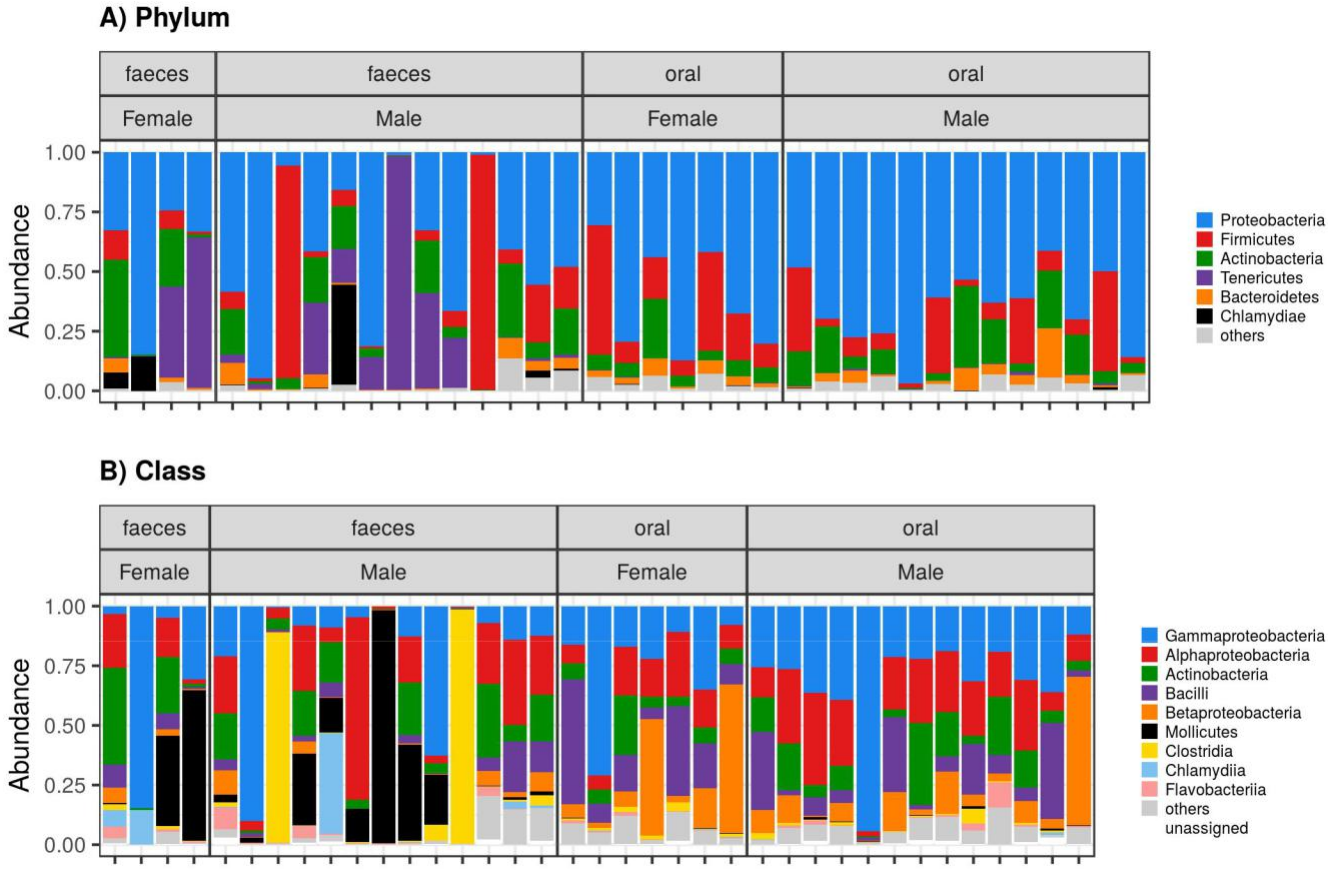

Fig 3. Barplots indicating oral and faecal microbiota composition of the great tit. Proportions of bacterial (*a*) phyla and (*b*) classes are shown.

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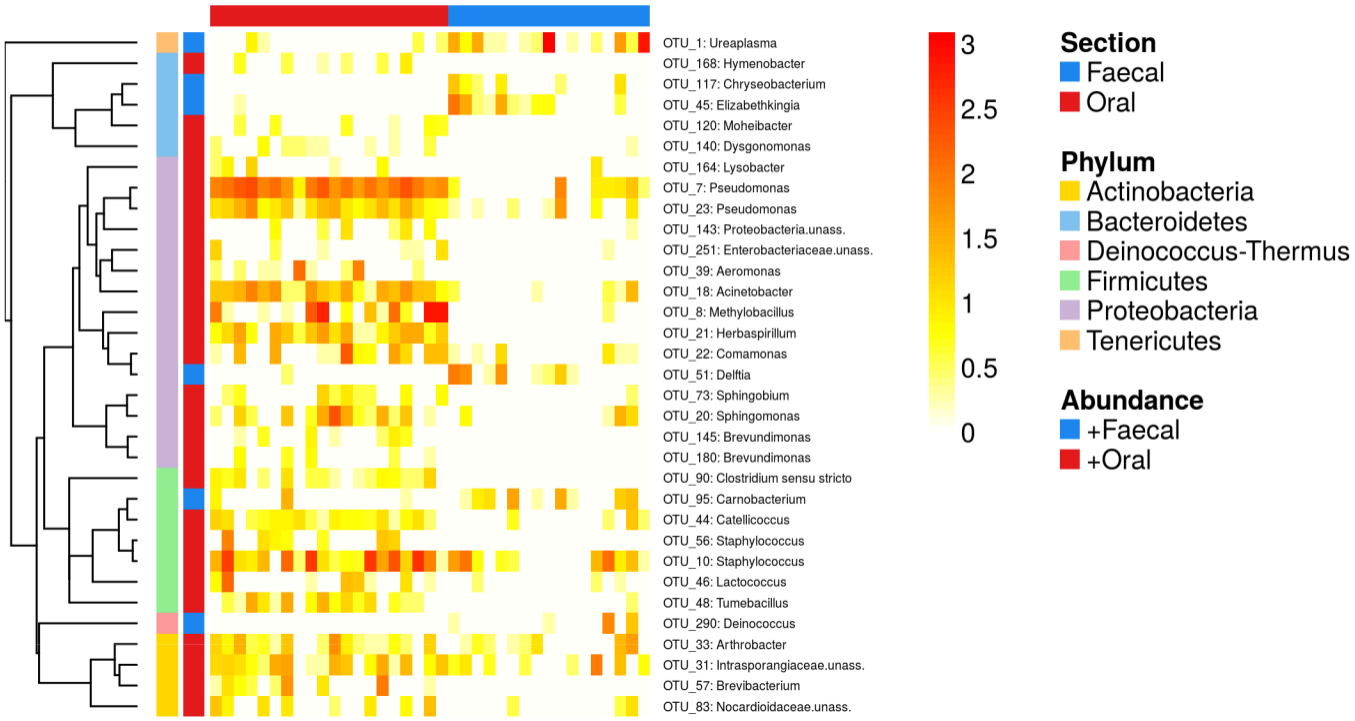

Fig 4. Heatmap for OTUs, whose abundance varied between the oral and faecal microbiota of the great tit. OTUs were identified according to permutations-based LMEs. Cell colours indicate OTU abundances in individual samples (log10 scaled values). Column annotations indicate GIT regions, whereas row annotations show Phylum-level assignations and if the OTU was overrepresented in oral or faecal samples (+oral or +faecal). OTUs are ordered according to their phylogeny (i.e. FastTree-based phylogeny for representative sequences).

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ANCOVA analyses did not reveal any effect of sex or scaled body mass index on the diversity of microbial communities associated with these two GIT regions ($p < 0.3$ in all cases). In addition, according to adonis analyses, there was no effect of these two variables on the composition of oral or faecal microbiota ($p > 0.2$, $R^2 < 0.02$ in all cases).

Analyses on the subset of individuals with both oral and faecal microbiota sampled did not reveal any correlation of the microbial structure between these two GIT regions at the within-individual level. First, alpha diversity estimates for these two GIT regions were not correlated (range of Pearson's $r = -0.22 \sim -0.26$, $p > 0.5$ for all types of diversity indexes). Furthermore, we did not detect any within-individual correlation in microbial composition between faecal and oral samples (Mantel test: $p > 0.4$, range of cor. coefs. = -0.25 ~ 0.19 for all four community dissimilarity indexes). Finally, the correlation of relative abundances of individual OTUs between oral swabs and faeces was negligible (mean of Spearman correlation coefficient = 0.05185, interquartile range = -0.21600 ~ 0.32870).

Discussion

Many previous studies that focused predominantly on captive bred mammals and humans have found pronounced differences in microbial structure between body sites as well as among different GIT compartments [37,81,82]. Our aim was to extend current knowledge on micro-bial divergence between GIT regions with data from the free-living population of a passerine bird, i.e. a taxonomic group that, to our knowledge, has not been studied in this context before. Consistent with previous research, we found pronounced differences between the proximal vs. distal GIT microbiota. According to our data, less than 30% of all OTUs were shared between

these two GIT compartments in the great tit. Differences in terms of OTU absence vs. presence reflected pronounced variation in the relative abundances of bacterial taxa that were detected in oral vs. faecal microbiota. Compared to faecal microbiota, oral samples were characterized by a higher proportion of the classes Bacilli (phylum Firmicutes) and Betaproteobacteria (phy-lum Proteobacteria). In addition, bacteria from the phyla Chlamydiae and Tenericutes as well as from the class Clostridia (phylum Firmicutes) were nearly absent in the great tit oral microbiota.

Altogether, the composition of the great tit faecal microbiota was comparable with previous studies on other passerine birds [29,30,60,83–86], where OTUs from the phyla Proteobacteria, Firmicutes and Actinobacteria represented the dominant components. On the other hand, knowledge on the taxonomic content of the oral microbiota in birds is currently very limited. However, our data indicate that there is pronounced variation between the great tit and other avian hosts. For example, a dominance of *Lactobacilli* was detected in a recent study on the quail (*Coturnix japonica*) [87], while this bacterial genus constituted only a low proportion of the oral microbiota in our population. Similarly, abundances of *Haemophilus* and *Streptococcus* that dominated the oral microbiota of the kakapo (*Strigops habroptilus*) [88] were low in the great tit. Compared to mammalian oral microbiota, where bacteria from the phyla Bacteroi-detes, Firmicutes and Proteobacteria typically dominate, the oral microbiota in our population was characterized by a low proportion of Bacteroidetes and increased abundances of Actino-bacteria [40,43,82,89]. In addition, the diversity of the oral microbiota was significantly increased compared to the faecal microbiota in our population. As studies on other vertebrate species commonly report both higher [1,40,43,90] and lower [82,91] values of alpha diversity in oral vs. faecal microbiota, further research should focus on factors driving this variation.

At the OTU level, the faecal microbiota was characterized by increased abundances of *Ureaplasma*, *Deinococcus*, *Carnobacterium*, *Chryseobacterium*, *Delftia* and *Elizabethkingia* OTUs. *Ureaplasma* together with another Tenericutes OTU corresponding to the genus *Mycoplasma* that tended to be increased in the faecal microbiota as well (qvalue ~ 0.08), are common inhab-itants of vertebrate gastrointestinal and urogenital tracts. Although these taxa are often asymp-tomatically present in birds, some of these species are involved in severe pathogenesis [92]. The *Deinococcus* OTU (phylum Deinococcus-Thermus) has previously been detected in sev-eral vertebrate species [85,93]; however, its effect on the host physiology is poorly known. The *Carnobacterium* OTU (phylum Firmicutes) is a lactic acid bacterium with putative probiotic properties providing protection against various bacterial pathogens [94,95]. On the other hand, *Chryseobacterium* and *Elizabethkingia* OTUs (both from the family Flavobacteriaceae) are related to several pathogenic species of human and other vertebrate taxa [96–98].

According to the OTU-level analyses, the oral microbiota was characterized by increased abundance of OTUs from genera that commonly colonize the oral cavity, skin or intestine of various vertebrate taxa (for example *Staphylococcus*, *Acinetobacter*, *Sphingomonas*, *Brevundi-monas*, *Dysgonomonas*, *Hymenobacter*, *Sphingobium*). Many OTUs exhibiting higher abun-dances in the oral cavity compared to faeces can be involved in interactions with their host's immune system and physiology, or can shape the community composition via interactions with other members of the oral microbiota. This applies, for example, to four Actinobacterial OTUs (from the genus *Arthrobacter*, *Brevibacterium* and the family Intrasporangiaceae, Nocardioidaceae). Actinobacteria produce a wide variety of bacteriocines and other com-pounds suppressing the proliferation of bacterial competitors. Therefore, their presence in the oral cavity could be crucial for the defence against bacterial pathogens as well as for the main-tenance of overall microbial structure [99]. In line with this possibility, *Arthrobacter* abun-dances in the lower intestine were positively associated with survival rates of passerine species closely related to the great tit [59]. Other bacteria associated with the oral cavity that may be

involved in interactions with invading pathogens and other community members included *Lysobacter*, *Pseudomonas* and *Herbaspirillum*. *Lysobacter* can shape microbial community through the production of bacteriocines and the active predation of other bacteria [100,101]. *Pseudomonas* and *Herbaspirillum* produce extracellular siderophores, i.e. iron chelating compounds, which provide them a competitive advantage over other bacteria by reducing the availability of iron in the environment [102]. In addition, *Pseudomonas* cells secrete exopolysaccharides that make them a difficult target for the host immune system [103], and some *Pseudomonas* species can be pathogenic for birds [104–106]. It is also worth noting the abundance increase of two lactic acid bacteria in the oral cavity, *Catellicoccus* and *Lactococcus*, which can shape the oral community structure by the modulation of abiotic environmental conditions or by direct interactions with host immune system or other community members [107,108].

Even though the sample size was limited, we did not observe any correlation between oral vs. faecal microbiota at the within-individual level. Consequently, we propose that microbial communities associated with the proximal and distal GIT are shaped by independent mechanisms. These can theoretically include (1) host-intrinsic mechanisms such as effects of the immune system and other biotic and abiotic factors operating within the GIT, or (2) extrinsic sources of variation including pools of bacteria present in the diet and other environmental sources.

Relatively low interindividual variation of oral microbiota suggests either that environmental bacterial pools colonizing the oral cavity exhibit high homogeneity in space and time, or that there is low interindividual variation in host-specific mechanisms regulating oral microbiota. As previous research has found only limited effects of environmental bacteria on oral microbiota in non-avian vertebrates [43,50], and as environmental microbial consortia typically exhibit high variation [109–111], we favor the latter explanation. High interindividual variation of faecal microbiota suggests that host-intrinsic factors driving its composition differ markedly among hosts. However, as the passage of food through the passerine gut is extremely fast [112,113] and thus the decomposition of bacteria from the external environment is probably not as effective as in mammals, we cannot exclude the possibility that the variation of faecal microbiota is also driven to certain extent by bacteria that get into the GIT with food. As knowledge of the factors driving within-species variability in the avian GIT microbiota remains limited [59,61,85], further research and specifically designed experiments are required to untangle the relative contribution of transient environmental bacteria to microbial compo-sition in different GIT regions of passerines and other vertebrate taxa.

In conclusion, our study is the first to characterize the oral microbial structure and compared it with the faecal microbiota in a free-living bird population. Our results show that the oral and faecal microbiota of passerines represent two distinct bacterial consortia that exhibit marked differences at all levels of community structure, and that the interindividual variation of these communities is likely to be shaped by independent mechanisms. We propose that aside from the effect of environmental bacteria, the structure of both the faecal and oral micro-biota is driven to a large extent by mutual interactions among community members or by the host vs. microbiota interactions including immunity. Consequently, given the putative effects of these two microbial communities on the host's heath status, further research focusing on the microbiota in wild vertebrate populations may benefit from simultaneous sampling of these two communities.

Supporting information

S1 Table. Detail listing of great tit samples including the GIT region (faecal or oral), indi-vidual identity (ID), accession number, sex and body weight.
(XLSX)

S2 Table. Taxonomic composition of the great tit GIT microbiota and its variation between oral and faecal samples.
(XLSX)

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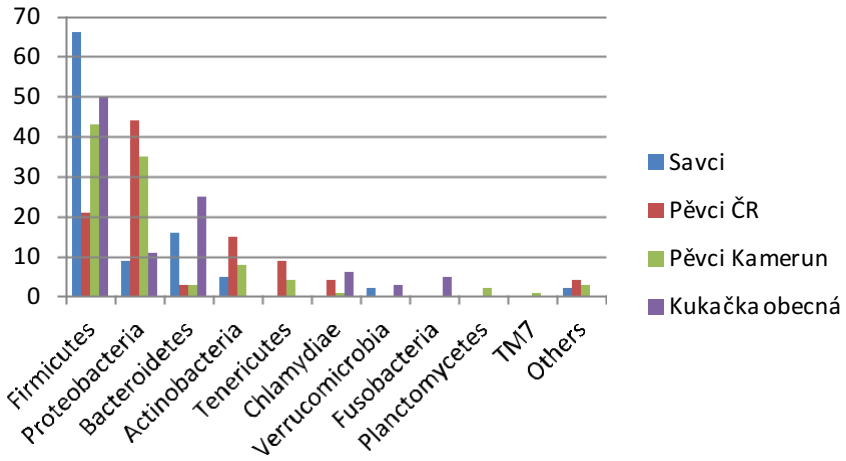
4. Výsledky a Diskuze

4.1. Taxonomické složení SM

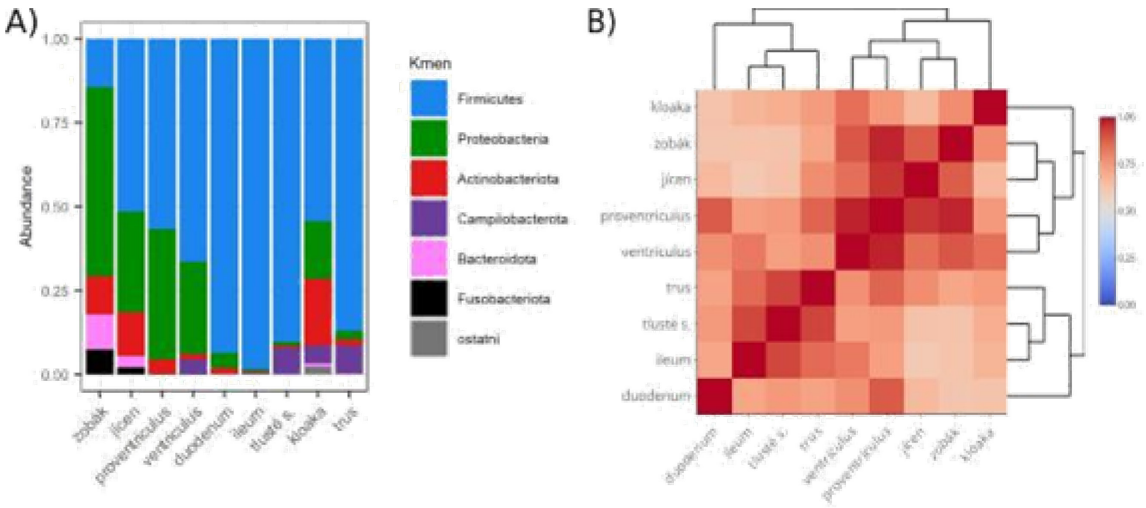
U pěvců v SM dominují bakteriální kmeny Proteobacteria, Firmicutes, Actinobacteria, Tenericutes, Bacteroidetes a Chlamydia **[Publikace A]**. Podobné výsledky byly získány ve většině mých publikací **[Publikace A–I].** Nicméně u mláďat kukačky obecné bylo složení SM mírně odlišné zejména kvůli většímu výskytu kmene Bacteroidetes **[Publikace H]**. Kukačka obecná patří do řádu kukaček (Cuculiformes), má dobře vyvinuté slepé střevo a je jediným druhem zahrnutým v této práci, který nepatří mezi pěvce. Obecně jsou výsledky složení SM u pěvců a kukačky v souladu i s ostatními publikacemi, které studovaly SM u ptáků (Hird *et al.*, 2015; Bodawatta *et al.*, 2018; Grond *et al.*, 2018, 2019). Potvrzuje se, že pěvčí SM je velmi odlišná ve srovnání s daleko více studovanými savci. Savci mají sice nejvíce zastoupený kmen Firmicutes, který je také hojný i u pěvců, ale i Bacteroidetes, který se u pěvců vyskytuje v daleko menší míře. Na druhou stranu u pěvců hojný kmen Proteobacteria je u savců reprezentován méně (Obrázek 2; Ley *et al.*, 2008) **[Publikace A]**. Tyto tři bakteriální kmeny Firmicutes, Proteobacteria a Bacteroidetes jsou v různé míře zastoupené i u ostatních skupin obratlovců, např. u plazů (Arizza *et al.*, 2019; Qin *et al.*, 2019; Tang *et al.*, 2019), obojživeníků (Shu *et al.*, 2019; Zhang *et al.*, 2019; Xu *et al.*, 2020) i u ryb (Singh *et al.*, 2019; Dulski, Kozłowski & Ciesielski, 2020; Gao *et al.*, 2020).



***Obrázek 2**. Průměrné podíly dominantních bakteriálnich kmenů (ukázány kmeny zastoupené víc jak v 1 % u dané skupiny) u savců (Ley et al., 2008), pěvců z ČR **[Publikace A]**, pěvců z Kamerunu během období dešťů **[Publikace B]** a kukačky obecné **[Publikace H]**.*

4.2. Srovnání mikrobioty napříč trávicím traktem

U pěvců se zdá, že nejsou tak velké rozdíly mezi jednotlivými částmi střeva (Bodawatta *et al.*, 2018, 2020) jako u jiných druhů ptáků (Grond, Guilani & Hird, 2020b) či savců (Suzuki & Nachman, 2016; Bendová *et al.*, 2020), což by mohlo být vysvětleno například absencí dobře vyvinutého slepého střeva u pěvců. U slavíka obecného a tmavého jsme detekovali signifikantní rozdíly mezi jednotlivými částmi tenkého střeva, ale tyto rozdíly vysvětlovaly pouze malou část variability mezi vzorky **[Publikace C]**, což je v souladu s výsledky předešlých studií (Bodawatta *et al.*, 2018, 2020). Mé doposud nepublikované výsledky u papoušků (řád Psittaciformes), kteří mají, obdobně jako pěvci, pouze rudimentární slepá střeva, ukazují nejen malé rozdíly mezi jednotlivými částmi střeva, ale také velmi podobnou mikrobiotu mezi vzorky zejména tlustého střeva a trusu, zatímco kloakální výtěry se od vzorků střev lišily podstatně více než trus (Schmiedová *et al.*, nepublikované výsledky, Obrázek 3). Tyto výsledky ukazují, že trus lépe odráží složení mikrobioty ve střevech než kloakální výtěry a je tedy lepším materiálem využitelným při neinvazivních metodách studia SM u volně žijících ptáků. U kukačky obecné jsme pozorovali odlišnou mikrobiotu mezi trusem a zastrašujícím sekretem, který pravděpodobně pochází ze slepého střeva **[Publikace H]**, což je konzistentní s výsledky u jiných druhů ptáků s vyvinutým slepým střevem (Grond *et al.*, 2020b). Dále jsme nalezli rozdíly ve složení mikrobioty mezi proximální (výtěry zobáku) a distální (vzorky trusu) částí trávicího traktu sýkory koňadry **[Publikace I]**. Odlišnosti mezi proximální a distální částí trávicího traktu nejsou překvapivé vzhledem k rozdílným podmínkám pro život bakterií v těchto částech. Velké rozdíly ve složení mikrobioty mezi proximální a distální částí trávicího traktu byly detekovány i u jiných druhů ptáků (Grond *et al.*, 2020b; Schmiedová *et al.*, nepublikované výsledky, Obrázek 3). V rámci tenkého střeva slavíků byla pozorována korelace mezi vzorky z různých jeho částí od stejného jedince **[Publikace C]**, což naznačuje homogenitu SM napříč tenkým střevem. Překvapivě u kukačky nebyla mezi vzorky trusu a zastrašujícím sekretem nalezena korelace ve složení mikrobioty **[Publikace H]**. Zatímco u sýkory koňadry jsme nalezli pouze velmi slabou korelaci mezi mikrobiotou z výtěru zobáku a mikrobiotou v trusu v rámci jedince **[Publikace I].** To ukazuje, že v různých částech trávicího traktu se podmínky pro život bakterií liší, což způsobuje rozdílné složení mikrobioty napříč trávicím traktem a variabilitu mikrobioty v rámci jedince.



***Obrázek 3**. A) Průměrné podíly dominantních bakteriálních kmenů („ostatní" jsou kmeny s abundancí menší než 1 %). B) Heatmapa párových Prokrustových korelací klastrovaných podle algoritmu „average linkage", který počítá průměrné vzdálenosti mezi klastry. V obrázcích je devět různých typů vzorků od andulky vlnkované (Melopsittacus undulatus): výtěr zobáku (zobák), jícen, žlaznatý žaludek (proventriculus), svalnatý žaludek (ventriculus), dvě části tenkého střeva dvanáctník (duodeum) a kyčelník (ileum), tlusté střevo (tlusté s.), kloakální výtěr (kloaka) a trus. (Schmiedová et al., nepublikované výsledky)*

4.3. Vliv klimatické zóny na SM

Napříč širokou škálou taxonů byl popsán pokles diverzity s rostoucí zeměpisnou šířkou (McCoy & Connor, 1980, ale Owen & Owen, 1974), včetně parazitů a patogenů asociovaných se zvířecími hostiteli (Guernier, Hochberg & Guégan, 2004). Diverzita SM byla dosud studovaná pouze velmi málo, přičemž relevantní data jsou jenom z výzkumu lidské populace (De Filippo *et al.*, 2010; Lee *et al.*, 2014; Suzuki & Worobey, 2014; Yatsunenko *et al.*, 2012; but Suzuki *et al.*, 2020). Zatímco u lidí byla pozorována větší alfa diverzita SM v tropech (De Filippo *et al.*, 2010; Lee *et al.*, 2014), tak u pěvců jsme nenašli podporu pro větší alfa diverzitu v tropech ve srovnání s temperátem **[Publikace B]**. U pěvců v tropech jsme pozorovali vysokou variabilitu ve složení SM mezi obdobím sucha a obdobím dešťů. Jedinci z temperátu se více lišili ve složení SM od jedinců odebíraných během tropického období dešťů než od jedinců odebíraných během tropického období sucha. Například kmen Firmicutes byl více zastoupený v tropech pouze během období dešťů, zatímco v období sucha byl jeho podíl srovnatelný s mírným pásem **[Publikace B]**. V lidské populaci byla pozorována pozitivní korelace kmene Firmicutes se zeměpisnou šířkou (Suzuki & Worobey, 2014) a podobný trend, i když nesignifikantní, byl pozorován i u volně žijících myší (Suzuki *et al.*, 2020). U lidí a

laboratorních myší byl nárůst Firmicutes a odpovídající pokles Bacteroidetes spojován se zvýšenou tvorbou krátkých mastných kyselin (short chain fatty acids, SCFA) a potažmo i energetickými zdroji, které je hostitel schopný využívat (Turnbaugh *et al.*, 2006; Jumpertz *et al.*, 2011). U lidí se zdá, že za změnami v alfa diverzitě i ve složení SM stojí rozdílný životní styl mezi rozvojovými a vyspělými zeměmi. Konkrétně vědci z vyspělých zemí, kteří po dobu 3–6 měsíců žili ve Středoafrické republice a přizpůsobili svůj životní styl tradičním zemědělcům, měli poté SM více podobnou místním populacím tradičních zemědělců i lovcům a sběračům než lidem z USA (Gomez *et al.*, 2019).

4.4. Vliv migrace na složení SM

Migrační chování souvisí u ptáků s komplexními změnami ekologických a fyziologických znaků. Například během migrace dochází ke změnám ve velikosti některých orgánů a svalů (Piersma, Gudmundsson & Lilliendahl, 1999; Schwilch *et al.*, 2002). Také během migrace dochází ke zhoršení imunity a kondice, což může vést k větší náchylnosti vůči nemocem a parazitárním infekcím (Owen & Moore, 2008). S ohledem na plasticitu SM a její tendenci se přizpůsobit novým ekologickým nikám a fyziologickým stavům hostitele (Suzuki & Worobey, 2014; Gomez *et al.*, 2019; Suzuki *et al.*, 2020) lze očekávat vliv migrace na její složení. Konkrétně bylo pozorováno u dvou poddruhů vlaštovky obecné, které se liší migračním chováním (jeden poddruh je rezident *H. r. transitiva*, druhý poddruh je migrant *H. r. rustica* a vyskytují se na stejném území ve stejný čas), odlišné složení SM (Turjeman *et al.*, 2020). Také u jespáka rudokrkého (*Calidris ruficollis*) byly pozorovány rozdíly mezi migrujícími jedinci a rezidenty (Risely *et al.*, 2017, 2018) a tento rozdíl se snižoval s dobou strávenou na hnízdišti (Risely *et al.*, 2018). U několika druhů ptáků byly pozorovány nejenom změny SM během migrace, ale také proměny SM mezi jarní a podzimní migrací či zimovištěm (Lewis, Moore & Wang, 2016a, 2016b; Wu *et al.*, 2018; Zhang *et al.*, 2020). Všechny tyto výsledky potvrzují fluktuaci SM v souvislosti s migrací a zdá se tedy, že je SM v krátkodobém časovém horizontu ovlivněna prostředím, ve kterém se jedinec aktuálně vyskytuje.

U pěvců z mírného pásu jsme se zajímali o rozdíl SM mezi druhy migrujícími během zimního období do tropických oblastí Afriky (trans-saharský migrant) a rezidentními druhy (zůstávajícími po celý rok na jednom místě) nebo těmi, kteří migrují pouze na krátkou vzdálenost (maximálně do Středomoří). Kvůli vystavení různému prostředí během migrace jsem očekávala vyšší alfa diverzitu u trans-saharských migranů než

u ostatních temperátních druhů. Tato predikce se však nepotvrdila, protože jsme pozorovali podobnou alfa diverzitu u všech temperátních druhů **[Publikace B]**. Vzhledem k předchozím studiím jsme očekávali, že během hnízdního období se trans-saharští migranti nebudou ve složení SM lišit od rezidentů a nebo migrantů na krátkou vzdálenost. Na získaných datech jsme pozorovali rozdíly ve složení SM mezi trans-saharskými migranty a rezidenty společně s migranty do Středomoří. Zároveň se však složení SM trans-saharských migrantů lišilo i od tropických druhů z obou sezon **[Publikace B]**.

Navíc u dvou druhů trans-saharských migrantů, pěnice slavíkové (*Sylvia borin*) a budníčka většího (*Phylloscopus trochilus*), se složení SM signifikantně lišilo mezi hnízdištěm a zimovištěm, přičemž tyto změny víceméně odrážely změny ve složení SM pozorované i mezi ostatními temperátními druhy pěvců a tropickými pěvci z období sucha **[Publikace B]**. Tyto výsledky jsou v souladu se studií sledující změny SM u vědců z vyspělých zemí pobývajících dlouhodobě v Africe (Gomez *et al.*, 2019). Stejně jako naše výsledky i předchozí studie detekovaly fluktuaci SM v souvislosti s migrací (Lewis *et al.*, 2016a, 2016b; Wu *et al.*, 2018; Zhang *et al.*, 2020). Tyto pozorované změny by mohly být způsobeny osídlením střeva bakteriemi z prostředí, ve kterém se jedinec aktuálně vyskytuje. K takovému osídlení může docházet během migrace či krátce po příletu na zimoviště či hnízdiště.

4.5. Vliv geografické vzdálenosti na SM

Naše výsledky na datovém souboru pěvců z mírného pásu ukazují na jistou míru korelace mezi rozdíly ve složení SM a geografickou vzdáleností mezi lokalitami **[Publikace A]**. Podobně i na vnitrodruhové úrovni mezi populacemi vlaštovky obecné jsme zjistili signifikantní, avšak slabé rozdíly mezi vzorkovanými lokalitami **[Publikace D].** I na základě dalších studií se ukazuje, že prostorová segregace hraje u ptáků určitou roli, nicméně její význam se liší mezi jednotlivými studiemi. Zatímco naše publikace zaznamenaly pouze menší význam prostorové segregace, což je v souladu s výsledky dalších studií (Hird *et al.*, 2015; Loo *et al.*, 2019a; Capunitan *et al.*, 2020; Bodawatta *et al.*, 2021b), výsledky jiných publikací pokládají prostorovou segregaci za nejvýznamnější testovaný faktor (Hird *et al.*, 2014; Gillingham *et al.*, 2019; Grond *et al.*, 2019). Podobně rozporuplné výsledky byly zaznamenány i u savců. Ve většině publikací byla prostorová segregace důležitým faktorem (Grieneisen *et al.*, 2019; Gaona *et al.*, 2020; Grond *et al.*, 2020a), ale v jiné publikaci nebyl efekt prostorové segregace pozorován (Baxter *et al.*,

2015). Rozdílné výsledky mohou být důsledkem odlišného geografického měřítka, na kterém výzkum probíhal. V některých studiích byly sledované lokality od sebe vzdálené jen několik kilometrů a v jiných se tato vzdálenost pohybovala v řádech stovek až tisíců kilometrů. Rozdílné měřítko na geografické škále může hrát roli například v tom, zda se studované populace/jedinci mohou potkávat či jsou izolované, a tedy si nemohou SM předávat. Není také jasné, do jaké míry hraje roli samotná prostorová izolovanost a do jaké míry jsou pozorované efekty spojené s dalšími faktory, které se v prostou mění jako např. rozdílnost obývaného prostředí. Například u pěvců byl pozorován rozdíl ve SM mezi jedinci z města a venkova (Phillips, Berlow & Derryberry, 2018; Teyssier *et al.*, 2018b). Naše výsledky ukazují, že i při poměrně velké vzdálenosti mezi studovanými populacemi z tropů a temperátu (okolo 5000 km) se může SM do určité míry překrývat **[Publikace B]**, a proto pravděpodobně geografická vzdálenost není nejdůležitějším faktorem.

4.6. Vliv ekologických faktorů na SM

U pěvců jsme na mezidruhové úrovni nepozorovali signifikantní vliv ekologických znaků a životních strategií na složení SM, které zahrnovaly i složení potravy **[Publikace A]**. V jiné studii provedené na ptácích pozorovali rovněž pouze slabý efekt některých ekologických faktorů (Hird *et al.*, 2015). Zajímavé je, že u tropických pěvců byla pozorována korelace mezi potravou a SM, která ale nebyla pozorována u temperátních druhů **[Publikace B]**. Tento rozdíl mezi pěvci z tropů a z temperátu by mohl být důsledkem větší potravní specializace tropických pěvců (Wheelwright, 1988). Na rozdíl od ptačích studií je u savců ekologie hostitele, a zejména pak složení potravy, naprosto klíčová (Ley *et al.*, 2008; Muegge *et al.*, 2011; Youngblut *et al.*, 2019). I u ptáků byl pozorován vliv potravní strategie na SM (Bodawatta *et al.*, 2018; Loo *et al.*, 2019a, 2019b; Capunitan *et al.*, 2020; Murray *et al.*, 2020), i když v některých publikacích není tato souvislost zcela jednoznačná (Michel *et al.*, 2018). Vliv potravy na složení SM byl také detekován u jednotlivců pomocí metody stabilních izotopů, která odráží zdroje uhlíku a trofickou úroveň potravy, a tedy udává výživovou hodnotu potravy (Loo *et al.*, 2019a, 2019b; Murray *et al.*, 2020). Experimentálně bylo pozorováno, že se liší složení SM u vrabce domácího (*Passer domesticus*) podle toho, jestli byl krmen městským, nebo venkovským typem potravy (Teyssier *et al.*, 2020). Navíc experimentálně byly pozorovány změny SM se změnou potravy u sýkory koňadry (Davidson *et al.*, 2020;

Bodawatta *et al.*, 2021a). Z tohoto důvodu jsme se rozhodli zaměřit na potravu v rámci vnitrodruhové úrovně u vlaštovky obecné, kde jsme pozorovali signifikantní, i když pouze slabou korelaci mezi složením SM a potravou **[Publikace E].** V souhrnu tyto výsledky naznačují, že potrava vysvětluje určitou, i když relativně omezenou část variability složení SM u pěvců. Na rozdíl od ptáků byla u savců potrava často hlavním faktorem vysvětlujícím variabilitu SM (Ley *et al.*, 2008; Muegge *et al.*, 2011; Gomez *et al.*, 2019). Ve srovnání se savci, kde byla pozorována korelace potravy a SM, u ptáků tato korelace signifikantní nebyla (Song *et al.*, 2020).

4.7. Vliv fylogenetické příbuznosti a příslušnosti k druhu na SM

Z našich výsledků vyplývá, že podstatně větší vliv než ekologie a geografie má na složení SM u temperátních pěvců jejich fylogenetická příbuznost **[Publikace A]**. Efekt fylogeneze se potvrdil i v datovém souboru rozšířeném o tropické pěvce **[Publikace B]**. Signifikantní vliv fylogeneze by teoreticky mohl být důsledkem transgeneračního přenosu SM mezi rodiči a dalšími členy komunity na potomky po mnoho generací, včetně speciačních událostí. Ten může vést ke korelované evoluci a fylogenezi mezi SM a jejich hostitelem. Naše výsledky a další doposud nepublikované analýzy na tomto datasetu (Kubovčiak *et al.*, v recenzním řízení) však naznačují, že spíše než transgeneračním přenosem SM byl daný jev způsobený znaky hostitele, které korelují s jeho fylogenezí, avšak nemohly být do analýz zahrnuté v důsledku absence relevantních dat. Jedním z těchto kandidátních znaků, na které by se měl výzkum v budoucnu více zaměřit, může být například variabilita v imunitních genech, které regulují složení SM. Jako první jsme se také pokusili posoudit potenciální roli SM při speciaci u ptáků. Konkrétně jsme srovnávali SM ze sympatrie a alopatrie u dvou blízce příbuzných druhů slavíků (slavíka tmavého a obecného), kteří příležitostně v sekundární kontaktní zóně hybridizují. Souvislost SM při tvorbě reprodukční bariéry zde nebyla prokázána **[Publikace C]**. Pouze slabou korelaci mezi složením SM a fylogenetickou vzdáleností mezi hostiteli (tzv. efekt fylosymbiózy) pozorovali u 15 druhů jeřábů chovaných ve stejných podmínkách a krmených stejnou potravou (Trevelline *et al.*, 2020). U tropických pěvců z Nové Guiney nebyl prokázán efekt fylosymbiózy, ale byl zde prokázán vliv příslušnosti k druhu (Bodawatta *et al.*, 2021b). Ve většině ostatních publikací netestovali přímo efekt fylogeneze, ale pouze příslušnost k druhu (jeho taxonomickou identitu), která často

ovlivňovala složení SM (Hird *et al.*, 2015; García-Amado *et al.*, 2018; Laviad-Shitrit *et al.*, 2019), zatímco v několika dalších publikacích nebyla příslušnost k druhu signifikantní nebo byl tento efekt méně podstatný než například lokalita či potrava (Hird *et al.*, 2014; Michel *et al.*, 2018; Grond *et al.*, 2019; Loo *et al.*, 2019b). Při analýzách SM napříč obratlovci je potrava důležitá společně s fylogenezí (Youngblut *et al.*, 2019; Song *et al.*, 2020). Efekt fylogeneze byl ale slabší u ptáků ve srovnání se savci (Song *et al.*, 2020).

4.8. Srovnání vlivu vnitřních regulačních mechanismů, environmentálních faktorů a sociálních kontaktů na SM

Zde jsme si kladli otázku, jaký je relativní efekt vnitřních regulačních mechanismů specifických pro daný druh hostitele v porovnání s vlivem environmentálních faktorů. V tomto konkrétním případě jsme porovnávali SM mláďat dvou druhů rákosníků se SM mláďat kukačky obecné, která byla vychovávána stejnými rodičovskými druhy. Efekt regulačních mechanismů bylo tudíž možné vysledovat na základě porovnání různých druhů mláďat vystavených stejnému prostředí. Efekt prostředí, zahrnující mimo jiné i odlišnosti v potravě a sociální přenos SM od rodičů na potomky, jsme zjistili na základě porovnání SM u stejného druhu vychovávaného různými druhy pěstounských rodičů. Mláďata kukaček a rákosníků chovaná v hnízdech dvou druhů rákosníků se signifikantně lišila ve složení SM, přestože byla vychovávána stejným druhem rákosníka **[Publikace H]**. Podobně u mláďat kukačky chocholaté (*Clamator glandarius*) v hnízdě obývaném společně s mláďaty straky obecné (*Pica pica*) bylo pozorováno rozdílné složení SM mezi mláďaty zmíněných druhů (Ruiz-Rodríguez *et al.*, 2009a, 2009b, 2018; Lee *et al.*, 2020). Na základě těchto publikací se zdá, že v rámci studovaného modelového systému je SM více ovlivněna vnitřními regulačními mechanismy hostitele než environmentálními faktory. Jedním z vysvětlujících faktorů u hostitele by mohla být odlišná stavba trávicího traktu kukaček a pěvců.

U zastrašujícího sekretu kukačky a u trusu rákosníků byla mikrobiota ovlivněna druhem sociálního rodiče (rákosníka velkého či obecného), což ale neplatilo pro trus kukačky **[Publikace H]**. Tato skutečnost naznačuje, že vliv sociálních rodičů spolu s variabilitou hnízdního prostředí může mít různý efekt na mikrobiotu v jednotlivých částech trávicího traktu. Výsledky cross-fostering experimentů u sýkory koňadry a modřinky (*Cyanistes caeruleus*) naznačují efekt identity hnízda, ve kterém mládě vyrůstalo, na složení SM, což

by mohlo naopak podporovat význam environmentálních faktorů (Lucas & Heeb, 2005; Teyssier *et al.*, 2018a). Podobně na vnitrodruhové úrovni u vlaštovky obecné si jsou mláďata z jednoho hnízda ve složení SM podobnější v porovnání s ostatními mláďaty **[Publikace D]** (také Ambrosini *et al.*, 2019), ale zde nelze jednoznačně bez dalších cílených experimentů konstatovat, zda je rozhodující vliv genotypu nebo prostředí hnízda.

U lidí byl zaznamenán přenos bakterií od matky na potomka během porodu a kojení (Bäckhed *et al.*, 2015). U myší byl také pozorován transgenerační přenos SM (Rosshart *et al.*, 2017). U savců, kteří jsou většinou živorodí a svá mláďata kojí, jsou lepší podmínky pro transgenerační přenos od matky na potomky než u vejcorodých ptáků. Proto není překvapivé, že v naší studované populaci vlaštovky obecné byla detekovaná pouze slabá korelace mezi složením SM sociální matky a mláďat. Současně s tím ale nebyl nalezen žádný efekt otce **[Publikace D].** V případě obdobné studie zaměřené na variabilitu kloakální mikrobioty u italské populace vlaštovek nebyl detekován paternální ani maternální vliv na kloakální mikrobiotu mláďat v hnízdě (Ambrosini *et al.*, 2019). Z těchto výsledků lze usuzovat, že transgenerační přenos SM z rodičů na potomky výsledné složení SM u ptáků příliš neovlivňuje. U vlaštovky obecné byla pozorována podobnější SM u sociálních párů v porovnání s ostatními jedinci, a to nejenom v naší studované populaci **[Publikace G]**, ale i v již zmíněné italské populaci (Ambrosini *et al.*, 2019). Tyto výsledky naznačují vliv sociálních kontaktů a kopulací v rámci páru na SM u ptáků. I u savců byl detekován sociální přenos SM (Tung *et al.*, 2015; Moeller *et al.*, 2016), ale tento přenos nebyl stejný pro všechny druhy bakterií (Moudra *et al.*, 2021). Zdá se tedy, že sociální kontakt ovlivňuje složení SM u savců i u ptáků.

4.9. Změny SM během ontogeneze a stabilita SM v čase

U lidí probíhají dynamické změny SM během ontogeneze, které jsou spojené zejména s kojením a jeho ukončením (Bäckhed *et al.*, 2015; Stewart *et al.*, 2018). Překvapivě jsme u vlaštovky obecné nenalezli významné změny SM asociované se stářím mláďat **[Publikace D].** Byl zde pozorován slabý nárůst alfa diverzity se stářím mláďat, ale taxonomické složení se signifikantně neměnilo. V italské populaci nebyly zjištěny žádné signifikantní změny kloakální mikrobioty spojené s věkem mláďat, a to ani na úrovni alfa diverzity (Ambrosini *et al.*, 2019). Nekonzistentní výsledky u alfa diverzity by mohly být způsobeny jiným typem vzorku (kloakální výtěr vs. trus). U sýkory koňadry byly zjištěny rozdíly SM u mláďat mezi 8 a 15 dnem od vylíhnutí (Teyssier *et al.*, 2018a) a u vrabce

domácího byly nalezeny marginálně nesignifikantní změny SM v souvislosti s věkem mláďat (Kohl *et al.*, 2019). Co stojí za různými výsledky mezi zmíněnými publikacemi není jasné. Na druhou stranu jsme objevili signifikantně rozdílné složení SM mezi mláďaty a dospělci **[Publikace D]**, které nemohou být vysvětleny rozdíly v potravě, která je v naší populaci u mláďat a rodičů stejná **[Publikace E]**. Rozdíly u vlaštovky obecné mezi mláďaty v hnízdě a dospělci byly pozorovány také v italské populaci (Ambrosini *et al.*, 2019), avšak SM mláďat vylétlých z hnízda se již nelišila od SM dospělců (Musitelli *et al.*, 2018). Tyto výsledky naznačují, že SM mláďat se pravděpodobně mění s vylétnutím mláďat z hnízda a poté se více podobá SM dospělců. Vzhledem k výsledkům z italské populace (Musitelli *et al.*, 2018) se nezdá být pravděpodobné alternativní vysvětlení rozdílů mezi dospělci a mláďaty, které předpokládá, že dospělci mají SM ovlivněnou zimovištěm a migrační cestou, zatímco u mláďat je SM definována především hnízdním materiálem a kontaktem s rodiči v průběhu krmení. Toto alternativní vysvětlení se nezdá být pravděpodobné i s ohledem na výsledky naší studie zahrnující dva druhy trans-saharských migrantů, kteří měli odlišné složení SM na zimovišti a na hnízdišti **[Publikace B]**. I zde by mohly být pozorované rozdíly v dynamice SM během ontogeneze u savců a ptáků vysvětleny živorodostí vs. vejcorodostí a s tím spojenou následnou péčí, jako je kojení u savců.

Dále jsme u mláďat i dospělců vlaštovky obecné objevili sice signifikantní, i když s velmi malým efektem, stabilitu složení SM v čase v rámci jedince **[Publikace D]**. Na rozdíl od pěvců je SM u lidí v dospělosti stabilní i v horizontu několika let (Faith *et al.*, 2013). Naproti tomu u volně žijících savců je podobně jako u pěvců u dospělců obvykle pozorováno kolísání SM v čase (Baxter *et al.*, 2015; Aivelo, Laakkonen & Jernvall, 2016; Ren *et al.*, 2016), i když existují i výjimky (Stevenson, Buck & Duddleston, 2014). Výrazná variabilita SM v čase by mohla znamenat, že vliv SM na fenotyp hostitele v dlouhodobějším časovém horizontu je pouze omezený, nebo dokonce žádný. Zde bych vyzdvihla, že v případě dospělců vlaštovky obecné je sice celkové složení SM v čase málo stabilní, nicméně část SM se vyznačuje značnou stabilitou. Konkrétně se jedná o 17 taxonomických jednotek, které by fenotyp hostitele mohly ovlivňovat a být tak zajímavé i z hlediska dalšího výzkumu **[Publikace D]**. U ptáků i savců byly pozorovány signifikantní změny SM během sezony (Sommer *et al.*, 2016; Xiao *et al.*, 2019; Zhang *et al.*, 2020). Také u tropických ptáků se zdá, že je složení SM sezonně proměnlivé, protože v období dešťů bylo složení SM u pěvců signifikantně odlišné od složení SM během

období sucha **[Publikace B]**.

4.10. Souvislost SM s imunitním systémem a role SM při obraně proti predátorům

Vliv SM na fenotyp hostitele jsme zkoumali u mláďat vlaštovky obecné. Testovali jsme asociaci SM s intenzitou reakce imunitního systému na PHA (fytohemaglutinin) otokový kožní test. PHA test detekuje buňkami zprostředkovanou imunitu pomocí velikosti otoku (Goto *et al.*, 1978; Tella *et al.*, 2008; Vinkler *et al.*, 2010, 2012). Na úrovni celkového složení SM jsme pozorovali souvislost s intenzitou reakce na indikaci PHA a dále jsme nalezli 10 konkrétních taxonomických jednotek souvisejících s touto reakcí **[Publikace F]**. Také u kukačky chocholaté (*Clamator glandarius*) a straky obecné (*Pica pica*) bylo pozorováno propojení mezi SM a imunitní odpovědí (Ruiz-Rodríguez *et al.*, 2009b). U kachen (rodu *Anas*) byla pozorována souvislost mezi složením SM a přítomností či nepřítomností viru chřipky (influenza A virus; Ganz *et al.*, 2017; Hird *et al.*, 2018). Bohužel se ze zjištěných výsledků nedá jednoznačně říct, zda je SM ovlivněna přítomností viru chřipky, či SM ovlivňuje náchylnost na nakažení virem chřipky, nebo zda platí oboje. Pro rozklíčování tohoto stavu by byl ideální odběr vzorků od stejných jedinců v delším časovém horizontu či experimentální infekce virem chřipky, na což by se mohl další výzkum zaměřit. Navíc složení kloakální mikrobioty u vlaštovky obecné mělo vliv na přežívání do další sezony (Ambrosini *et al.*, 2019). Z těchto výsledků se dá usuzovat, že složení SM by mohlo hrát roli při reakci imunitního systému a přežívání hostitele.

V zastrašujícím sekretu kukačky obecné, který může poskytnout ochranu proti čichem se orientujícím predátorům, jsme pozorovali vyšší podíl bakteriálních rodů spojovaných s produkcí volatilních (těkavých) molekul **[Publikace H]**, které byly již dříve v zastrašujícím sekretu detekovány (Röder *et al.*, 2016). Tyto výsledky ukazují na příspěvek mikrobioty v rámci evoluce chemických antipredačních mechanismů u mláďat kukačky. Také u pěvců v sekretu z uropygiální žlázy byly pozorovány bakterie produkující volatilní látky související s chemickou signalizací (Whittaker & Theis, 2016; Whittaker *et al.*, 2019). Přispěvek mikrobioty k tvorbě chemických signálů byl také zjištěn u hyeny žíhané a skvrnité (*Hyaena hyaena* a *Crocuta crocuta*; Theis *et al.*, 2013), surikat (*Suricata suricatta*; Leclaire *et al.*, 2017) a bengálské kočky (*Felis catus* ×

Prionailurus bengalensis; Yamaguchi *et al*., 2019). U obratlovců se zdá, že symbiotické bakterie napomáhají tvorbě chemických signálů, nicméně i zde je mnoho prostoru pro další výzkum.

5. Závěr

Většina variability SM u pěvců zůstává stále nevysvětlená i přes celou řadu výše popsaných a testovaných parametrů **[Publikace A–I]**. Stejně tak tomu je i v případě ostatních publikací uskutečněných na ptácích (Ambrosini *et al.*, 2019; Grond *et al.*, 2019; Laviad-Shitrit *et al.*, 2019). Podle komparativní studie, která zahrnuje savce i ptáky, fylogeneze u ptáků vysvětluje méně variability než fylogeneze u savců a zároveň je variabilita složení SM u ptáků vyšší než u savců (Song *et al.*, 2020). Zajímavé je pozorování konvergence mezi ptačí a netopýří SM (Song *et al.*, 2020), která by mohla být spojena s adaptacemi k letu. Létající ptáci i savci mají například redukovanou délku střeva, kratší retenční dobu, po kterou prochází potrava trávicím traktem a mají lepší střevní paracelulární absorpci (což znamená, že více živin je vstřebáváno hostitelem, a tedy to může snižovat důležitost symbiotických bakterií, Caviedes-Vidal *et al.*, 2007). Toto je jen část z možných vysvětlení této konvergence a další výzkum je žádoucí. Velká variabilita ve složení SM ptáků v porovnání se savci je pozorovatelná i v našich publikacích, kde došlo ke stejnému nebo velmi podobnému laboratornímu zpracování vzorků **[Publikace A–I]** (savci viz Bendová *et al.*, 2020; Matějková *et al.*, 2020; Moudra *et al.*, 2021; Čížková *et al.*, 2021). Velké množství nevysvětlené variability společně s velkou variabilitou mezi jedinci a poměrně slabou stabilitou SM u ptáků by mohlo být způsobeno výraznou fluktuací mikrobioty, která trávicím traktem pouze prochází pravděpodobně společně s potravou, ale trávicí trakt trvale neosídluje, což potvrzuje i přítomnost symbiotických bakterií hmyzu detekovaných ve SM různých pěvců **[Publikace A–I]**. Mezi detekované symbionty hmyzu patří například *Arsenophonus*, *Buchnera*, *Pseudonocardia*, *Rickettsia*, *Rickettsiella*, *Serratia* či *Spiroplasma* (Baumann, 2005; Moran *et al.*, 2005; Braig, Turner & Perotti, 2008; Nováková, Hypša & Moran, 2009; Anbutsu & Fukatsu, 2011). Konkrétně byla například nalezena pozitivní korelace mezi klopuškou (*Lygus*) v potravě vlaštovek a bakterií z rodu *Rikettsia* **[Publikace E]**. Zda bakterie trávicím traktem pouze prochází nebo jsou-li v něm aktivní, nelze používanými metodami zatím odlišit. Z našich dat se zdá, že v trávicím traktu je část SM stabilní, a proto by se měl další výzkum zaměřit na studium souvislosti mezi fenotypem

hostitele a těmito konkrétními bakteriálními taxony. Zároveň je třeba se zaměřit na stabilitu SM i u dalších skupin ptáků, aby se potvrdilo, zda námi pozorované bakteriální taxony jsou stabilní napříč ptáky/pěvci. Každopádně lze konstatovat, že výsledky studií realizovaných na savcích nejsou obecně aplikovatelné na ptáky a pravděpodobně ani ostatní obratlovce a je tedy důležité věnovat se také studiu složení SM u dalších skupin obratlovců. Navíc by se kromě studia bakterií mohl budoucí výzkum věnovat více i jiným složkám mikrobioty jako jsou houby a viry a jejich vzájemným interakcím.

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7. Přílohy

Příloha 1 k Publikaci B

Table S1: Sample metadata (code = sample identity, species, category= temperate trans-Saharan migrants [Temperate_Transaharan], temperate residents/short-distance migrant [Temperate_Resid.Short], tropical rainy season [Tropical_Wet] and tropical dry season [Tropical_Dry].

code	species	category	diet
ZA24483	Acrocephalus_arundinaceus	Temperate_Transaharan	0.9
ZA35700	Acrocephalus_arundinaceus	Temperate_Transaharan	0.9
ZA50401	Acrocephalus_arundinaceus	Temperate_Transaharan	0.9
ZA58051	Acrocephalus_arundinaceus	Temperate_Transaharan	0.9
ZA58058	Acrocephalus_arundinaceus	Temperate_Transaharan	0.9
CZ-14-311	Acrocephalus_palustris	Temperate_Transaharan	0.8
CZ-14-432	Acrocephalus_palustris	Temperate_Transaharan	0.8
CZ-14-433	Acrocephalus_palustris	Temperate_Transaharan	0.8
CZ-14-436	Acrocephalus_palustris	Temperate_Transaharan	0.8
CZ-14-441	Acrocephalus_palustris	Temperate_Transaharan	0.8
TP61366	Acrocephalus_palustris	Temperate_Transaharan	0.8
TP61487	Acrocephalus_palustris	Temperate_Transaharan	0.8
TP61494	Acrocephalus_palustris	Temperate_Transaharan	0.8
TR27144	Acrocephalus_palustris	Temperate_Transaharan	0.8
S640540	Acrocephalus_schoenobaenus	Temperate_Transaharan	0.7
TP52638	Acrocephalus_schoenobaenus	Temperate_Transaharan	0.7
TP52640	Acrocephalus_schoenobaenus	Temperate_Transaharan	0.7
TP52648	Acrocephalus_schoenobaenus	Temperate_Transaharan	0.7
TP52650	Acrocephalus_schoenobaenus	Temperate_Transaharan	0.7
TP67321	Acrocephalus_schoenobaenus	Temperate_Transaharan	0.7
TP46133	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TP52626	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TP52649	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TP61499	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TP61500	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TP67323	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TR27097	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
TR67328	Acrocephalus_scirpaceus	Temperate_Transaharan	0.7
F157035	Aegithalos_caudatus	Temperate_Resid.Short	0.6
F157036	Aegithalos_caudatus	Temperate_Resid.Short	0.6
F157902	Aegithalos_caudatus	Temperate_Resid.Short	0.6
F160964	Aegithalos_caudatus	Temperate_Resid.Short	0.6
F160965	Aegithalos_caudatus	Temperate_Resid.Short	0.6
F160985	Aegithalos_caudatus	Temperate_Resid.Short	0.6
CAM-14-015	Alethe_diademata	Tropical_Wet	1
CAM-14-016	Alethe_diademata	Tropical_Wet	1
CAM-14-025	Alethe_diademata	Tropical_Wet	1
CAM-14-064	Alethe_diademata	Tropical_Wet	1
CAM-14-155	Alethe_diademata	Tropical_Wet	1
CAM-14-031	Alethe_poliocephala	Tropical_Wet	1
CAM-14-082	Alethe_poliocephala	Tropical_Wet	1

CAM-14-084	Alethe_poliocephala	Tropical_Wet	1
CAM-14-100	Alethe_poliocephala	Tropical_Wet	1
CAM-14-101	Alethe_poliocephala	Tropical_Wet	1
CAM-14-141	Alethe_poliocephala	Tropical_Wet	1
CAM-14-148	Alethe_poliocephala	Tropical_Wet	1
CAM-14-167	Alethe_poliocephala	Tropical_Wet	1
CAM-14-187	Alethe_poliocephala	Tropical_Wet	1
CAM-14-177	Andropadus_latirostris	Tropical_Wet	0.5
CAM-14-027	Apalis_cinerea	Tropical_Wet	1
CAM-14-018	Bathmocercus_rufus	Tropical_Wet	1
CAM-14-032	Bathmocercus_rufus	Tropical_Wet	1
CAM-14-019	Bleda_syndactylus	Tropical_Wet	0.9
CAM-14-073	Bleda_syndactylus	Tropical_Wet	0.9
CAM-14-198	Bradypterus_lopezi	Tropical_Wet	1
CAM-14-006	Camaroptera_chloronota	Tropical_Wet	1
S673719	Carduelis_carduelis	Temperate_Resid.Short	0.1
S679108	Carduelis_carduelis	Temperate_Resid.Short	0.1
S679110	Carduelis_carduelis	Temperate_Resid.Short	0.1
S679126	Carduelis_carduelis	Temperate_Resid.Short	0.1
S694410	Carduelis_carduelis	Temperate_Resid.Short	0.1
S726633	Carduelis_carduelis	Temperate_Resid.Short	0.1
TRO3039	Carduelis_carduelis	Temperate_Resid.Short	0.1
N699079	Carduelis_chloris	Temperate_Resid.Short	0
N699083	Carduelis_chloris	Temperate_Resid.Short	0
N707051	Carduelis_chloris	Temperate_Resid.Short	0
CZ-14-345	Carduelis_flammea	Temperate_Resid.Short	0.3
F157034	Certhia_familiaris	Temperate_Resid.Short	0.6
F160993	Certhia_familiaris	Temperate_Resid.Short	0.6
F160996	Certhia_familiaris	Temperate_Resid.Short	0.6
J84508	Certhia_familiaris	Temperate_Resid.Short	0.6
K495170	Cinclus_cinclus	Temperate_Resid.Short	1
K514695	Cinclus_cinclus	Temperate_Resid.Short	1
K514745	Cinclus_cinclus	Temperate_Resid.Short	1
K514759	Cinclus_cinclus	Temperate_Resid.Short	1
K514760	Cinclus_cinclus	Temperate_Resid.Short	1
K514818	Cinclus_cinclus	Temperate_Resid.Short	1
K514819	Cinclus_cinclus	Temperate_Resid.Short	1
ZX02897	Coccothraustes_coccothraustes	Temperate_Resid.Short	0.1
CAM-14-102	Cossypha_isabellae	Tropical_Wet	0.8
CAM-14-172	Cossypha_isabellae	Tropical_Wet	0.8
CAM-14-181	Cossypha_isabellae	Tropical_Wet	0.8
CAM-14-017	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-075	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-078	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-114	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-116	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-153	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-156	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-166	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-173	Cryptospiza_reichenovii	Tropical_Wet	0.2
CAM-14-188	Cryptospiza_reichenovii	Tropical_Wet	0.2
S704259	Cyanistes_caeruleus	Temperate_Resid.Short	0.5
TP61453	Cyanistes_caeruleus	Temperate_Resid.Short	0.5
TP61454	Cyanistes_caeruleus	Temperate_Resid.Short	0.5

CAM-14-022	Cyanomitra_olivacea	Tropical_Wet	0.4
CAM-14-093	Cyanomitra_olivacea	Tropical_Wet	0.4
S733906	Delichon_urbicum	Temperate_Transaharan	1
TP61430	Delichon_urbicum	Temperate_Transaharan	1
TP61473	Delichon_urbicum	Temperate_Transaharan	1
CAM-14-057	Dyaphorophyia_castanea	Tropical_Wet	0.7
CAM-14-026	Dyaphorophyia_concreta	Tropical_Wet	1
CAM-14-105	Dyaphorophyia_concreta	Tropical_Wet	1
CAM-14-106	Elminia_albiventris	Tropical_Wet	1
CAM-14-190	Elminia_albiventris	Tropical_Wet	1
N707061	Emberiza_citrinella	Temperate_Resid.Short	0.3
N708621	Emberiza_citrinella	Temperate_Resid.Short	0.3
N718829	Emberiza_citrinella	Temperate_Resid.Short	0.3
N718830	Emberiza_citrinella	Temperate_Resid.Short	0.3
N728971	Emberiza_citrinella	Temperate_Resid.Short	0.3
NP708617	Emberiza_citrinella	Temperate_Resid.Short	0.3
NP708619	Emberiza_citrinella	Temperate_Resid.Short	0.3
TP79907	Emberiza_citrinella	Temperate_Resid.Short	0.3
TP79918	Emberiza_citrinella	Temperate_Resid.Short	0.3
TP79943	Emberiza_citrinella	Temperate_Resid.Short	0.3
TP79958	Emberiza_citrinella	Temperate_Resid.Short	0.3
CZ-14-300	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
CZ-14-302	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
CZ-14-315	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
CZ-14-320	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
TP52637	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
TP79925	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
TP79985	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
TR27181	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
TR27195	Emberiza_schoeniclus	Temperate_Resid.Short	0.3
TP61410	Erithacus_rubecula	Temperate_Resid.Short	0.6
TP61422	Erithacus_rubecula	Temperate_Resid.Short	0.6
TP63289	Erithacus_rubecula	Temperate_Resid.Short	0.6
TP79913	Erithacus_rubecula	Temperate_Resid.Short	0.6
TP79930	Erithacus_rubecula	Temperate_Resid.Short	0.6
TP79939	Erithacus_rubecula	Temperate_Resid.Short	0.6
TP90655	Erithacus_rubecula	Temperate_Resid.Short	0.6
P62883	Ficedula_albicollis	Temperate_Transaharan	0.9
P62885	Ficedula_albicollis	Temperate_Transaharan	0.9
P62886	Ficedula_albicollis	Temperate_Transaharan	0.9
P62888	Ficedula_albicollis	Temperate_Transaharan	0.9
S673733	Ficedula_albicollis	Temperate_Transaharan	0.9
TP62887	Ficedula_albicollis	Temperate_Transaharan	0.9
TP90658	Ficedula_albicollis	Temperate_Transaharan	0.9
TP90661	Ficedula_albicollis	Temperate_Transaharan	0.9
CZ-14-90	Fringilla_coelebs	Temperate_Resid.Short	0.6
N708635	Fringilla_coelebs	Temperate_Resid.Short	0.6
N718833	Fringilla_coelebs	Temperate_Resid.Short	0.6
TP61371	Fringilla_coelebs	Temperate_Resid.Short	0.6
TP61413	Fringilla_coelebs	Temperate_Resid.Short	0.6
TP79934	Hippolais_icterina	Temperate_Transaharan	0.8
TP79952	Hippolais_icterina	Temperate_Transaharan	0.8
TP79967	Hippolais_icterina	Temperate_Transaharan	0.8
TP79968	Hippolais_icterina	Temperate_Transaharan	0.8

TP79975	Hippolais_icterina	Temperate_Transaharan	0.8
TP61451	Hippolais_icterina	Temperate_Transaharan	0.8
TP79987	Hippolais_icterina	Temperate_Transaharan	0.8
TR03047	Hippolais_icterina	Temperate_Transaharan	0.8
TR27107	Hippolais_icterina	Temperate_Transaharan	0.8
S645205	Hirundo_rustica	Temperate_Transaharan	0.8
S677687	Hirundo_rustica	Temperate_Transaharan	0.8
s704248	Hirundo_rustica	Temperate_Transaharan	0.8
CAM-14-024	Illadopsis_cleaveri	Tropical_Wet	1
CAM-14-098	Illadopsis_cleaveri	Tropical_Wet	1
CAM-14-028	Illadopsis_rufipennis	Tropical_Wet	1
CAM-14-029	Illadopsis_rufipennis	Tropical_Wet	1
CAM-14-086	Kakamega_poliothorax	Tropical_Wet	1
CAM-14-088	Kakamega_poliothorax	Tropical_Wet	1
CAM-14-115	Kakamega_poliothorax	Tropical_Wet	1
CAM-14-136	Laniarius_atroflavus	Tropical_Wet	1
CAM-14-189	Laniarius_poensis	Tropical_Wet	1
ZA42995	Lanius_collurio	Temperate_Transaharan	0.9
ZA50561	Lanius_collurio	Temperate_Transaharan	0.9
ZA50563	Lanius_collurio	Temperate_Transaharan	0.9
CAM-14-196	Linurgus_olivaceus	Tropical_Wet	0.1
TP90657	Locustella_fluviatilis	Temperate_Transaharan	1
xxx	Locustella_fluviatilis	Temperate_Transaharan	1
TK61634	Locustella_luscinioides	Temperate_Transaharan	1
TN42826	Locustella_luscinioides	Temperate_Transaharan	1
TP52603	Locustella_luscinioides	Temperate_Transaharan	1
TP61490	Locustella_luscinioides	Temperate_Transaharan	1
TP79924	Locustella_luscinioides	Temperate_Transaharan	1
N678579	Luscinia_megarhynchos	Temperate_Transaharan	0.7
TP61361	Luscinia_svecica	Temperate_Transaharan	0.7
TP61382	Luscinia_svecica	Temperate_Transaharan	0.7
TP61438	Luscinia_svecica	Temperate_Transaharan	0.7
CAM-14-003	Macrosphenus_flavicans	Tropical_Wet	0.8
CAM-14-040	Macrosphenus_flavicans	Tropical_Wet	0.8
S679113	Motacilla_cinerea	Temperate_Resid.Short	1
S679114	Motacilla_cinerea	Temperate_Resid.Short	1
S679116	Motacilla_cinerea	Temperate_Resid.Short	1
S679119	Motacilla_cinerea	Temperate_Resid.Short	1
S679924	Motacilla_cinerea	Temperate_Resid.Short	1
TP52631	Motacilla_cinerea	Temperate_Resid.Short	1
TP61342	Motacilla_cinerea	Temperate_Resid.Short	1
S673727	Muscicapa_striata	Temperate_Transaharan	0.8
S679115	Muscicapa_striata	Temperate_Transaharan	0.8
S679121	Muscicapa_striata	Temperate_Transaharan	0.8
S704257	Muscicapa_striata	Temperate_Transaharan	0.8
TP79932	Muscicapa_striata	Temperate_Transaharan	0.8
CAM-14-133	Neocossyphus_poensis	Tropical_Wet	1
CAM-14-174	Neocossyphus_poensis	Tropical_Wet	1
N691000	Parus_major	Temperate_Resid.Short	0.4
N717226	Parus_major	Temperate_Resid.Short	0.4
N717229	Parus_major	Temperate_Resid.Short	0.4
N717232	Parus_major	Temperate_Resid.Short	0.4
N717239	Parus_major	Temperate_Resid.Short	0.4
N717240	Parus_major	Temperate_Resid.Short	0.4

N717246	Parus_major	Temperate_Resid.Short	0.4
N717248	Parus_major	Temperate_Resid.Short	0.4
N717253	Parus_major	Temperate_Resid.Short	0.4
N717256	Parus_major	Temperate_Resid.Short	0.4
N708660	Passer_domesticus	Temperate_Resid.Short	0.1
N718828	Passer_domesticus	Temperate_Resid.Short	0.1
N718847	Passer_domesticus	Temperate_Resid.Short	0.1
N718854	Passer_domesticus	Temperate_Resid.Short	0.1
N719010	Passer_domesticus	Temperate_Resid.Short	0.1
N719013	Passer_domesticus	Temperate_Resid.Short	0.1
N723982	Passer_domesticus	Temperate_Resid.Short	0.1
N723983	Passer_domesticus	Temperate_Resid.Short	0.1
N724009	Passer_domesticus	Temperate_Resid.Short	0.1
TP63273	Passer_domesticus	Temperate_Resid.Short	0.1
CZ-14-443	Passer_montanus	Temperate_Resid.Short	0.4
CZ-14-456	Passer_montanus	Temperate_Resid.Short	0.4
N719027	Passer_montanus	Temperate_Resid.Short	0.4
TP52651	Passer_montanus	Temperate_Resid.Short	0.4
TP61448	Passer_montanus	Temperate_Resid.Short	0.4
TP61478	Passer_montanus	Temperate_Resid.Short	0.4
TP61479	Passer_montanus	Temperate_Resid.Short	0.4
TP61481	Passer_montanus	Temperate_Resid.Short	0.4
TP79928	Periparus_ater	Temperate_Resid.Short	0.4
TP80137	Periparus_ater	Temperate_Resid.Short	0.4
TP80138	Periparus_ater	Temperate_Resid.Short	0.4
TP80139	Periparus_ater	Temperate_Resid.Short	0.4
TP80140	Periparus_ater	Temperate_Resid.Short	0.4
TP80141	Periparus_ater	Temperate_Resid.Short	0.4
TP61447	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP61456	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP61458	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP61470	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP63288	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP79922	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP79933	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP79965	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP79977	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP79982	Phoenicurus_ochruros	Temperate_Resid.Short	0.6
TP61377	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
TP61446	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
TP79931	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
TP79945	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
TP79950	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
TP79969	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
TR03038	Phoenicurus_phoenicurus	Temperate_Transaharan	0.8
CAM-14-059	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-062	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-111	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-127	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-130	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-132	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-157	Phyllastrephus_poensis	Tropical_Wet	0.8
CAM-14-109	Phyllastrephus_poliocephalus	Tropical_Wet	1
CAM-14-195	Phyllastrephus_poliocephalus	Tropical_Wet	1

F130012	Phylloscopus_collybita	Temperate_Resid.Short	0.8
F160938	Phylloscopus_collybita	Temperate_Resid.Short	0.8
F160961	Phylloscopus_collybita	Temperate_Resid.Short	0.8
F160975	Phylloscopus_collybita	Temperate_Resid.Short	0.8
F160992	Phylloscopus_collybita	Temperate_Resid.Short	0.8
J89029	Phylloscopus_collybita	Temperate_Resid.Short	0.8
F139420	Phylloscopus_trochilus	Temperate_Transaharan	0.8
F160971	Phylloscopus_trochilus	Temperate_Transaharan	0.8
F160977	Phylloscopus_trochilus	Temperate_Transaharan	0.8
P62998	Phylloscopus_trochilus	Temperate_Transaharan	0.8
CAM-14-122	Ploceus_melanogaster	Tropical_Wet	0.8
CAM-14-152	Ploceus_melanogaster	Tropical_Wet	0.8
CZ-14-473	Poecile_palustris	Temperate_Resid.Short	0.5
CZ-14-474	Poecile_palustris	Temperate_Resid.Short	0.5
S679730	Poecile_palustris	Temperate_Resid.Short	0.5
TP61469	Poecile_palustris	Temperate_Resid.Short	0.5
CAM-14-054	Poliolais_lopezi	Tropical_Wet	1
CAM-14-128	Poliolais_lopezi	Tropical_Wet	1
CAM-14-145	Poliolais_lopezi	Tropical_Wet	1
CAM-14-159	Poliolais_lopezi	Tropical_Wet	1
CAM-14-183	Poliolais_lopezi	Tropical_Wet	1
CAM-14-039	Prinia_bairdii	Tropical_Wet	1
CAM-14-061	Prinia_bairdii	Tropical_Wet	1
CAM-14-063	Prinia_bairdii	Tropical_Wet	1
CAM-14-129	Prinia_bairdii	Tropical_Wet	1
CAM-14-134	Prinia_bairdii	Tropical_Wet	1
CAM-14-171	Prinia_bairdii	Tropical_Wet	1
CAM-14-182	Prinia_bairdii	Tropical_Wet	1
TP61343	Prunella_modularis	Temperate_Resid.Short	0.5
TP61412	Prunella_modularis	Temperate_Resid.Short	0.5
TP61433	Prunella_modularis	Temperate_Resid.Short	0.5
TP61434	Prunella_modularis	Temperate_Resid.Short	0.5
TP61466	Prunella_modularis	Temperate_Resid.Short	0.5
TP79914	Prunella_modularis	Temperate_Resid.Short	0.5
TP79915	Prunella_modularis	Temperate_Resid.Short	0.5
CAM-14-139	Pseudoalcippe_abyssinica	Tropical_Wet	0.7
CAM-14-158	Pseudoalcippe_abyssinica	Tropical_Wet	0.7
CZ-14-078	Regulus_ignicapilla	Temperate_Resid.Short	1
F127777	Regulus_ignicapilla	Temperate_Resid.Short	1
F127779	Regulus_ignicapilla	Temperate_Resid.Short	1
F127780	Regulus_ignicapilla	Temperate_Resid.Short	1
F127781	Regulus_ignicapilla	Temperate_Resid.Short	1
F160946	Regulus_ignicapilla	Temperate_Resid.Short	1
F160959	Regulus_ignicapilla	Temperate_Resid.Short	1
F160980	Regulus_ignicapilla	Temperate_Resid.Short	1
F171601	Regulus_ignicapilla	Temperate_Resid.Short	1
F160152	Regulus_regulus	Temperate_Resid.Short	1
F160958	Regulus_regulus	Temperate_Resid.Short	1
F160973	Regulus_regulus	Temperate_Resid.Short	1
F160979	Regulus_regulus	Temperate_Resid.Short	1
J89456	Regulus_regulus	Temperate_Resid.Short	1
J89457	Regulus_regulus	Temperate_Resid.Short	1
J89459	Regulus_regulus	Temperate_Resid.Short	1
J89460	Regulus_regulus	Temperate_Resid.Short	1

J89461	Regulus_regulus	Temperate_Resid.Short	1
J89462	Regulus_regulus	Temperate_Resid.Short	1
J89855	Regulus_regulus	Temperate_Resid.Short	1
S726622	Remiz_pendulinus	Temperate_Resid.Short	0.6
S599794	Serinus_serinus	Temperate_Resid.Short	0
S679109	Serinus_serinus	Temperate_Resid.Short	0
CAM-14-056	Sheppardia_bocagei	Tropical_Wet	1
CAM-14-069	Sheppardia_bocagei	Tropical_Wet	1
CAM-14-076	Sheppardia_bocagei	Tropical_Wet	1
CAM-14-079	Sheppardia_bocagei	Tropical_Wet	1
CAM-14-080	Sheppardia_bocagei	Tropical_Wet	1
CAM-14-185	Sheppardia_bocagei	Tropical_Wet	1
N708636	Sitta_europaea	Temperate_Resid.Short	0.7
N708672	Sitta_europaea	Temperate_Resid.Short	0.7
N717258	Sitta_europaea	Temperate_Resid.Short	0.7
N718831	Sitta_europaea	Temperate_Resid.Short	0.7
N718862	Sitta_europaea	Temperate_Resid.Short	0.7
N718880	Sitta_europaea	Temperate_Resid.Short	0.7
N719001	Sitta_europaea	Temperate_Resid.Short	0.7
N728954	Sitta_europaea	Temperate_Resid.Short	0.7
N728963	Sitta_europaea	Temperate_Resid.Short	0.7
CAM-14-096	Smithornis_sharpei	Tropical_Wet	1
CAM-14-068	Spermophaga_haematina	Tropical_Wet	0.2
CAM-14-072	Spermophaga_haematina	Tropical_Wet	0.2
CZ-14-446	Sturnus_vulgaris	Temperate_Resid.Short	0.3
TK18822	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP61352	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP61357	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP61378	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP79903	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP79916	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP79929	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP79956	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP79957	Sylvia_atricapilla	Temperate_Resid.Short	0.5
TP79964	Sylvia_atricapilla	Temperate_Resid.Short	0.5
N718835	Sylvia_borin	Temperate_Transaharan	0.5
TP61409	Sylvia_borin	Temperate_Transaharan	0.5
TP61414	Sylvia_borin	Temperate_Transaharan	0.5
TP61416	Sylvia_borin	Temperate_Transaharan	0.5
TP61450	Sylvia_borin	Temperate_Transaharan	0.5
TP61467	Sylvia_borin	Temperate_Transaharan	0.5
TP61363	Sylvia_communis	Temperate_Transaharan	0.6
TR03044	Sylvia_communis	Temperate_Transaharan	0.6
S677634	Sylvia_curruca	Temperate_Transaharan	0.6
TP61418	Sylvia_curruca	Temperate_Transaharan	0.6
TP63297	Sylvia_curruca	Temperate_Transaharan	0.6
TP79920	Sylvia_curruca	Temperate_Transaharan	0.6
TP79921	Sylvia_curruca	Temperate_Transaharan	0.6
TP79938	Sylvia_curruca	Temperate_Transaharan	0.6
TP79976	Sylvia_curruca	Temperate_Transaharan	0.6
TP90675	Sylvia_curruca	Temperate_Transaharan	0.6
TR03045	Sylvia_curruca	Temperate_Transaharan	0.6
TR03055	Sylvia_curruca	Temperate_Transaharan	0.6
CAM-14-037	Trochocercus_nitens	Tropical_Wet	1