

MTA doktori értekezés

Mikrobiális genomikai vizsgálatok: a metagenomtól az
antimikrobiális rezisztencia-potenciálig

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1. Köszönetnyilvánítás

A dolgozatomban összefoglalt eredmények a 2017-ben az Állatorvostudományi Egyetemen létrejött Bioinformatikai Központban végzett bioinformatikai kutatásaink egy részét mutatja be. Az ide vezető éveimben számosan támogattak, alakítottak, tanítottak, neveltek. Közülük kiemelkedő szerepet játszottak, természetesen, szüleim, akik minden szükséges körülményt biztosítottak számomra. Nagybátyám, Matula Péter, aki először mutatta meg, az 1980-as évek elején, a biológia és az informatika kapcsolatában rejlő lehetőségeket, és támogatott számítógépekkel, programozási szakirodalommal. Általános és középiskolai éveim során szerencsém volt több remek tanárral találkozni, akik bizonyos pontokon meghatározók voltak szellemi fejlődésemben: Antal Emília, Horváth Elemérné, Meizl Józsefné és Vajda Istvánné. Nagyon nagy szerencsémnek érzem, hogy kamaszkoromtól szerzetesek közelében lehettem. Közülük Dolmány Vendel O.Cist. különösen fontos szerepet játszott világnézeti, erkölcsi alakulásomban. Korai halála után Keszthelyi Vazul O.Cist., Köveshegyi Rajnald O.Cist., Égi Konrád O.Cist. és Bán Elizeus O.Cist. élete, jelleme és tanítása mutatott példát. Intellektuális formálódásomban szintén fontos volt egyetemi éveim alatt a Szent Ignác Szakkollégium, ahol a jezsuiták interdiszciplináris szemlélete mellett Kamarás István és Sárosi Attila gyakorolt rám jelentős hatást. Az Állatorvostudományi Egyetemen oktatóim közül biológiai, orvosi gondolkodásomat alapvetően alakította Medveczky István, Rózsa Lajos, Süveges Tibor, Szita Géza és Tuboly Sándor.

Geoepidemiológiai kutatásaimban fontos segítséget nyújtottak Barcza Zoltán, Bódis Katalin, Dövényi Péter és Nagymarosy András. A PhD-mhoz kapcsolódó járványügyi térinformatikai rendszer fejlesztésem a Csongrád megyei kollégák, köztük Fodor László és Szigeti Sándor segítségével nélkül nem jöhetett volna létre. Sokat tanultam a Békési Lászlóval, Dubecz Attilával, Farkas Róberttel, Földi Zsolttal, Gábor Györggyel, Lakos Andrással, Makrai Lászlóval, Patai Árpáddal, Reibling Tamással, Franz Rubellel és Valcz Gáborral közös munkákból.

Hálás vagyok azért, hogy Molnár Béla meghívására bioinformatikai kutatásokba kapcsolódhattam be, amelyekben Spisák Sándorral együtt tanulhattunk. Liposits Zsolt jóvoltából neuroendokrinológiai bioinformatikai elemzésekben is részt vehettem. A két évtizedes együttműködés Csabai Istvánnal és Vattay Gáborral szintén meghatározó volt pályámban. Biztosan állíthatom, hogy a Csabai István teremtette, fenntartott szellemi közeg nélkül sem a bemutatott, sem más munkáim nem születtek volna meg. A Bioinformatikai Központ létrehozása lehetővé tette, hogy teljes egészében erre a területre összpontosítsak, amiben Gálfi Péter segítségével, Farkas Róbert támogatásáért is hálás vagyok. Saját mintáink szekvenálásával kapcsolatban Maróti Gergely nyújtott nélkülözhetetlen támogatást. Szakdolgozóim, tudományos diákköröseim és doktoranduszaim inspiráló hatása nélkül sok munkát nem fejeztem volna be, vagy nem publikáltam volna. Utóbbiak közül különösen hálás vagyok Nagy Sára Ágnesnek és Tóth Adrienn Grétának, akik a pályát megfutva is maradtak munkatársaim.

Szűkebb és tágabb családom elfogadó szeretete nélkül nem végezhettem volna szabadon a rövidebb vagy hosszabb távon teljes figyelmemet elvonó kutatómunkámat. Gyermekeim és különösen feleségem hosszan tűrő megértését nem feledem. OAMDG!

...az értelem sohasem ragadhatja meg olyan pontosan az igazságot, hogy ne lehetne pontosabban megragadni. Úgy viszonyul az igazsághoz, mint sokszög a körhöz: minél több szöge van a beírt sokszögnek, annál hasonlóbb a körhöz, de növelje bár a végtelenségig szögeinek számát, mégsem lesz soha egyenlő vele, csak ha a körrel való azonosságban feloldódik.

Nicolaus Cusanus: A tudós tudatlanság

2. Bevezetés

Cusanus gondolatát a mikrobiológia tudománytörténetében számos mozzanat támogatja. Az élővilágról, annak gazdagságáról alkotott nézeteinket jelentősen tágitotta a mikroszkóp nyújtotta lehetőség, amely a mikroorganizmusok megismerésének sokáig egyetlen eszköze volt. A baktériumok kimutatása, tenyésztése, azok kórképhez kapcsolása szintén javította a fertőző betegségekről alkotott modellünk valóságához közelítését. Az összetett okú betegségek már további modellfrissítést igényeltek, mivel a nagy megbetegítőképeségű kórokozók oktani tanulmányozására kialakított modelljeinket (pl. Koch-féle posztulátumok) szétfeszítették. A diagnosztikai tudásunkkal párhuzamosan a betegségmegelőzési és terápiás lehetőségek bővülése szintén átrajzolta a fertőző betegségekről alkotott modelljeinket. Az antibiotikumok felfedezése, majd alkalmazása óriási paradigmaváltást jelentett a bakteriális betegségek elleni küzdelemben. Amit nem sokkal később követett az antimikrobiális rezisztencia (AMR) okozta modellkorrekciós igény. Ha ezeket a példákat átgondoljuk, minden modellváltás során részletesebb lett, bővült és egyben komplexebbé is vált a valóságról alkotott képünk. Minden modellfrissítéshez eszközök megjelenése, használata kapcsolódik, némelyek megjelenése kikényszeríti a változást, mások létrehozása következménye a változásnak. A klasszikus mikrobiológiai eszközöket az utóbbi években egyre többször bioinformatikai megoldásokkal egészítjük ki.

Egy összetett mikrobiológiai közösségben, legyen az környezetben vagy kórfolyamatban együtt élő közösség, az alkotó mikroorganizmusok egymásra hatást gyakorolnak, így egymáshoz viszonyított arányuk lényeges lehet. Feltételezhető az is, hogy a kórfolyamat lefolyásában ennek is szerepe lehet. Ahhoz, hogy az életközösségen belül leírassuk ezeket az arányokat és dinamikájukat minden összetevő mennyiségét szükséges ismernünk. A hagyományos mikrobiológiai vizsgálatok során a különböző baktériumok kimutatása céljából gyakran baktériumtenyésztést alkalmazunk. Amely természetesen az egyes fajoknak, nemzetségeknek megfelelő többé-kevésbé speciális táptalajon történik. Ha a mikrobiológiai mintában sok baktériumfaj fordult elő, akkor sokféle igénynek megfelelő tenyésztést kell végeznünk. Emellett fontos megjegyezni, hogy a baktériumok igen jelentős része nem, vagy nagyon különleges feltételek mellett tenyészhető. Ugyanakkor ezzel a célzott, vagy ún. modellvezérelt megközelítéssel a baktériumok eredeti, mintabeli részaránya nem rekonstruálható. További nehézséget jelent az, hogy a kimutatás során minden olyan baktériumra célzott tenyésztést kell végeznünk, amely a mintában előfordulhat. Azonban nem is ritkán, előfordulhatnak olyan baktériumfajok is, amelyekkel joggal nem számoltunk és

így a jelenlétüket nem is vizsgáltuk. E megközelítésre alapozott modellünk érthető módon részlegesen írja le valóság teljességét. (George E. P. Box: „*minden modell rossz, de vannak, amelyek használhatók*”). A vázolt megközelítés számos területen igen hasznosnak bizonyult, az ismert korlátaival együtt is. Az újgenerációs szekvenálás (next generation sequencing, NGS) megjelenése és a metagenomikai eszköztár, módszerek azonban lehetőséget nyújtanak a több fajból álló életközösségek mikrobiológiai összetételének pontosabb megismerésében. A mikrobióta nukleinsav-szekvenciáinak, a mikrobiom NGS-alapú vizsgálata lehetőséget ad modellünk valósághoz közelítésében, ami a korábbival szemben nem modell-, hanem ún. adatvezérelt megközelítés. Ennek során az adott minta nukleinsav-molekuláit szekvenáljuk, az így létrehozott digitális szekvenciákat pedig taxonadatbázisokhoz hasonlítjuk. E taxonklasszifikáció eredményeként a szekvenciákhoz taxonokat rendelünk, ami alapján taxonabundanciát becsülhetünk. Az abundancia tényezők, csoportok szerinti változását, eltérését ún. *profilvizsgálatokkal* elemezzük.

Mivel a klasszikus mikrobiológiai megközelítés, ahogy láttuk, célzott vizsgálatokon nyugszik, minden eredményhez hipotézisre, modellre van szükségünk. Azonban a kérdéses mintában lehetnek olyan mikroorganizmusok is, amelyekre senki sem gondolt, így azok vizsgálatát sem célozta meg. Az ún. shotgun metagenomikai szekvenálás lehetőséget nyújt arra, hogy a mintában jelen lévő minden organizmusra vonatkozóan információhoz jussunk. Ez a profilvizsgálatok mellett lehetőséget nyújt olyan organizmusok azonosítására, amelyek szintén fontosak lehetnek biológiai vagy klinikai szempontból (kórokozó-azonosítás).

Az AMR igen jelentős, globális egészségügyi kihívás, mivel egyre több antibiotikummal (AB) szemben, egyre gyakrabban fordul elő, így csökkentve az antibakteriális kezelések hatékonyságát. A hatékony terápia feltétele, hogy a kórokozó baktériumok érzékenyek legyenek az alkalmazott AB-ra. Célzott AB-terápia esetén azt megelőzi az adott kórfolyamatban szerepet játszó baktériumok AB-érzékenységi vizsgálata (antimicrobial susceptibility test, AST). Ez a klasszikus mikrobiológiai gyakorlatban, nagy vonalakban, úgy történik, hogy a betegből mintát véve tenyésztjük az abban lévő baktériumokat. A korábban említetteknek megfelelően különböző tenyésztési feltételek mellett azonosíthatjuk az eredeti mintában jelen lévő baktériumfajok egy részét (nem az összeset). A folyamat során létrejött baktériumtelepekből néhányat (nem az összeset) kiválasztva ún. szintenyészeteket készítünk, amelyeken elvégezhetjük az AST-t. Erre különböző eljárásokat használhatunk, amelyekben mindenképpen közös, hogy modellalapú, néhány AB-ra kiterjedő célzott vizsgálat. E fenotípusos rezisztenciavizsgálat során kiválasztjuk azokat az AB-ket, amelyekre érzékenynek mutatkoztak a baktériumok, és alkalmazzuk a kórfolyamat kezelésében. A fenotípusos AMR hátterében ún. antimikrobiális rezisztenciagének (ARG) állnak. Ezek hosszú evolúciós folyamat során létrejött, többé-kevésbé speciális gének, illetve ide értjük azokat a génváltozatokat is, amelyek polimorfizmusok révén játszanak szerepet az AMR-ben. Az AB-k széles körben való használata szelekciós nyomást gyakorolva a velük találkozó baktériumokra, olyan törzsek kiválogatódását eredményezi, amelyekkel szemben kevés-

bé vagy egyáltalán nem hatékonyak. Baktériumközösségek, vagy -színtenyészetek shotgun szekvenálása lehetővé teszi, hogy akár a teljes genom(ok) szekvenciáját leírassuk. Így a mintánkban jelen lévő ARG-k összességének, a rezisztom vizsgálatát is lehetővé tevő, adatvezérelt megközelítést jelent. Mivel az említett taxonklasszifikációhoz hasonlóan itt is adatbázishoz, ARG-adatbázishoz hasonlítva keresünk egyező szekvenciális mintázatokat. A baktériumok között gének, így ARG-k is átadódhatnak az ún. horizontális géntranszfer (HGT) során. A HGT megvalósulásának valószínűségét növelik bizonyos mobilis genetikai elemek (MGE): plazmidok, profágok és integrált mobilis elemek (iMGE). Amennyiben a baktérium genomjában az ARG-k MGE-hez kapcsolhatóan vannak jelen, akkor a HGT-re nagyobb az esélyük. A shotgun szekvenálás a rezisztom mellett a mobilom, vagyis az MGE-k összességének vizsgálatát is lehetővé teszi.

Dolgozatomban olyan munkáink eredményeit foglalom össze, amelyek során a fentiekben vázolt megközelítéssel igyekeztünk a klasszikus mikrobiológia valóság-megragadásának lehetőségeit bővíteni. A sort azzal nyitom, amelynek során az országos, klimatikusan reprezentatív mintavételre alapozott mézelő méh (*Apis mellifera*) és *Ixodes ricinus* minták metagenomikai vizsgálatát végeztük. A bemutatott eredményeink másik csoportja rezisztom-, és mobilomvizsgálatokból származnak. Ezek során olyan lehetséges forrásokat tanulmányoztunk, amelyekből közvetlenül vagy közvetve az emberi szervezetbe juthatnak ARG-k. A rezisztomvizsgálatok kérdéseket vetnek fel annak klinikai értelmezési lehetőségeit, korlátait illetően. Dolgozatom zárásaként a rezisztom és a fenotípusos AMR kapcsolatára vonatkozó elemzéseink eredményeit mutatom be, valamint az abból következő antimikrobiális rezisztencia-potenciállal kapcsolatos megfontolásokat.

3. Célkitűzések

A bemutatott munkáink során különböző mikrobiológiai kérdésekre nagy áteresztőképességű szekvenálással generált adatok genomikai elemzésével kerestünk válaszokat. Saját vizsgálatainkból és mások eredményeiből is láttuk, hogy a mikrobiom nagy változatosságot mutat még egészséges állatokban, emberekben is. Ugyanakkor ahhoz, hogy az egészségestől való eltéréseket értékelhessük – akár diagnosztikai célból –, szükséges lenne az egészséges állapot természetes variabilitásának ismerete. Mivel a mézelő méhek (*Apis mellifera*) bélmikrobiótáját aránylag kis számú mikroorganizmus alkotja, és e fajjal kapcsolatban már korábban is végeztünk epidemiológiai vizsgálatokat, valamint rendkívüli ökológiai/gazdasági szerepet játszó, de ugyanakkor az utóbbi években jelentős pusztulásoknak kitett háziállat, a kérdés tanulmányozásához megfelelő modellállatnak találtuk. A mézelőméh-bélbakterioma természetes variabilitásának vizsgálata céljából országos, klimatikus reprezentatív, ismételt mintavételezésen alapuló felmérést végeztünk.

Epidemiológiai elemzéseket végezve számos ízeltlábú vektorral, vektor-közvetítette fertőző betegséggel kapcsolatos kutatásban vettem részt.^{1–7} Mivel a klímaváltozás^{8,9} következtében ezek a betegségek egyre gyakrabban, egyre nagyobb területen fordulnak elő, e vektorok mikrobiomkutatása is hangsúlyosabbá vált. Országos, klimatikusan reprezentatív mintavételre alapozott felmérés során gyűjtött *Ixodes ricinus* kullancsok metagenomikai vizsgálatát végeztük a fejlődési állapotok és klimatikus környezet szerinti bakterioma profil leírása céljából. A profilvizsgálat mellett mind a mézelő méhek, mind a kullancsok metagenom-elemzéseiben a mintákban jelenlévő, esetleg kórokozó organizmusok keresése is célunk volt.

Annak a pontosabb ismerete, hogy az ARG-k, az azokat hordozó baktériumok honnan és hogyan kerülhetnek ez emberi szervezetbe, fontos lehet az AMR elleni küzdelemben. Korábbi metagenomikai munkánk során vizsgáltuk a sertésbélisárban lévő baktériumok¹⁰ rezisztomját.¹¹ Az ottani eredmények inspirálták olyan, állatokhoz köthető ARG-források vizsgálatát, amelyekből ARG-hordozó baktériumok kerülhetnek az emberi szervezetbe. Ebből a célból rezisztom-, és mobilomvizsgálatokat végeztünk nyerstej-, probiotikus tejtermék-, probiotikum-, probiotikus, illetve kullancs-terjesztette baktériumtörzs-, és kutyanyál-minták felhasználásával. A nyers tej és probiotikus tejtermékek eredményei irányították figyelmünket a szilázs rezisztom-, és mobilomvizsgálatára. Hiszen a szilázs, a probiotikus tejtermékekhez hasonlóan erjesztéssel készül, így a fogyasztásával nagy

menyiségű baktérium juthat a tejtermelő szarvasmarhák emésztőrendszerébe, onnan más szerveikbe, így akár emberi fogyasztásra szánt élelmiszerekbe is.

A rezisztomvizsgálataink kapcsán újra és újra felmerült a kérdés, hogy az ARG-k jelenléte hogyan értelmezhető a fenotípusos AMR vonatkozásában. A válaszhoz adatokat gyűjtendő nagyszámú *Escherichia coli* szintenyészetből származó fenotípusos és genotípusos rezisztencia eredményeket vetettünk össze.

4. Módszerek

A bemutatott munkáinkban új-, és harmadik generációs szekvenálási adatokat elemeztünk bioinformatikai módszerekkel. Egyes vizsgálatainkban a felhasznált adatokat részben (a 45. lapon a VI., VII., VIII. és XIII. közlemény) vagy egészben (I., II., III., IV. és V. közlemény) saját mintagyűjtésre alapozott szekvenálással generáltuk. Más munkáinkban kizárólag mások által szekvenált adatokat használtunk fel (IX., X., XI. és XII. közlemény). A mások által szabadon elérhetővé tett adatokat a National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repozitóriumból töltöttük le. E minták beválogatásának részletes szempontjai, illetve az összes mintára vonatkozó részletes adatok a dolgozat alapját képező, mellékelt közleményekben olvashatók. Az egyes vizsgálatok eredményeinek értelmezéséhez szükséges mintaleírásokat rövid bevezetőként foglaltam össze az Eredmények fejezetben. A bioinformatikai adatfeldolgozást, elemzést az alább bemutatott lépésekben és eszközökkel végeztük. Adott vizsgálatban felhasznált programok verziószámát, illetve a futtatási beállításokat a közleményekben részletesen leírtuk, itt nem ismételtem meg.

Azoknál a metagenomikai vizsgálatoknál, amelyekben abundancia-összehasonlítást is végeztünk, az elemzéseket azzal kezdtük, hogy az Illumina paired-end readeket PEAR¹² segítségével egyesítettük. Az így egyesített szekvenciákat, illetve a további vizsgálatokban használt Illumina readeket minőségi alapon Adapterremoval¹³, TrimGalore (<https://github.com/FelixKrueger/TrimGalore>) vagy Trimmomatic¹⁴ programokkal szűrtük. A gazda eredetű szennyeződések eltávolítása¹⁵ céljából a szekvenciákat Bowtie2-vel¹⁶ illesztettük a gazda referenciagenomjára. Azokban a vizsgálatokban, amelyekben abundancia-különbségeket vizsgáltunk, a duplikátumok szűrését VSEARCH-el¹⁷ végeztük.

Taxonklasszifikációra a Kraken2¹⁸ szoftvert használtuk, úgy, hogy a taxonadatbázisokat az NCBI-ről letöltött adatok (NT¹⁹, RefSeq²⁰) alapján mindig magunk készítettük, azért, hogy a lehető legfrissebb gyűjteményre alapozottan végezhesük a vizsgálatokat. Ez alól kivételt jelentett a *Vairimorpha ceranae* célzott vizsgálata, amikor az EuPathDB46-Clean adatbázist is használtuk.²¹ A taxonklasszifikáció eredményeként kapott abundancia-táblázatokat R-környezetben²², a phyloseq²³ és a microbiome²⁴ csomag függvényeit használva dolgoztuk fel. Az α -diverzitás csoportok szerinti eltéréseit lineáris modellel, míg a β -diverzitás esetén PERMANOVA-val²⁵ vizsgáltuk a vegan²⁶ csomag segítségével. A taxononkénti abundancia-eltéréseket a DESeq2 R-csomaggal elemeztük.²⁷

A méhbél-metagenom szezonális hatás elemzése során ún. kevert modellt alkalmaztunk az ismételt mérések kezelésére, amelyben a méhészetek, mint random tényezők szerepeltek.

A rövid leolvasási szekvenciák hosszabb, összefüggő szakaszokká (kontig) történő összeillesztését (de novo assembly) MEGAHIT²⁸ segítségével végeztük, ettől egy esetben térünk el, amikor a metaSPAdes-t²⁹ használtuk. A *Vairimorpha ceranae* vizsgálatában a kontigok nukleotidsorrendjének pontosítására (polishing) a POLCA-t használtuk.³⁰ Az *I. ricinus* metagenomvizsgálata során, kórokozó baktériumok keresése céljából a kontigokat is taxonklasszifikáltuk. A kontigokat, amelyeket a Kraken2 kórokozó baktérium nemzetiségbe sorolt, BLAST³¹ segítségével reprezentatív prokarióta genomokon ellenőriztük.

A filogenetikai elemzések során a többszörös illesztést (multiple sequence alignment, MSA) a MAFFT³² programmal végeztük. A legjobb szubsztitúciós modellt a phangorn³³ R-csomag függvényeivel választottuk ki, a bayes-i információs kritérium (Bayesian information criterion, BIC) alapján. A generált neighbor-joining fákat maximum likelihood módszerrel optimalizáltuk. A bootstrap-értékeket 100 iterációval állítottuk elő. Ezt a *Vairimorpha ceranae* esetén a *polar tube protein 1* (PTP1), az *Ixodiphagus hookeri* esetén 28S riboszomális RNS gén alapján végeztük.

Az antimikrobiális rezisztenciagének (ARG) vizsgálatához a kontigokban a nyitott leolvasási kereteket (open reading frame, ORF) a Prodigal³⁴ felhasználásával prediktáltuk. Ezek aminosav-szekvenciáját a Resistance Gene Identifier (RGI) segítségével hasonlítottuk a Comprehensive Antibiotic Resistance Database (CARD) adatbázis ARG-ihez.^{35,36} Ettől a XIII. tanulmányban térünk el, amikor az ARG-ket a ResFinder-rel³⁷ prediktáltuk. Az ARG-k mobilitásának vizsgálatában az azokat hordozó kontigok plazmid- és profág-eredetét a PlasFlow³⁸, illetve a VirSorter2³⁹ segítségével elemeztük. Az ARG-k integrált mobilis genetikai elemekkel (iMGE) való kapcsoltságát távolság alapon vizsgáltuk.^{40,41}

5. Eredmények

Az alább bemutatott eredmények az utóbbi néhány évben végzett mikrobiális genomikai munkáinkból származnak. Ezek alapját képező tizenhárom közlemény mindegyikében meghatározó (első vagy utolsó, egyben levelező) szerző voltam. Az egyes szakaszcímekben, zárójelben látható római szám arra utal, hogy az ott bemutatottak mely forrásközleményből származnak (45. lap). A fajneveket a szakaszonkénti első megjelenéskor írtam ki teljesen, utána rövidítve. Az ARG-k, AB-k és AB-hatóanyagcsoportok angol nevét használtam, egységesítve a különböző közleményekben esetleg némileg eltérő ARG-neveket. Az eredeti ábrák angol nyelvű feliratait magyarra fordítottam.

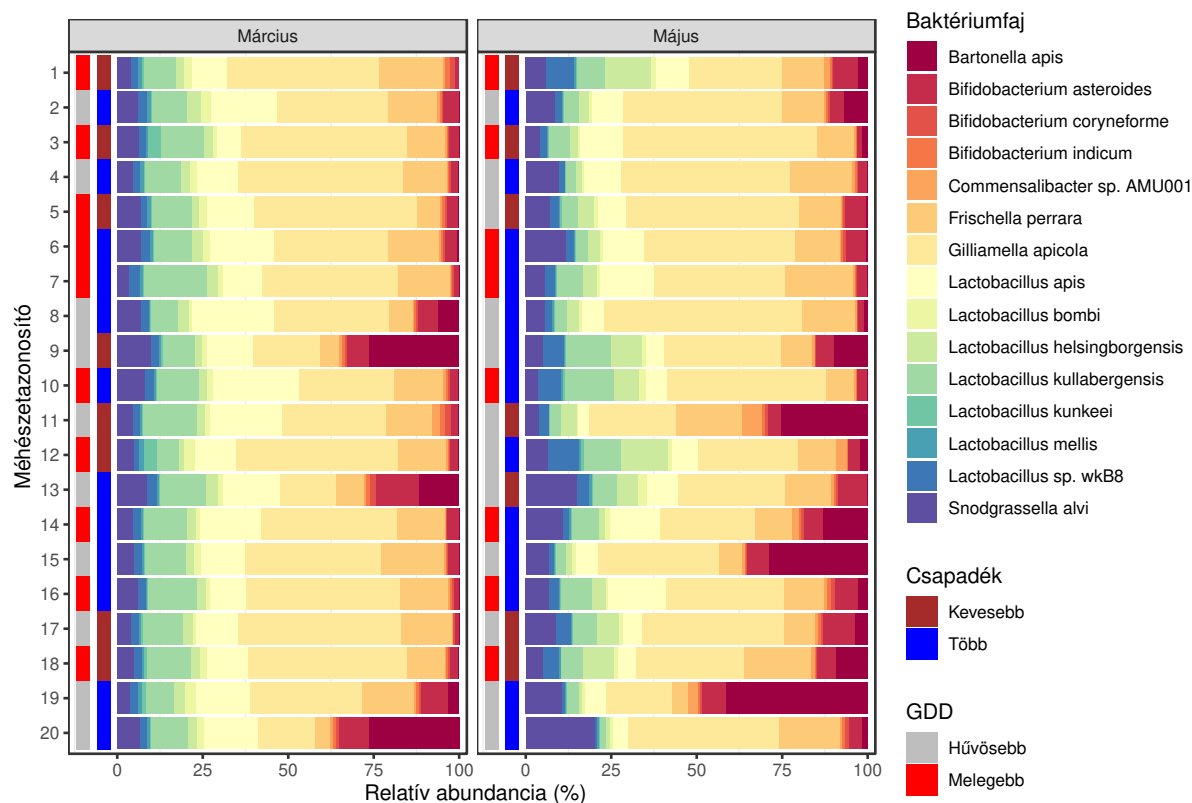
5.1. *Apis mellifera* bélmetagenom (I-III.)

A vizsgálatokhoz országos, ismételt mintavételezést végeztünk 2019-ben, a méztermelési időszak kezdetén (március) és csúcspontján (május). A 20 méhészetet úgy választottuk ki, hogy klimatikus környezetük szerint reprezentatív mintát kapjunk. Az éghajlati környezetet az éves növekedési foknapokkal (growing degree day, GDD) és az éves csapadékösszeggel jellemeztük. A környezeti változók szerint két-két kategóriát hoztunk létre: hűvösebb-melegebb, csapadékosabb-szárazabb. A klinikailag egészséges családokból vett mintákból generált short readek alapján végzett metagenomikai vizsgálattal vizsgáltuk a bakteriom természetes variabilitását. Emellett a kórokozó *Vairimorpha* (*Nosema*) *ceranae* és a méhek fonalas vírusának (*Apis mellifera* filamentous virus, AmFV)⁴² vizsgálatát is lehetővé tették a shotgun szekvenált minták.

Bakteriom (I.)

A márciusban gyűjtött, hűvösebb környezetből származó mintákban a α -diverzitás szignifikánsan ($p=0,0215$) magasabb volt, mint a márciusban melegebbjárásokból vett mintákban. A α -diverzitásban nem volt szignifikáns különbség a márciusban gyűjtött minták csapadékkategóriái között ($p=0,178$). A májusban gyűjtött mintákban nem volt szignifikáns különbség a GDD vagy a csapadékkategóriák között ($p=0,463$ és $p=0,456$). A β -diverzitás elemzésében a baktériumfajok összetételének vizsgálatával szignifikáns ($p=0,002$) különbséget találtunk a márciusi és a májusi minták között. A márciusi minták bakterioma hasonlóan szignifikáns ($p=0,02$) eltérést mutatott a hűvösebb és a melegebbjárások között.

Ugyanebből az időszakból származó mintáknál a csapadékszintek alapján nem azonosítható különbség ($p=0,155$). A májusban gyűjtött mintákban nem volt szignifikáns eltérés sem a GDD- ($p=0,277$), sem a csapadékkategóriák ($p=0,849$) között. A magbakteriombeli fajok mintavételenként relatív abundanciáját méhészetenként az 5.1. ábra mutatja be.



5.1. ábra. Mézelő méhek bélmintáinak magbakteriom-összetétele. A mintavételenkénti relatív abundancia mellett a méhészetek környezetének klimatikus (növekedési foknapok (GDD) és csapadék) jellemzőit színek jelölik.

A *Bartonella apis* (FC: 15,41, $q<0,001$ (q : korrigált p)), *Bifidobacterium asteroides* (FC: 1,61, $q=0,008$), *Commensalibacter* sp. AMU001 (FC: 2,46, $q<0,001$), *Lactobacillus helsingborgensis* (FC: 1,7, $q=0,008$) és *Snodgrassella alvi* (FC: 1,49, $q=0,011$) abundanciája szignifikánsan növekedett márciustól májusig. Ugyanebben az összehasonlításban a *Lactobacillus apis* (FC: 0,64, $q=0,007$), *Lactobacillus bombi* (FC: 0,64, $q=0,005$), *Lactobacillus kullabergensis* (FC: 0,57, $q<0,001$) és a *Lactobacillus mellis* (FC: 0,64, $q=0,005$) abundanciája szignifikánsan csökkent. A márciusban gyűjtött mintákban a *Lactobacillus kunkeei* (FC: 3,86, $q=0,094$) szignifikánsan magasabb abundanciájú volt a melegebb járásokban, mint a hűvösebbekben. Ugyanebben az időszakban a *B. apis* (FC: 0,02, $q<0,001$) és a *B. asteroides* (FC: 0,47, $q=0,003$) szignifikánsan alacsonyabb abundanciájú volt a melegebb járásokban, mint a hűvösebbekben. A márciusi mintákban a *L. kunkeei* (FC: 0,13, $q=0,011$) szignifikánsan alacsonyabb abundanciájú volt a csapadékosabb járásokban, mint a szárazabbakban. A májusban gyűjtött mintákban egyik magbakteriomfaj sem mutatott szignifikáns eltérést sem a GDD-, sem a csapadékkategóriák szerint összehasonlításban.

A szakirodalmi adatok alapján a mézelő méhek viszonylag egyszerű bélbakteriótával rendelkeznek, amelyet kisszámú faj alkot. A bakteriótának vannak olyan tagjai, amelyek mindig megtalálhatók a bélben, így a *S. alvi*, a *G. apicola*, illetve *Lactobacillus* és *Bifidobacterium* fajok. Ezek mellett gyakran előforduló, de nem nélkülözhetetlen fajok pl. *B. apis* és *F. perrara*.^{43–47} Eredményeink összhangban vannak ezekkel a korábbi eredményekkel, mivel a magbakteriom tartalmazott különböző *Snodgrassella*, *Gilliamella*, *Lactobacillus* és *Bifidobacterium* fajokat (*B. asteroides*, *B. coryneforme*, *B. indicum*, *L. apis*, *L. helsingborgensis*, *L. kullabergensis*, *L. mellis*).

A bélbakteriom normál összetételének és természetes variabilitásának megismerése fontos alapot jelenthet annak értékelésében, hogy a különböző kóros állapotok hogyan változtathatják meg az összetételét. Várakozásainknak megfelelően jelentős különbségeket figyeltünk meg a szezonális és a környezeti állapotok között. A bélbakteriom jelentősen különbözött a márciusi és a májusi mintáinkban.

***Vairimorpha ceranae* (II.)**

Az összes mintában azonosítani tudtunk *V. ceranae* eredetű readeket, amelyek nagyobb számban fordultak elő a májusi mintákban, mint a márciusi mintákban (FC: 3,13, 95%CI: 1,61-6,11, $p < 0,001$). A márciusi minták esetében a hőmérséklet és csapadék tekintetében nem tudtunk szignifikáns különbségeket feltárni, azonban a melegebb területekről gyűjtött mintákban nagyobb mennyiségben azonosítottuk a *V. ceranae* eredetű szekvenciákat, mint a hűvösebb területekről gyűjtött minták esetében (FC: 1,66, 95%CI: 0,44-6,21, $p = 0,451$). Ugyanezen mintavétel esetén a csapadékosabb területekről gyűjtött mintákban nagyobb arányban azonosítottuk a *V. ceranae*hoz sorolt readeket, mint a szárazabb területekről gyűjtött mintákban (FC: 2,98, 95%CI: 0,81-10,97, $p = 0,101$). Májusban a hőmérséklet esetében szintén nem figyeltünk meg szignifikáns különbséget, azonban a melegebb területekről gyűjtött mintákban kisebb mennyiségben tudtunk *V. ceranae* eredetű readeket azonosítani (FC: 0,44, 95%CI: 0,16-1,20, $p = 0,107$). Csapadékosabb területekről származó májusi minták szignifikánsan nagyobb arányban tartalmaztak *V. ceranae* eredetű readeket, mint a szárazabb területekről gyűjtöttek (FC: 3,77, 95%CI: 1,42-10,00, $p = 0,008$).

A májusi minták közül a 19. méhészetből, de novo assembly segítségével létre tudtuk hozni a *PTP1* gén egy 649 bp hosszúságú darabját. E szakasz alapján végzett filogenetikai elemzés során thaiföldi, spanyolországi és hawaii mintákkal mutatta a legnagyobb szekvenciális hasonlóságot.

Fonális vírus (III.)

A taxonklasszifikáció során víruseredetűnek minősített readok mintánkénti minimális, maximális és medián száma 443, 72 010 és 1074 volt. Ezek között a méhek fonális vírusának genomjához illeszkedő readek domináltak, minden minta tartalmazott ebből a fajból

származó readeket. Az *AmFV*-ból származó readek nagyobb mennyiségben voltak jelen a márciusi mintákban, mint a májusi mintákban (FC: 5,53, 95%CI: 2,38-12,84, $p < 0,001$). Márciusban a melegebb területekről gyűjtött mintákban nagyobb mennyiségben azonosítottunk *AmFV*-eredetű readeket, mint a hűvösebb területekről gyűjtött mintákban (FC: 26,05, 95%CI: 7,31-92,81, $p < 0,001$). A nedvesebb területekről márciusban gyűjtött mintákban kisebb arányban azonosítottunk *AmFV*-eredetű readeket, mint a szárazabb területekről gyűjtött mintákban (FC: 0,33, 95%CI: 0,13-0,8, $p = 0,014$). A májusban gyűjtött mintákban talált *AmFV*-readek gyakorisága nem mutatott szignifikáns különbséget a csoportok között, sem a környezeti hőmérséklet, sem a csapadék alapján (FC: 1,44, 95%CI: 0,61-3,39, $p = 0,40$; FC: 1,85, 95%CI: 0,78-4,37, $p = 0,16$).

Négy mintából (márciusi 10., 16., 18. és májusi 13.) gyakorlatilag a teljes *AmFV* genomot össze lehetett állítani de novo assemblyvel. A négy minta readjeit a KR819915.2 referenciagenomra illetve 99,8-99,6%-ban lefedték azt. Az így létrehozott genomok (OR270109.1, OR644609.1, OR644610.1, OR644611.1) a referenciagenomhoz hasonlítva legalább 97,7%-os átlagos nukleotid-azonosságot mutattak. A DNS-polimeráz filogenetikai elemzése alapján svájci szekvenciához hasonlítottak leginkább a magyar minták.

Összefoglalás

Mivel a mézelő méhek bélbakteriótája kevés számú fajból áll, jó modell lehet a bélbakterióta természetes variabilitásának vizsgálatában. Ennek az ismerete azért fontos, hogy viszonyítási alapunk legyen a kóros eltérések azonosításában. Eredményeink azt mutatják, hogy szezonálisan és környezeti körülményektől függően nagy különbségek figyelhetők meg az egészséges méhek bélbakteriomjában. A *V. ceranae* hazai előfordulási gyakoriságában évszakos és klimatikus eltéréseket találtunk. Elsőként állítottunk össze fonalas vírusgenomokat hazai mintákból.

5.2. *Ixodes ricinus* metagenom (IV-V.)

A mézelő méhekkel kapcsolatos vizsgálatához hasonló módon a klimatikus környezetet figyelembe vevő, országosan reprezentatív mintavételezés során 20 helyről gyűjtöttünk *I. ricinus* kullancsokat. Ezek közül 17 helyről lett a szekvenáláshoz elegendő számú nimfát és nőtényt tartalmazó mintánk.

Bakteriom (IV.)

Az α -diverzitás nem mutatott szignifikáns különbséget sem a nőtények és nimfák ($p = 0,138$), sem a nőtényeken belül a hidegebb és melegebb ($p = 0,562$), sem a szárazabb és nedvesebb környezet ($p = 0,577$) összehasonlításában. A nimfákon belül ugyanezek az összehasonlítások $p = 0,174$ és $p = 0,309$ értéket adtak. A nemzetségszintű β -diverzitás

szignifikáns különbséget ($p=0,005$) mutatott a nőstények és nimfák között. A nőstényeken belül nem mutatott szignifikáns eltérést sem a hőmérséklet- ($p=0,444$), sem a csapadék-kategóriák ($p=0,244$) közötti összehasonlítás, ahogy a nimfákon belül sem (hőmérséklet $p=0,108$, csapadék $p=0,722$).

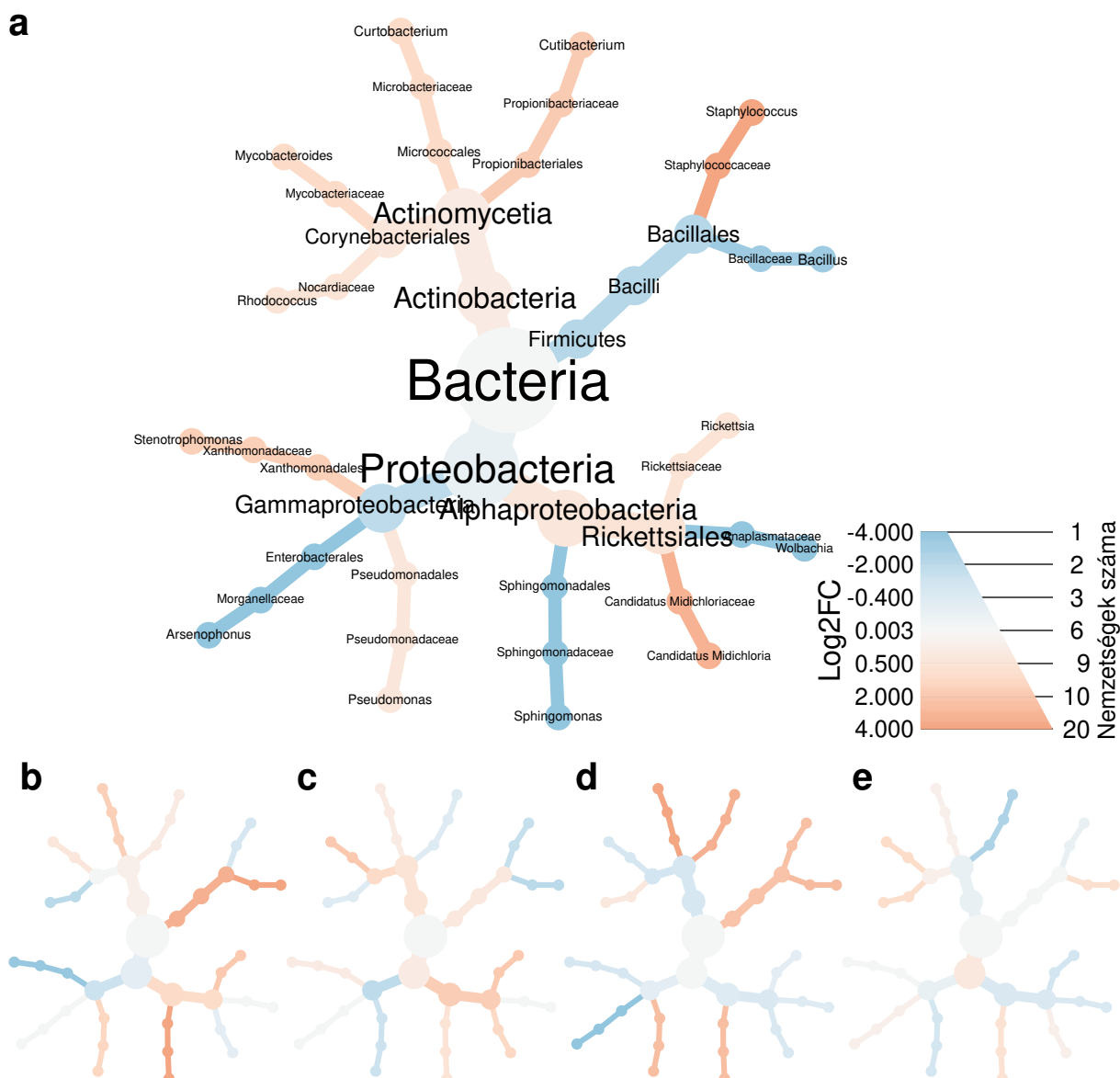
A csoportok taxononkénti összehasonlítás szerinti abundancia-eltéréseit az 5.2. ábra foglalja össze. A nőstények és nimfák összehasonlításakor a következő nemzetségek mutatnak szignifikáns ($q<0,05$) abundancia-különbségeket: *Arsenophonus*, *Bacillus*, *Candidatus Midichloria*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*, *Wolbachia*. A nőstény mintákban a hőmérséklet szerint a következőket találtuk differenciálónak: *Curtobacterium*, *Pseudomonas*, *Sphingomonas*. A nőstényeknél nem volt olyan nemzetség, amely szignifikáns különbséget mutatott volna a csapadékkategóriák között. A nimfáknál a *Curtobacterium* szignifikáns eltérést mutatott a hőmérsékleti csoportok között, valamint a *Bacillus* és *Curtobacterium* a csapadékkategóriák között. Az *Arsenophonus*, *Candidatus Midichloria* és *Wolbachia* nemzetség tagjai kullancs-asszociáltak, míg a *Bacillus*, *Curtobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus* nemzetségbeliek talaj-, víz-, növény-, gerinces eredetű, így inkább a környezetből jelenhettek meg a mintákban.^{48–51}

Az *Arsenophonus* a nőstényekben szignifikánsan alacsonyabb abundanciát mutatott, mint a nimfákban, mely eltérés felvetette, hogy a nimfák egy része *Ixodiphagus hookeri*-fertőzött lehetett. Ez a parazitoid a lárvá és a nimfa kullancsokba petézik, petéi csak a vérszívott vagy a vért szívó nimfákban tudnak kifejlődni.^{52,53} Ha a kullancs immunrendszere nem képes elhatárolni a petéket, akkor azok kikelnek, a lárvák a kullancs szöveteit fogyasztják, és így annak pusztulását okozzák.⁵² A mintáinkban talált *Arsenophonus nasoniae*, amely az *I. hookeri* endoszimbiontája, Bohacsova et al.⁵⁴ szerint csak a parazitoiddal fertőzött kullancs nimfákban mutatható ki. Így az *A. nasoniae*-t hordozó nimfák a parazitoid miatt gyakran nem érik el a kifejlett stádiumot. A parazitoid-találatok hiánya a kifejlett nőstényekben az azzal fertőzött nimfák elpusztulása miatt lehet.

A nőstények és nimfák között látható jelentős *Candidatus Midichloria* abundancia-eltéréshez hasonlóra vannak irodalmi adatok. Így kifejlett *I. ricinus* nőstényekben inkább mutatható ki a *Candidatus Midichloria*, mint hímekben.⁵⁵

A *Wolbachia* nemzetség abundanciája a nimfákban magasabb volt. Az egyik azonosított faj, a *W. pipientis* jelenléte erős kapcsolatot mutat az *I. hookeri*-ével.^{56,57} Így a nimfák magasabb *Wolbachia* abundanciájának oka lehet ugyanaz, mint a *Arsenophonus* nemzetség esetében.

A magbakteriom tagjai mellett további, viszonylag kisebb gyakoriságú, de nagy klinikai jelentőségű baktériumnemzetségekből (*Anaplasma*, *Bartonella*, *Borrelia*, *Borreliella* és *Ehrlichia*) származó szekvenciákat is kimutattunk. Az *I. ricinus* kullancsok a Lyme-borreliózist okozó *Borrelia burgdorferi* sensu lato komplex vektora.^{58–60} Mintáinkban a *B. burgdorferi*-t nem mutattuk ki, azonban más *Borrelia* és *Borreliella* azonosított fajok (*B. garinii*, *B. valaisiana*, *B. afzelii*, *B. coriaceae*, *B. miyamotoi*) is szerepet játszhatnak a betegség kialakításában.

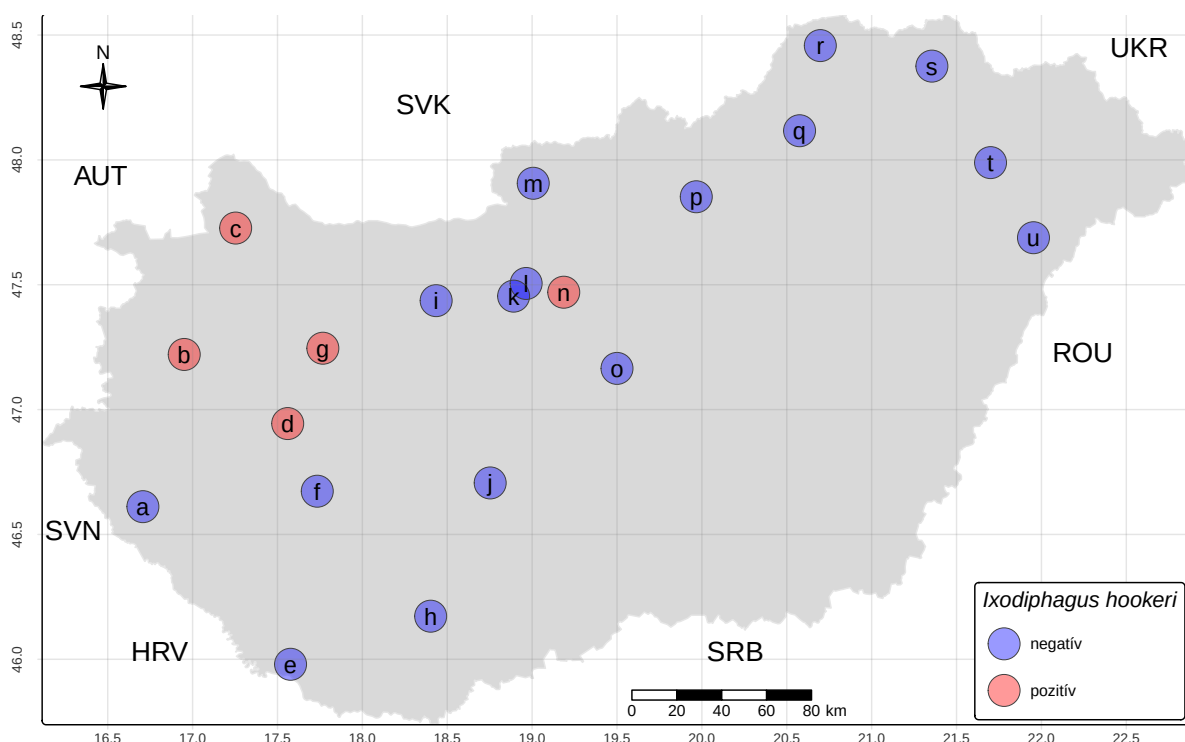


5.2. ábra. A magbakteriom taxononkénti abundancia-eltérései. A színek az összehasonlított csoportok medián abundanciájának változását jelölik. **a)** A nőstények nimfákhoz (referencia) hasonlítva. **b)** A nősténymintákon belül a melegebb területekről származók a hűvösebb területekről származókhoz hasonlítva. **c)** A nősténymintákon belül a szárazabb területekről származók a nedvesebb területekről származókhoz hasonlítva. **d)** A nimfamintákon belül a melegebb területekről származók a hűvösebb területekről származókhoz hasonlítva. **e)** A nimfamintákon belül a szárazabb területekről származók a nedvesebb területekről származókhoz hasonlítva.

Ixodiphagus hookeri (V.)

Az *Arsenophonus* és *Wolbachia* nemzetség relatív abundanciájának nimfák és nőstények közötti szignifikáns eltérése okán célzott vizsgálatot végeztünk az *I. hookeri* esetleges jelenlétének genomikai igazolására. Mivel a 17 mintánkkal átfedő gyűjtési időszakból származó, további négy-négy *I. ricinus* nimfa és nőstény metagenom-szekvenálási adatai is rendelkezésre álltak, az *I. hookeri* jelenléti vizsgálatába ezeket is bevontuk.

A 21 felnőtt nőstény minta egyikében sem azonosítottunk olyan kontigokat, amelyek egyértelműen *I. hookeri* eredetűek lehettek. A 21 nimfamintából ötben (*b*, *c*, *d*, *g*, *n*) találtunk *I. hookeri*-ből származó kontigokat (5.3. ábra). A hazai mintákból létrehozott kontigok *I. hookeri* 28S rRNS génjével (MH077537.1) átfedő szakaszainak szekvenciális egyezősége 378/386 (97,9%, *b* minta, GenBank id: OQ316579), 556/559 (99,4%, *c*: OQ316577), 439/447 (98,2%, *d*: OQ316581), 445/453 (98,2%, *g*: OQ316578) és 300/308 (97,4%, *n*: OQ312115).



5.3. ábra. Az *I. ricinus* minták gyűjtési helyei. A piros pontok azokat a mintavételi helyeket jelölik, ahol találtunk *I. hookeri* szekvenciákat, a kék pontok pedig azokat, ahol nem. A *k*, *l*, *n*, *t* jelölésű helyekről származott a kiegészítő négy-négy nimfa-, és nőstényminta.

Vizsgálatunkban egy kivétellel az összes olyan mintavételi pont, ahol bizonyítottan *I. hookeri* található, Nyugat-Magyarországon volt. A négy nyugat-magyarországi mintavételi pont alkotta klaszter közel fekszik az osztrák és szlovák határhoz. Az *I. hookeri* ausztriai előfordulásáról nem találtunk adatokat. A fajt Szlovákiában, korábban három helyen azonosították; Soponya közelében (*Haemaphysalis concinna*),⁶¹ Pozsonyhoz közel (*I. ricinus*)^{62,63} és a Szlovák Karsztban (*I. ricinus*, és *H. concinna*).⁶⁴ Eredményeink közlésekor a magyarországi *d* mintavételi pont jelentette a *I. hookeri* legdélebbi kimutatási pontját Közép-Európában, ami felveti annak a lehetőségét, hogy akár a Balkán-félszigeten is előfordulhat a faj. Annak ellenére, hogy az *m*, *p*, *q*, *r* és *s* mintavételi pontok olyan területek közelében helyezkednek el, ahol az *I. hookeri* jelen van Szlovákiában, az ezekről a helyekről gyűjtött mintákban nem találtunk *I. hookeri* szekvenciákat.

Összefoglalás

A bakteriom csoportonkénti eltérései mellett kórokozó baktériumfajokat is azonosítottunk. Az *Arsenophonus* és *Wolbachia* nemzetség relatív abundanciájának a kifejllett nőstényekhez viszonyított nimfákban tapasztalt magasabb volta miatt végzett célzott vizsgálattal azonosítottuk az *I. hookeri*-t, Magyarországon először.

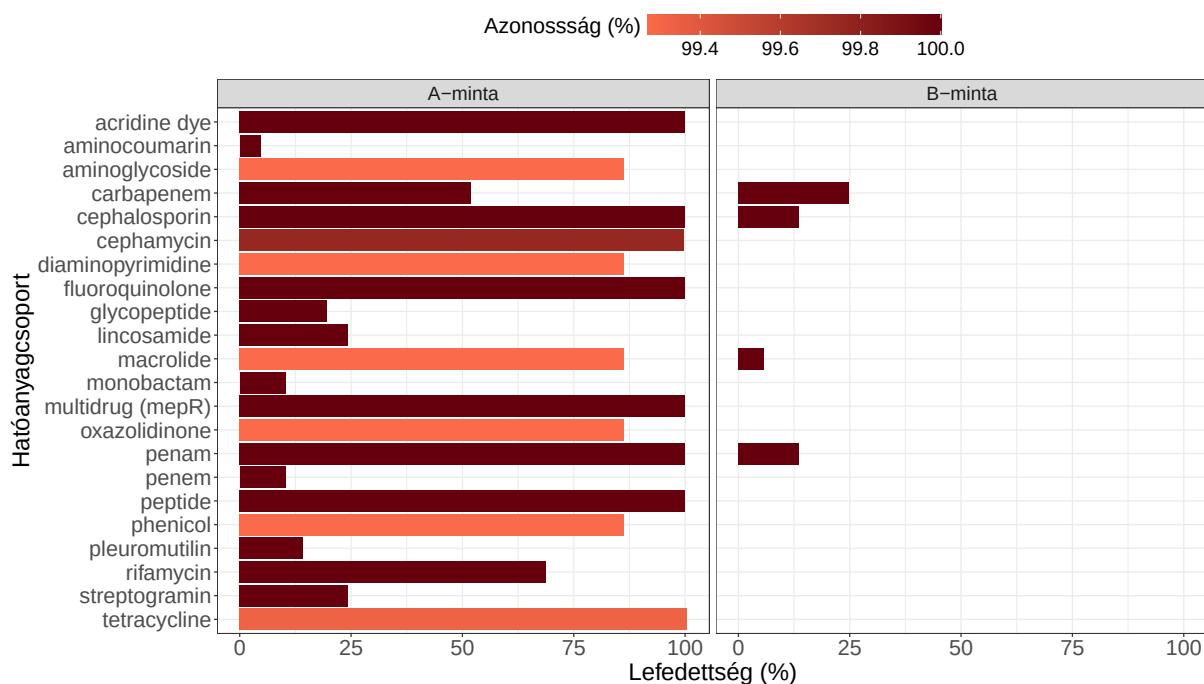
5.3. Nyerstej-rezisztom (VI.)

Két, piacon vásárolt nyerstej-minta metagenomikai, rezisztom és mobilom elemzését végeztük el. A két minta közül budapesti piacon vásárolt, nagylétszámú állományból származó A-mintában három „tökéletes” ARG-találatot kaptunk: *mepR*, *mgrA* és *Staphylococcus aureus* *norA*. Az ezeket az ORF-eket tartalmazó kontigok taxonómiai besorolása alapján a legvalószínűbb eredetük a *Staphylococcus* nemzetséghez tartozó baktérium lehetett. Az MGE-elemzés azt mutatta, hogy ezen ORF-ek egyike sem mobil. Ebben a mintában a „szigorú” találatok szekvencia-lefedettsége 2,12% és 100% között mozgott, átlagosan 36,61% volt. Az ORF-ek és az ARG referenciaszekvenciák azonossága 95,02% és 100% között volt, átlagosan 99,59%. Az ARG-t tartalmazó kontigok a következő nemzetségekhez sorolhatók: *Acinetobacter* (2,86%), *Carnobacterium* (11,43%), *Chryseobacterium* (2,86%), *Corynebacterium* (2,86%), *Kocuria* (11. 43%), *Lactococcus* (8,57%), *Leuconostoc* (2,86%), *Macrococcus* (2,86%), *Moraxella* (5,71%), *Staphylococcus* (37,14%) és *Streptococcus* (11,43%). A legalább 90%-os lefedettségű és 90%-os szekvenciaazonosságú ARG-találatok (és azokat feltételezhetően hordozó baktériumok): a *DHA-1* (*S. aureus*), *mepA* (*S. aureus*), *tet(38)* (*S. aureus*) és *blaZ* (*Staphylococcus* nemzetség tagja). Ezek közül a *blaZ*, illetve a *tet(38)* gént hordozó kontigot plazmid-eredetűként prediktáltuk. A *blaZ* ARG-nek további mobilitást biztosíthat, hogy az azt hordozó kontig tíz ORF távolságon belül tartalmazott egy fág integráz ORF-et.

A B-mintában nem volt tökéletes találat, a „szigorú” találatok szekvencia-lefedettsége ebben a mintában 5,71% és 24,83% között mozgott, az átlag 13,31% volt. Az ORF-ek és az ARG referenciaszekvenciák közötti azonosság 100,00% volt minden egyes kimutatott ARG esetében. Az ARG-t tartalmazó kontigok a *Acinetobacter* (50%) és *Delftia* (50%) nemzetségekhez sorolhatók. Az azonosított ARG-k egyike sem volt kapcsolatba hozható mobilis genetikai elemekkel.

Az 5.4. ábra foglalja össze, hogy a prediktált ARG-k mely hatóanyagcsoport hatását befolyásolhatják. Mivel egy hatóanyagcsoport több ORF-hez is társulhat, azt a találatot választottuk ki, amely az adott hatóanyagcsoportban a legnagyobb lefedettséget és szekvenciaazonosságot mutatta.

A két mintánk különbözik mind a magbakteriom összetételében, mind ARG-tartalmában. Az A-mintában a bakteriomot a Gram-pozitív baktériumok dominálták. Továbbá az ARG-t tartalmazó kontigok többsége Gram-pozitív baktérium-eredetűnek



5.4. ábra. A nyerstej-mintákban talált ARG-k maximális lefedettsége és szekvenciális azonossága hatóanyagcsoportonként.

klasszifikálódott. A B-mintában a Gram-negatív baktériumok uralták a bakteriomot. Ebben a mintában nemcsak a kimutatott ARG-k száma volt alacsonyabb, hanem az ARG-k maximális lefedettsége is. Ennek a jelenségnek az okát a B-minta alacsonyabb szekvenálási mélységében látjuk. Mivel az ORF-eknek a referencia ARG-kkel való szekvenciális azonossága magas, feltételezhetjük, hogy az prediktált ORF-ek ARG-kból származnak. Ennek megfelelően az ARG-k alacsonyabb lefedettségének lehetséges oka az lehet, hogy a teljes ORF-ek összeállításához nem volt elegendő a szekvenálási mélység. Az A- és B-minta közötti ARG-különbség további lehetséges oka lehet az, hogy az egészségügyi problémák (pl. tüdőgyulladás) viszonylag gyakran fordulnak elő a nagyüzemi gazdaságokban. Mivel az antibiotikumok használata az állatorvosi gyakorlatban – az embergyógyászatához képest – megengedőbb a bakteriális fertőzések kezelésében, ez szelekciós nyomást gyakorol az állományok baktériumaira, ami növelheti az ARG-k gyakoriságát.

Összefoglalás

A haszonállattartásban létrehozott élelmiszerek fogyasztásával – a többi tápanyag mellett – az emberi szervezetbe tömegesen érkeznek genomok, az azokat alkotó gének. Mivel ezek a termékek nem csíramentesek, ezért mikroorganizmusokkal együtt fogyasztjuk azokat. Eredményeink alapján a nyers tejben ARG-t hordozó baktériumok is lehetnek, amelyek a tej elfogyasztásával az emésztőrendszerünkbe juthatnak.

5.4. Probiotikus rezisztomok (VII-IX.)

Joghurt és kefir (VII.)

A nyers tej rezisztomjának eredményei felvetik, hogy erjesztett tejtermékek – mint a joghurt és a kefir – előállítása során, ha az alapanyag tartalmaz ARG-t hordozó baktériumokat, akkor azok szaporodása révén az ARG-k mennyisége is megnőhet. Ennek vizsgálata céljából kefir-oltókultúrák (n=4), kefirtermékek (n=15), kefirkészítéséhez használt baktériumtörzsek (n=7), joghurttermékek (n=5), egy joghurt-oltókultúra (n=1) és tejminták (n=3) szekvenálási adatait használtuk fel.

A kefir oltókultúrákban a domináns baktériumfajok relatív abundancia szerint csökkenő sorrendben: *Lactobacillus kefiranofaciens* (57,7%), *Lactobacillus kefir* (15,7%), *Streptococcus thermophilus* (15,4%), *Lactococcus lactis* (6,8%), *Leuconostoc mesenteroides* (1,7%), *Leuconostoc pseudomesenteroides* (1%) és *Lactobacillus helveticus* (1%). A kefir termékekben található legjelentősebb fajok átfedésben voltak a oltókultúrákban azonosított fajokkal, bár relatív gyakoriságukban voltak különbségek (*L. kefiranofaciens* (55,4%), *L. mesenteroides* (35,7%), *Acetobacter ghanensis* (2,1%), *L. helveticus* (2,1%), *L. kefir* (1,8%), *Acetobacter orientalis* (0,6%), *Acetobacter oryzoeni* (0,2%)). A joghurt oltókultúrát a *Streptococcus thermophilus* (92,8%), a *Bifidobacterium animalis* (3,6%) és a *Lactobacillus delbrueckii* (3,5%) uralta, míg a joghurttermékek baktériomját a *S. thermophilus* (83,9%), *L. delbrueckii* (10,1%), *Lactobacillus acidophilus* (4,6%) és *B. animalis* (1,2%).

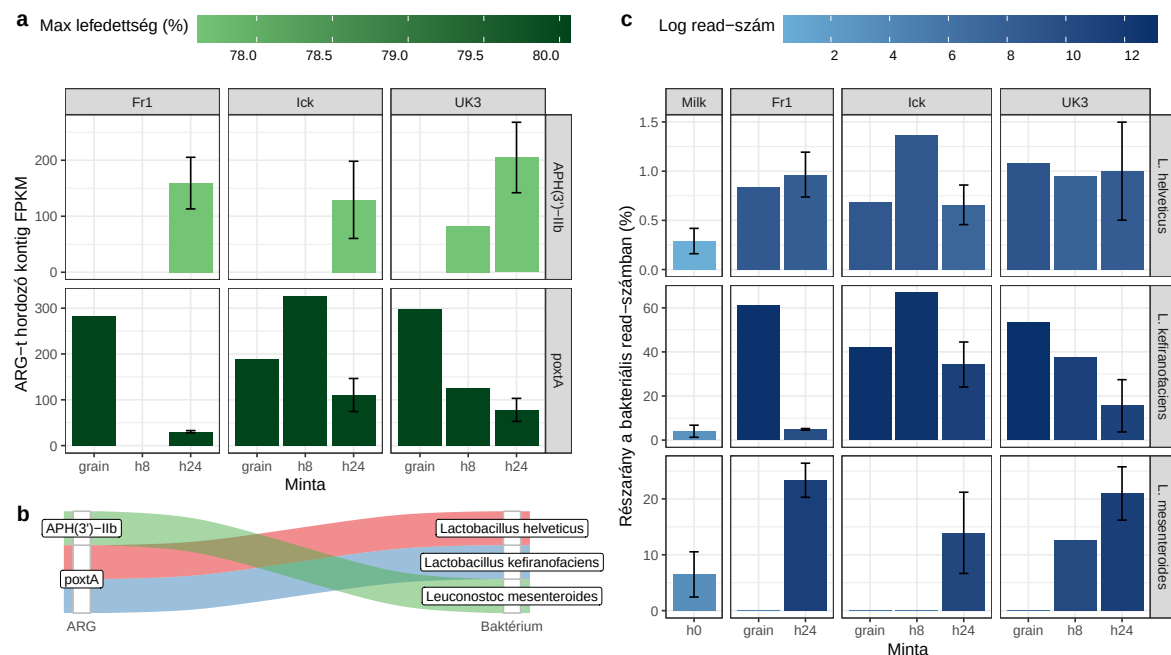
A legnagyobb ARG-abundancia a kefirtörzs mintáiban figyelhető meg (Fragments Per Kilobase per Million, FPKM átlag: 282, szórás: 51,1), ezt követték a kefirtermékek (240 ± 78,6) és a kefir oltókultúrák (209 ± 106). A joghurtmintáknál alacsonyabb abundanciát láttunk, az egyetlen oltókultúrában az FPKM 17,9 volt, míg a termékekben 45,7 ± 32,2. Ennek a jelenségnek egyik lehetséges oka lehet, hogy a kefir oltókultúrákban gombák is vannak. Mivel a gombák antibakteriális toxinokat termelhetnek, az ARG-kel rendelkező baktériumok versenyelőnyre tehetnek szert a gombákkal való együttélés során.

A kefirmintáinkban huszonkét ARG-t azonosítottunk termékben, kettőt az oltókultúrában és egyet a törzsben. A joghurtban egy ARG volt termékben és kettő oltókultúrában. Plazmidredetű ARG-tartalmú kontigot nem találtunk, az iMGE-vizsgálatunk azt mutatta, hogy egy mintában az *lmrD* gén mobil lehet. A kefirtermékben *Enterobacter hormaechei* genomjához klasszifikálódó kontigok a következő géneket tartalmazták: *acrB*; *acrD*; *ACT-36*; *bacA*; *baeR*; *CRP*; *emrB*; *emrR*; *Escherichia coli marR* mutant conferring antibiotic resistance; *E. coli soxS* with mutation conferring antibiotic resistance; *E. coli UhpT* with mutation conferring resistance to fosfomycin; *FosA2*; *marA*; *mdtB*; *mdtC*; *msbA*; *oqxA*; *oqxB*; *ramA*. *L. helveticus* és *L. kefiranofaciens* eredetű *poxtA*), valamint *L. mesenteroides* eredetű *APH(3')-IIb* gént azonosítottunk. A kefir oltókultúrában a *L. kefiranofaciens* eredetű *poxtA*, *L. lactis* eredetű *lmrD* gént azonosítottunk. A kefirtörzsek *L. kefiranofaciens*-hez és *L. plantarum*-hoz kapcsolható *poxtA* gént tartalmaztak. A jog-

hurokban a *L. acidophilus*-hoz és az *L. delbrueckii*-hez köthetően azonosítottunk *portA* gént. A joghurt oltókultúrában *B. animalis*-hoz klasszifikálható *Bifidobacterium adolescentis* *rpoB* mutans conferring resistance to rifampicin, illetve *L. delbrueckii*-hez kapcsolható *portA* gént azonosítottunk. Minden baktériumot, amelyeket az ARG-t tartalmazó kontigokhoz klasszifikáltunk, széles körben használják erjesztett tejtermékek előállítása során.^{65,66} Erjesztett italokból és joghurtokból izolált baktériumok ARG-vizsgálata során az *APH(3'')-III*, *APH(6')-APH(2'')*, *sul1*, *tet(M)* kimutatható volt a *Lactobacillus bulgaricus* törzsekben, míg a *APH(3'')-II*, *sul1*, *sul2*, *strA*, *strB*, *tet(M)* a *S. thermophilus*-ból származott.⁶⁷ Vizsgálatunkban az APH géncsaládba tartozó *APH(3')-Iib* gén feltehetően *L. mesenteroides*-ből származik. Hasonlóképpen, Carr et al.⁶⁸ együttes előfordulást találtak az *APH(3')-Ia* és a *L. mesenteroides* között kínai nyálmintákban. További hasonlóság Carr et al.⁶⁸ eredményeivel, hogy az *lmrD* gént az *L. lactis*-ban azonosítottuk. Guo et al.⁶⁹ ARG-eket mutatott ki *Lactobacillus* törzsekben, fermentált tejtermékekből, így az *erm(B)*, *gyrA*, *rpoB*, *vanE*, *vanX* géneket a *Lactobacillus casei*-ben, *APH(3'')-III*, *dfrD*, *erm(B)*, *gyrA*, *tet(W)*, *vanX* géneket *L. helveticus*-ban, *erm(B)* és *vanX* gént *L. plantarum*-ban.

ARG-abundancia változása kefirerjesztés során

A Walsh et al.⁷⁰ által közzétett, három oltókultúrával (Fr1, Ick, UK3) végzett fermentáció során, különböző időpontokban vett mintákat elemeztük. Az ő bakteriom-elemzésükhöz⁷⁰ hasonlóan eredményeink azt mutatják, hogy az ARG-abundancia változik a kefir erjedési folyamata során (5.5/a. ábra). Mindhárom oltókultúra esetén az *APH(3')-Iib* jelen van



5.5. ábra. Változások a kefir erjedése során. **a)** Az ARG-abundancia oltókultúrából (grain), 8 (h8) és 24 (h24) órás fermentálás után vett mintákban. **b)** ARG-k és legvalószínűbb eredetük. **c)** A valószínűsíthető ARG-t hordozó baktériumok relatív abundanciája.

a 24 órán keresztül erjesztett kefirben, míg a többi időpontban hiányzik, kivéve az UK3-mintában, ahol 8 óra után is detektálható volt. Az *PortA* a 8 órás Fr1-minta kivételével minden mintában kimutatható volt. Az abundancia 24 órán át követve az oltókultúra 10%-ára (Fr1), 59%-ára (Ick) és 26%-ára (Uk3) csökkent. Az Ick-mintákban a 24 órás minta *portA* abundanciája a 8 órásénak 34%-ára, a UK3 erjesztési láncban 62%-ra csökkent.

Az ARG-ket tartalmazó kontigok taxonklasszifikációjának eredményét az 5.5/b. ábra foglalja össze. A *APH(3')-IIb* gént tartalmazó összes kontig a *L. mesenteroides*-hez, a *portA* kontigok a *L. helveticus* és a *L. kefiranofaciens* fajokhoz rendelhetők. E baktériumok genomjára illeszkedő readok részaránya az összes bakteriális readok között az 5.5/c. ábrán látható. A csökkenő trendet mutató *L. kefiranofaciens*-vel ellentétben a *L. mesenteroides* relatív abundanciája nő az idővel. Az *L. helveticus*-ra illesztett readok abundanciája nem mutat tendenciaszerű növekedést vagy csökkenést az idő múlásával. Az *APH(3')-IIb* abundancia növekedése pozitív összefüggést mutat a *L. mesenteroides* relatív abundanciájával. Hasonlóképpen, a *portA* abundanciája csökken a *L. kefiranofaciens* részarányával.

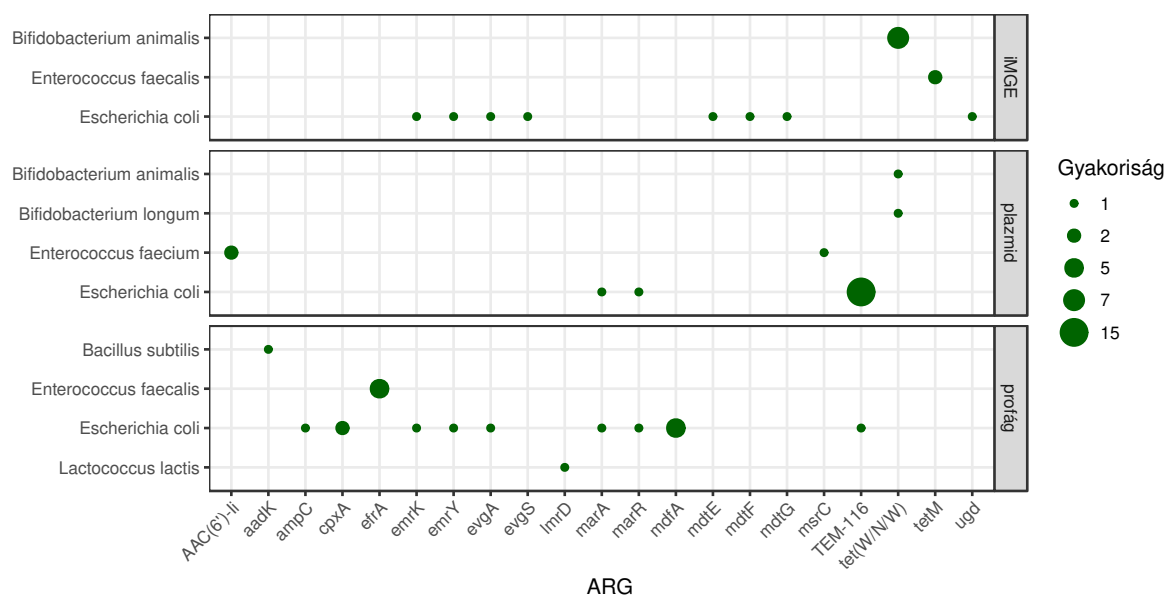
Probiotikumok rezisztomja (VIII.)

A joghurt-, és kefir minták vizsgálatából származó eredmények felvetik azt a kérdést, hogy egyéb probiotikus termékek tartalmaznak-e ARG-ket, illetve azok milyen mértékben mobilisak. Ennek megválaszolásához 27 probiotikus termék és 20 ilyen termékekből izolált baktériumtörzs rezisztomját és mobilomját elemeztük.

A termékek domináns nemzetségei átlagos relatív abundanciájuk csökkenő sorrendjében a *Lactobacillus* (40%), *Enterococcus* (35%), *Bifidobacterium* (34%), *Limosilactobacillus* (34%), *Lactococcus* (32%), *Lacticaseibacillus* (31%), *Bacillus* (26%), *Weizmannia* (22%), *Ligilactobacillus* (19%), *Streptococcus* (18%), *Lactiplantibacillus* (12%) és *Sphingobacterium* (2%) voltak.

Míg minden metagenomikai minta tartalmazott, az izolátumok többsége (n=11) nem tartalmazott ARG-t. Nem volt kimutatható ARG a *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Pseudomonas* sp. *RGM2144* vagy *Streptococcus thermophilus* izolátumokban. A legtöbb ARG-t olyan hat *E. coli* izolátumban találtuk, amelyeket ugyanabból a probiotikus termékből izoláltak. Az ARG-k jelentős része mobilis, amelyek iMGE-khez kapcsolódnak, plazmidokban vagy profágokban helyeződnek (5.6. ábra) össze.

A *Bacillus subtilis*-ben azonosított ARG-k: *aadK*, *B. subtilis mprF*, *B. subtilis pgsA* with mutation conferring resistance to daptomycin, *bmr*, *lmrB*, *mphK*, *vmlR*, *ykkC*, *ykkD*. Ezek mindegyikét azonosították már ebben a fajban, sőt többük esetén található adat arra vonatkozóan, hogy a fajra vagy a *Bacillus* nemzetségre jellemzők.⁷¹⁻⁷⁷ A *Bifidobacterium animalis*-ban talált *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin és *tet(W/N/W)* ARG-ket már mások is leírták a fajban.⁷⁸⁻⁸¹ A *Bifidobacterium bifidum*-ban *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *B. bifidum*



5.6. ábra. A probiotikumbeli mobil ARG-gyakoriságok baktériumfajonként. A pontméret az adott ARG iMGE-hez, plazmidhoz vagy profághoz kapcsolható előfordulási gyakoriságát jelzi.

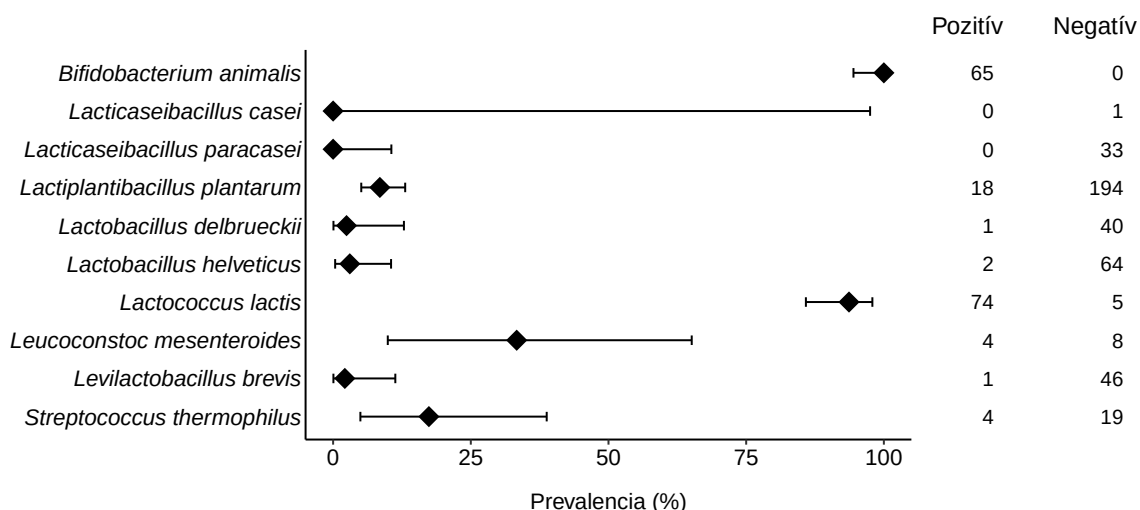
ileS conferring resistance to mupirocin és *tet(W/N/W)* géneket találtunk. Ezek közül a másodikat leírták már a fajban.⁸² *Bifidobacterium breve*-hez kapcsolhatóan *B. adolescentis* *rpoB* mutants conferring resistance to rifampicin és *tetW*, míg *Bifidobacterium longum* eredetűként *B. adolescentis* *rpoB* mutants conferring resistance to rifampicin és *tet(W/N/W)* ARG-eket azonosítottunk. Az *Enterococcus faecalis*-hoz köthetően több ARG-t találtunk: *dfrE*, *efrA*, *efrB*, *emeA*, *lsaA*, *tetM*. A *dfrE* gént először az *E. faecalis*-ban azonosították,⁸³ de mutatnak arra adatok, hogy nem kizárólag erre a fajra jellemző.⁸⁴ Az *efrA* és *efrB* géneket már szintén leírták az *E. faecalis* és *E. faecium* fajokban.^{85,86} Ismereteink szerint az *emeA* gént csak az *E. faecalis*-ban azonosították eddig.⁸⁵ A *E. faecalis* mellett az *lsaA* gént azonosították *Streptococcus agalactiae*-ban, míg a *tetM* baktériumfajok széles körében előfordul.^{87–91} Az *Enterococcus faecium*-ban azonosított *AAC(6')-II*, *eatAv*, *msrC* már leírtak ebben a fajban.^{92–95} *Escherichia coli*-hoz köthetően nagy számú ARG-t azonosítottunk: *acrB*, *acrD*, *acrE*, *acrF*, *acrS*, *bacA*, *baeR*, *baeS*, *cpxA*, *CRP*, *emrA*, *emrB*, *emrK*, *emrR*, *emrY*, *eptA*, *E. coli* *acrA*, *E. coli* *acrR* with mutation conferring multidrug antibiotic resistance, *E. coli* *ampC* beta-lactamase, *E. coli* *ampC1* beta-lactamase, *E. coli* *ampH* beta-lactamase, *E. coli* *emrE*, *E. coli* *GlpT* with mutation conferring resistance to fosfomycin, *E. coli* *marR* mutant conferring antibiotic resistance, *E. coli* *mdfA*, *E. coli* *soxR* with mutation conferring antibiotic resistance, *E. coli* *soxS* with mutation conferring antibiotic resistance, *evgA*, *evgS*, *gadW*, *gadX*, *kdpE*, *marA*, *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtF*, *mdtG*, *mdtH*, *mdtM*, *mdtN*, *mdtO*, *mdtP*, *msbA*, *PmrF*, *TEM-116*, *TolC*, *ugd*, *YojI*, amelyek mindegyikét leírták már a fajnál.^{35,36} Vizsgálatunkban minden *TEM-116* gén tartalmú minta ugyanabból a BioProjektből származott. Így felvethető, hogy ez a találatunk a minta előkészítésének során bekövetkezett kontamináció eredménye. Mivel

a minta-előkészítésre vonatkozó részletesebb információk nem állnak rendelkezésre, ezt a kérdést nem tudjuk megválaszolni. A *Lactococcus lactis* beli *lmrD* gén előfordulása a fajban szintén ismert.^{96,97} Az *AAC(3)-IV Streptomyces albulus*-ban való előfordulásáról nem ismerünk szakirodalmi adatot.

Probiotikus baktériumok rezisztomja (IX.)

A probiotikus eredetű minták vizsgálata során azt láttunk, hogy az egyes baktérium-fajok rezisztomja és mobilomja jelentős eltérést mutat. Abból a célból, hogy ebben a változatosságban további ismereteket szerezzünk, egy nagyobb mintaelemszámra épülő felmérő vizsgálatot végeztünk 12, gyakran használt probiotikus baktériumfaj (*Bifidobacterium animalis*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus kefirano-faciens*, *Lactobacillus kefir*, *Lactococcus lactis*, *Leucoconstoc mesenteroides*, *Levilactobacillus brevis*, *Streptococcus thermophilus*) izolátumainak szekvenálási adatait felhasználva.

Az 579 mintából 169 volt ARG-pozitív (29%; 95%CI: 26-33), ezek gyakorisága fajonként nagy eltérést mutatott (az 5.7. ábra). Az elemzett minták közül sem a *L. casei* faj egyetlen mintájában, sem a *L. paracasei* faj 33 mintájában nem azonosítottunk ARG-t. A *B. animalis* és *L. lactis* minták gazdagabbnak tűntek ARG-kben, előző esetében ARG-eket két gén, a *tet(W)* és a *B. adolescentis rpoB* mutations conferring rifampicin resistance dominálta. A *B. animalis* mind a 65 mintájában 15 mutáció (V516E, E525V, D532E, A533V, E543K, K552E, Q554E, A557V, V559D, G560A, E561A, E562G, V565E, S570E, S571M) volt jelen a *B. adolescentis rpoB* mutants conferring resistance to rifampicin gén rezisztenciában szerepet játszó 16 mutációjából, az egyetlen hiányzó az A443V.⁷⁹

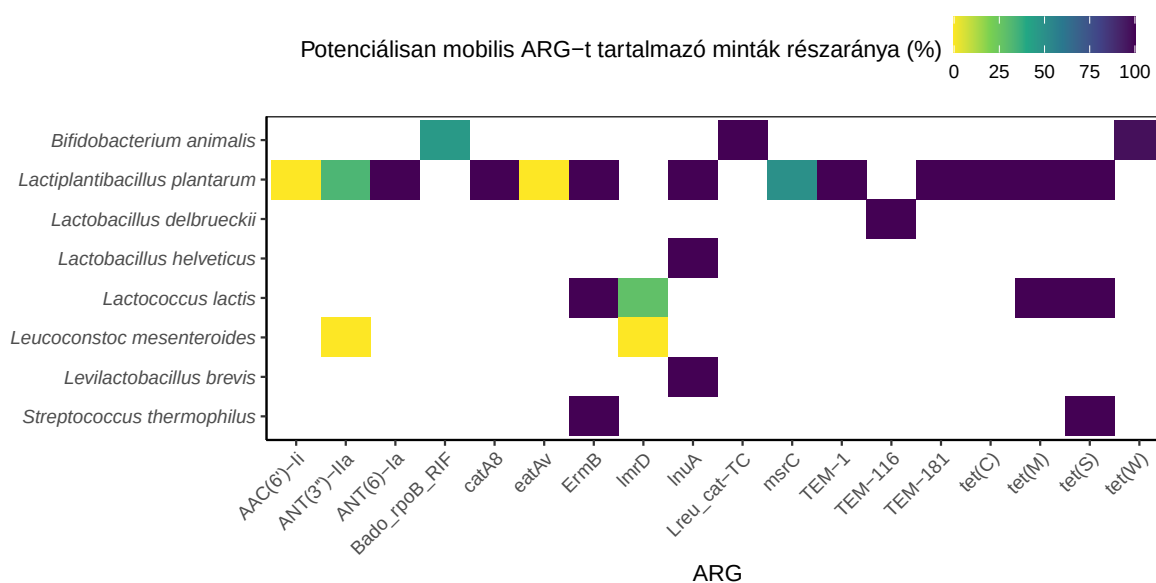


5.7. ábra. Az ARG-pozitív probiotikus baktériumminták fajonkénti részaránya. Az egyes fajok ARG-pozitív, és -negatív mintáinak száma a prevalencia (négyzet) és annak 95%-os CI-je (vízszintes egyenes) mellett szerepel.

A *L. lactis* esetében az *lmrD* volt a leggyakoribb. Ennek a génnek, az *lmrC*-vel együtt, nagy jelentőséget tulajdonítanak a fenotípusos multidrog-rezisztens *L. lactis* törzsekben.⁹⁸ A mintákban az *lmrD* előfordulás nagy része (51/71) kromoszomális volt, ami összhangban van a szakirodalmi adatokkal,⁹⁹ de 20 mintában plazmidon hordozott *lmrD* gént prediktáltunk.

A *L. delbrueckii*, *L. helveticus* és *L. brevis* törzsekben viszonylag kevés ARG-t azonosítottunk. Az alacsony ARG-szám és diverzitás mellett ezek mindegyike plazmid-asszociáltnak tűnik. A *L. plantarum*, *L. mesenteroides* és *S. thermophilus* fajok talán tekinthetők közepes ARG-gazdagságúnak. Míg a *L. mesenteroides* genomjai nem tartalmaztak mobilis genetikai elemhez kapcsolódó ARG-eket, addig a *S. thermophilus* rezisztenciája nagymértékben mobilnak tűnik. Az ARG-eket (pl. *ErmB*) hordozó plazmidok nem példa nélküliek a *Streptococcus* fajokban.¹⁰⁰ A nagy számú *L. plantarum* minta lehet annak az oka, hogy ebben a fajban tapasztaltuk a legnagyobb ARG-diverzitást, illetve legnagyobb mobilitási potenciált.

Egyetlen mintában sem találtunk bakteriofágokhoz köthető ARG-t. Ezzel szemben az ARG-t tartalmazó minták 66%-ában (112/169) legalább egy gén plazmidhoz vagy iMGE-hez kapcsolható volt. Az egyes baktériumfajokra vonatkozóan az 5.8. ábra mutatja a mobilis ARG-t tartalmazó minták részarányát, az ARG-pozitív minták között.



5.8. ábra. Potenciálisan mobilis ARG-eket tartalmazó minták részaránya az ARG-pozitív (n=169) probiotikus baktériumminták között. Rövidített ARG-nevek: Bado_rpoB_RIF: *Bifidobacterium adolescentis* *rpoB* mutants conferring resistance to rifampicin gén; Lreu_cat-TC: *Limosilactobacillus reuteri* *cat-TC* gén.

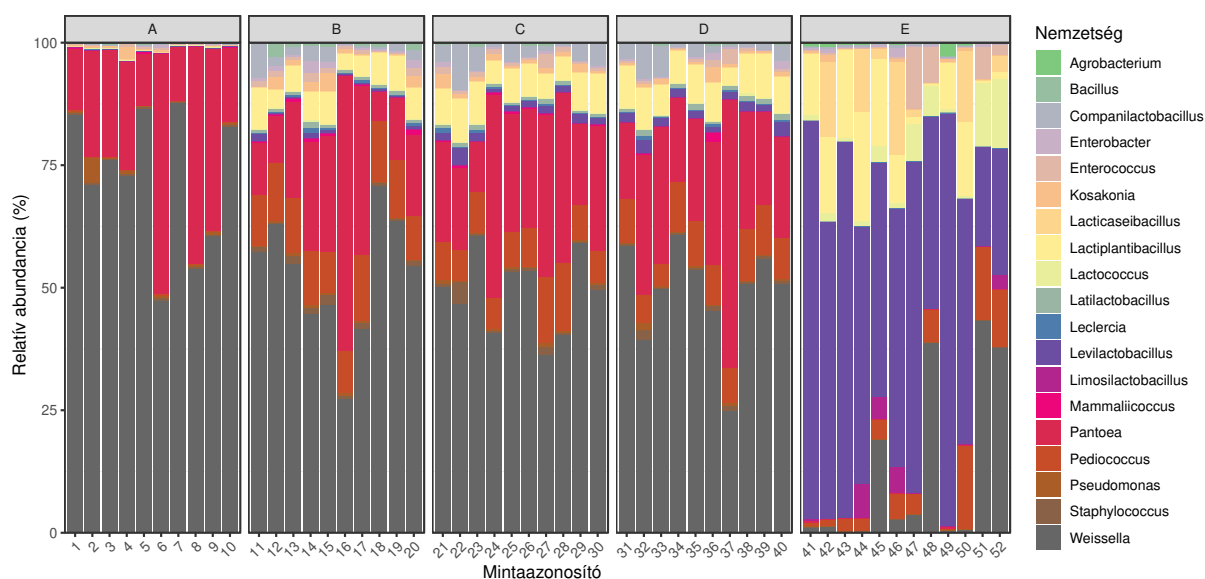
Összefoglalás

A probiotikus baktériumok fajonként eltérően több-kevesebb ARG-t tartalmaznak, melyek nem elhanyagolható mértékben könnyebben vehetnek részt HGT-ben. Az erjesztett

tejtermékekben vagy a tejből vagy az oltókultúrából származó ARG-k mennyisége az azokat hordozó baktériumok szaporodásával együtt változik. A probiotikus termékek, készítmények fogyasztásával az ARG-hordozó baktériumok az ember szervezetébe kerülhetnek.

5.5. Szilázs-rezisztom (X.)

A nyers tej rezisztomja, illetve az erjesztett tejtermékek vizsgálata során látott ARG-abundanciabeli változások irányították a figyelmünket a szilázsra. Ennek a szarvasmarhatartásban központi jelentőségű, erjesztett tömegtakarmánynak rezisztomjára vonatkozóan nem találtunk irodalmi adatokat. Ugyanakkor a joghurthoz és kefirhez hasonlóan feltételezhető volt, hogy az erjedése során szintén elszaporodhatnak ARG-t hordozó baktériumok, amelyek a takarmányfelvétel után akár a tehének tejében is megjelenhetnek. A szilázs-rezisztom tanulmányozásához megfelelő, vizsgálatunkkor elérhető, két BioProjektből származó 52 szilázminta magbakteriomjának nemzetségenkénti relatív abundanciája az 5.9. ábrán látható. A szakirodalmi adatok alapján szilázsra jellemző baktériu-



5.9. ábra. A szilázminták nemzetségszintű magbakteriomja. A PRJNA495415 BioProjektben a silózás 0., 7., 14. és 28. napján vették a mintákat (A, B, C, D csoport), PRJNA764355 BioProjekt összes mintáját az E csoportba soroltuk.

mok, mint a *Bacillus subtilis*,¹⁰¹ *Enterococcus faecium*,¹⁰² *Escherichia coli*,¹⁰³ *Lactiplantibacillus plantarum*,¹⁰⁴ *Lactococcus lactis*,¹⁰⁵ *Levilactobacillus brevis*,¹⁰⁶ *Ligilactobacillus acidipiscis*,¹⁰⁵ *Weissella paramesenteroides*.¹⁰⁷ jelentősen átfednek eredményeinkkel. Az azonosított *Cronobacter* sp. JZ38 növényi eredetű lehet.¹⁰⁸ Mivel az alábbi fajoknak szilázsban való előfordulására vonatkozóan nem találtunk adatokat, lehet, hogy szennyeződésként vannak jelen a mintákban: *Amylolactobacillus amylophilus*, *Enterobacter hormaechei*, *Enterococcus faecalis*, *Gracilibacillus* sp. SCU50, *Lactocaseibacillus manihotivorans*, *Lactocaseibacillus paracasei*, *Providencia rettgeri*, *Staphylococcus aureus*, *Staphylococcus*

carnosus, *Staphylococcus pseudoxylosum*, *Staphylococcus saprophyticus*, *Streptococcus suis*, *Tetragenococcus halophilus*. Bár egyesek a *Lactobacillaceae* családba, a *Leuconostoc* vagy az *Enterobacter* nemzetségekhez tartoznak, melyek számos fajtát használgják erjesztett élelmiszer-, és takarmány-előállítás során.

A vizsgált minták közül 20-ban azonosítottunk összesen 16 féle ARG-t, az azokathordozó kontigok a következő fajokhoz klasszifikálódtak: *Acinetobacter baumannii*: *aadA2*. *A. amylophilus*: *ant(6)-Ia*. *B. subtilis*: *aph(3')-IIa*. *Cronobacter* sp. JZ38: *qnrS1*. *Enterobacter hormaechei*: *aadA2*, *sul1*. *E. faecalis*: *aph(3')-IIIa*. *E. faecium*: *portA*. *E. coli*: *sul2*. *Gracilibacillus* sp. SCU50: *dfrG*. *L. manihotivorans*: *ant(6)-Ia*. *L. paracasei*: *portA*. *L. plantarum*: *aph(3')-IIIa*, *portA*, *vatE*. *L. lactis*: *lmrD*. *L. brevis*: *portA*. *L. acidiphiscis*: *ant(9)-Ia*. *P. rettgeri*: *qnrD1*. *S. aureus*: *ant(6)-Ia*, *tet(K)*. *S. carnosus*: *erm(44)v*. *S. pseudoxylosum*: *erm(44)v*. *S. saprophyticus*: *erm(44)v*. *S. suis*: *lsaE*. *T. halophilus*: *aph(3')-IIIa*. *W. paramesenteroides*: *ant(6)-Ia*.

Az ARG-k 54 példányban fordultak elő, amelyek közül 53 esetben feltételezhetjük, hogy mobilisak lehetnek. Ezek közül tíz ARG iMGE-vel kapcsolt, kettőt profágokban és 41-t plazmidokon detektáltunk. Öt mintában és öt fajban (*B. subtilis*, *E. hormaechei*, *E. faecium*, *L. paracasei*, *L. plantarum*) találtunk iMGE-vel kapcsolt ARG-eket. A *B. subtilis*-ban a *aph(3')-IIa*, *E. faecium*-ban, *L. paracasei*-ben és *L. plantarum*-ban a *portA* gént azonosítottuk iMGE-hez kapcsolódó génként. *E. hormaechei*-ben a *sul1* és a *aadA2* szintén iMGE-hez kapcsolt volt. Két profághoz kapcsolható ARG-t azonosítottunk, egy *erm(44)v*-t tartalmazó *S. pseudoxylosum*hoz eredetű, *S. suis*-hoz klasszifikált *lsaE* gént hordozó kontigon. Plazmidhoz kapcsolt ARG-et 19 mintában találtunk.

Az *aadA2* leírták már *A. baumannii*-ban.^{109,110} Az *ant(6)-Ia* számos fajban előfordul, beleértve *Lactobacillus* fajokat is.¹¹¹ Ugyanakkor az *A. amylophilus*-ban való előfordulására vonatkozóan nincsenek szakirodalmi adatok. Az *aph(3')-IIa* gént ismereteink szerint eddig nem mutatták ki *B. subtilis*-ban. A *qnrS1* gént eredményeinkkel összhangban *Cronobacter* fajban is leírták.¹¹² Az *E. hormaechei*-ben azonosított *aadA2* és *sul1* előfordul Gram-negatív baktériumokban. A mienkhez hasonló eredményeket közöltek *Enterobacter* spp. és *E. hormaechei* baktériumokban való előfordulásukról.^{113,114} A *Enterococcus* nemzetségen belül két ARG-t azonosítottunk, az *aph(3')-IIIa* gént *E. faecalis*-ban és a *portA* gént *E. faecium*-ban. Az *aph(3')-IIIa* rendszerint a *S. aureus*-ban és a *Enterococcus* fajokban fordul elő.¹¹⁵ A *portA* gént több fajban, így *E. faecium*-ban¹¹⁶ is leírták. A *sul2* gyakori *E. coli*-ban.^{36,117} A *dfrG* gént tudomásunk szerint eddig még nem írták le *Gracilibacillus* fajban, de előfordul a *Bacillaceae* családban.¹¹⁸ Az *ant(6)-Ia* gén számos baktériumban előfordul, köztük *Lactobacillus* fajokban,¹¹¹ de *L. manihotivorans*-ban előfordulására nem találtunk adatot. A szilázsmintákban a *PortA*-t kimutattuk *L. paracasei*-ben, *L. plantarum*-ban és *L. brevis*-ben is, az irodalomban ezekben a fajokban való előfordulására nem találtunk leírást, de más *Lactobacillaceae* fajban, a *L. acidophilus*-ban igen.¹¹⁹ Az *aph(3')-IIIa*-t *L. plantarum* genomhoz kapcsoltan is azonosítottuk, ami összhangban van korábbi eredményekkel.¹²⁰ *L. plantarum*-ban azonosítottuk a *vatE*

gént, amit leírtak *Lactobacillaceae* fajokban is,¹²¹ de nem *L. plantarum*-ban. Az *ant(9)-Ia* *L. acidipiscis*-ben való azonosítása az első ilyen adat. A *qnrD1* gén előfordulása ismert *Providencia* fajokban.¹²² Az *S. aureus*-hoz kapcsolhatóan találtuk az *ant(6)-Ia* és *tetK* géneket, mindkettő gyakori *Staphylococcus* fajokban.^{123,124} Az *erm(44)v* gént először a *S. saprophyticus*-ban¹²⁵ mutatták ki, amiben mi is azonosítottuk, de a *S. carnosus* vagy *S. pseudoxylus* fajokban való előfordulására nem találtunk adatokat. A *lsaE* gyakori *Streptococcus* fajokban,⁸⁹ *S. suis*-ban is leírták.¹²⁶ A már említett baktériumfajokon kívül a *aph(3')-IIIa* kimutatható volt *T. halophilus*-ban is. Ez az ARG ugyan gyakran jelenik meg *Enterococcaceae* fajokban,³⁶ de ennél a fajnál még nem írták le. Nem ismerünk közleményt, ami a *W. paramesenteroides*-ben azonosította az *ant(6)-Ia* gént.

Több ARG-vel kapcsolatban találtunk azok mobilitására utaló MGE-eket, így iMGE-kapcsolt *aph(3')-IIa*-t találtunk *B. subtilis*-ben, amit *E. coli*-ban leírtak már korábban.³⁶ Míg a *aadA2* és *sul1* gének plazmidon való előfordulására vannak adatok *E. hormaechei*-ben,¹²⁷ mi iMGE-khez társítva találtuk őket. A *portA* mellett található iMGE-kre vonatkozó eredményünk *E. faecium*-ban összhangban van mások eredményével.¹²⁸ Ugyanezt az együttes előfordulást találtuk, *L. paracasei*-ben, amire vonatkozóan nem találtunk irodalmi adatot. Az *erm(44)v* és az *lsaE* gén profágokkal kapcsolható *S. pseudoxylus* és *S. suis* fajokban. Míg a szakirodalomban hasonló összefüggés található az előbbi génre,¹²⁹ az utóbbival kapcsolatban nem ismerünk ilyen adatot.¹²⁶ A többi mobil ARG-t olyan kontigokon detektáltuk, amelyeket plazmidként prediktáltunk. Az *AdA2* *A. baumannii*, az *aadA2* és *sul1* *E. hormaechei*, az *aph(3')-IIIa* *E. faecalis*, a *sul2* *E. coli*, az *aph(3')-IIIa* *L. plantarum*, a *qnrD1* *P. rettgeri*, az *ant(6)-Ia* *S. aureus*, az *erm(44)v* *S. saprophyticus* fajban való plazmidhoz kapcsolt előfordulása ismert.^{36,129,130} Tudomásunk szerint még nem írták le az *aph(3')-IIa* *B. subtilis*, a *dfrG* *Gracilibacillus* sp. SCU50, az *ant(9)-Ia* *L. acidipiscis*, a *lsaE* *S. suis*, a *qnrS1* *Cronobacter* sp. JZ38, az *erm(44)v* *S. carnosus* és *S. pseudoxylus* fajban, plazmidon való előfordulását. Korábbi eredmények megerősítik a *vatE* előfordulását plazmidokon.¹³¹ Annak ellenére, hogy gyakran található így *Enterococcus*-fajokban,¹³² ott nincs korábbi adat arra vonatkozóan, hogy ugyanígy előfordulna *L. plantarum*-ban. Az *aph(3')-IIIa* gén *T. halophilus*-ban, plazmidon való előfordulása összhangban van azzal, hogy leírták *Enterococcus* fajokban, plazmidon.¹³³ Legjobb tudásunk szerint ezt az együttes előfordulást ebben a fajban még nem közölték.

Összefoglalás

A lucernaszilázsban azonosított baktériumokhoz köthetően több, túlnyomó részben mobil ARG-t találtunk. Ezek a takarmány felvételével az állatok emésztőrendszerébe jutnak, onnan a szervezetük más részeibe kerülhetnek. Mivel az erjesztett tömegtakarmányokat igen nagy mennyiségben állítják elő a világban, a bennük lévő ARG-hordozó baktériumok globális antimikrobiális rezisztenciában betöltött szerepének vizsgálata célszerű lenne.

5.6. Kullancs-terjesztette kórokozó baktériumok rezisztomja (XI.)

Előző vizsgálatainkban azt láttuk, hogy nagy számú ARG fordul elő olyan forrásokban, melyekből ARG-hordozó baktériumok juthatnak az állati vagy emberi szervezetbe. Ízeltlábú vektorok, pl. kullancsok számos bakteriális fertőzés terjesztői. A probiotikus baktériumokhoz hasonlóan, kullancs-terjesztette kórokozó baktériumfajok ARG-spektrumának leírása céljából végeztünk kiterjedt vizsgálatot. Ennek során 7 baktérium nemzetségből (*Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella* és *Rickettsia*) származó fajok, 1550 mintáját használtuk fel.

Annak ellenére, hogy nagyszámú mintát tudtunk bevonni az elemzésünkbe, meglepően kevés ARG-t sikerült azonosítanunk. Ráadásul az ARG-k csak néhány minta között oszlottak meg (5.1. táblázat). Az ARG-pozitív minták aránya 0% volt az *Anaplasma* és *Borrelia* nemzetségek esetében, 1,5% a *Bartonella* nemzetségben, 11,0% a *Coxiella* nemzetségben, 7,7% az *Ehrlichia* nemzetségben és 4,7% az *Rickettsia* nemzetségben. Kivételt képezett a *Francisella* nemzetség, ahol majdnem minden minta legalább egy ARG-t hordozott (98,9 %). Bár e nemzetségből származó ARG-pozitív minták száma nagy, túlnyomó részük csak egy ARG-t tartalmazott, az *FTU-1* gén-t, amely szinte minden *Francisella tularensis* genomban megtalálható,¹³⁴ beleértve a faj referenciagenomját is (ASM898v1). Ha ezt a gént kihagyjuk a számításból, és úgy elemezzük a *Francisella* minták ARG-tartalmát, akkor a pozitív minták részaránya 16,3%, ami még mindig magasabb mint a többi nemzetségnél.

Az ARG-pozitív baktériumfajok és a bennük azonosított ARG-k: *Bartonella henselae*: *blaZ*. *Bartonella quintana*: *APH(3')-IIIa*, *SAT-4*. *Coxiella burnetii*: *AcrE*, *AcrS*, *APH(3'')-Ib*, *APH(6)-Id*, *ArmR*, *bacA*, *bcr-1*, *CARB-16*, *cpxA*, *emrA*, *emrB*, *emrK*, *emrY*, *ErmC*, *Escherichia coli* *AcrAB-TolC* with *MarR* mutations conferring resistance to ciprofloxacin and tetracycline, *E. coli* *EF-Tu* mutants conferring resistance to pulvomycin, *E. coli* *emrE*, *E. coli* *mdfA*, *E. coli* *soxR* with mutation conferring antibiotic resistance, *E. coli* *soxS* with mutation conferring antibiotic resistance, *E. coli* *UhpT* with mutation conferring resistance to fosfomycin, *evgA*, *FosA*, *gadW*, *gadX*, *marA*, *mdtE*, *mdtG*, *mdtP*, *MexI*, *OXA-488*, *Pseudomonas aeruginosa* *emrE*, *P. aeruginosa* *soxR*, *TEM-1*, *TEM-116*, *tet(44)*, *tet(H)*, *TolC*, *ugd*. *Ehrlichia ruminantium*: *APH(3'')-Ib*, *APH(6)-Id*. *Francisella tularensis*: *AAC(6')-Ii*, *aad(6)*, *acrB*, *acrD*, *AcrE*, *AcrF*, *AcrS*, *APH(3')-IIIa*, *bacA*, *baeR*, *baeS*, *cpxA*, *efmA*, *emrA*, *emrB*, *emrK*, *emrY*, *Enterococcus faecium* chloramphenicol acetyltransferase, *eptA*, *ErmB*, *E. coli* *acrA*, *E. coli* *AcrAB-TolC* with *AcrR* mutation conferring resistance to ciprofloxacin, tetracycline, and ceftazidime, *E. coli* *AcrAB-TolC* with *MarR* mutations conferring resistance to ciprofloxacin and tetracycline, *E. coli* *ampC* β -lactamase, *E. coli* *EF-Tu* mutants conferring resistance to pulvomycin, *E. coli* *emrE*, *E. coli* *gyrA* conferring resistance to fluoroquinolones, *E. coli* *mdfA*, *E. coli* *soxR* with mutation conferring antibiotic resistance, *E. coli* *soxS* with mutation conferring antibiotic resistance, *evgA*, *FTU-1*, *gadW*, *gadX*, *kdpE*, *Klebsiella pneumoniae* *KpnH*, *marA*, *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtF*, *mdtG*, *mdtH*, *mdtM*, *mdtN*, *mdtO*, *mdtP*, *msbA*, *msrC*, *PmrF*,

SAT-4, *tet(L)*, *tet(M)*, *TolC*, *ugd*, *vanA*, *vanH*, *vanR*, *vanS*, *vanX*, *vanY*, *vanZ*, *YojI*. *Rickettsia conorii*: *OXA-192*. *Rickettsia japonica*: *APH(3')-Ia*. A potenciálisan mobilis és nem mobilis ARG-k nemzetségenkénti száma az 5.1 táblázatban olvasható.

5.1. táblázat. Kullancs-terjesztette baktériumfajok ARG-pozitív, és -negatív mintáinak, illetve mobilis és nem mobilis ARG-inek száma nemzetség szerint.

Nemzetség	Minták száma			ARG-k száma	
	negatív	pozitív	összes	mobilis	nem mobilis
<i>Anaplasma</i>	20	0	20	0	0
<i>Bartonella</i>	129	2	131	1	2
<i>Borrelia</i>	311	0	311	0	0
<i>Coxiella</i>	65	8	73	24	31
<i>Ehrlichia</i>	12	1	13	2	0
<i>Francisella</i>	11	948	959	327	1359
<i>Francisella</i> *	803	156	959	303	434
<i>Rickettsia</i>	41	2	43	2	0

*: *FTU-1*-t hordozók nélkül

Általánosan elfogadottnak tekinthető, hogy baktériumok rezisztenciája stresszorok (pl. AB, nehézfémek) szelekciós nyomására alakul ki, mutációkon vagy HGT-vel felvett ARG-ken keresztül.^{135–137} Következésképpen fontos figyelembe venni a mikroorganizmusok ökológiai környezetét a megfigyelt rezisztenciagén-hiány hátterének értelmezésekor. Az általunk vizsgált mikroorganizmusok többsége szigorúan vektorokhoz és gazdaszervezetekhez kötött, obligát intracelluláris baktérium.^{138–146} Kivételt képeznek a *Borrelia* fajok, amelyek extracelluláris patogének,¹⁴⁷ valamint a *Francisella* és *Coxiella* nemzetségek, ahol a vektor nélküli terjedési utak is fontosak.^{148,149} Ennek megfelelően limitált az a tér, amelyben a szelekciós nyomás és az ARG-k jelenléte együttesen jelentkezne. Elméletileg kullancsok találkozhatnak AB-okkal (pl. vérszívás során) vagy egyéb toxikus anyagokkal, de kérdéses, hogy ez a hatás milyen mértékben jelenthet valódi szelekciós nyomást a bennük élő baktériumokra. Emellett a rendelkezésre álló ARG-k jelenléte is korlátozott lehet, mivel a kullancsok saját bakteriótája korlátozza a környezetből származó mikroorganizmusok kullancsbeli kolonizációját.¹⁵⁰

A gazdaszervezetekben lévő baktériumokra nagyobb antibiotikum-nyomás nehezedhet. Természetesen ez nem elsősorban a vadon élő állatokban jelentkezik, hanem akkor, ha a baktériumokat háziállatokba, vagy akár emberekbe juttatják be a vektorok. Feltételezhetjük, hogy az emberi fertőzések jelentős részét antibiotikumokkal kezelik, és még ha ez az ilyen törzsek ARG-jének felvételével járna is, nem valószínű, hogy az ARG-ket szerzett baktériumok újra bekerülnek a kullancsba. A háziállatok sokkal nagyobb jelentőséggel bírhatnak ebből a szempontból. Mivel esetükben az AB-kezelés szintén általános, de a kezelés során nagyobb valószínűséggel maradhat élő kullancs az állaton, mint az embereknél. A haszonállatokból még kevésbé valószínű, hogy eltávolítják az összes kullancsot. Vizsgálatunkban csak a *Coxiella burnetii* esetében voltak ARG-pozitívak a haszonállatokból

származó minták. Függetlenség-vizsgálatot végezve a minta eredete és ARG-pozitivitása vonatkozásában, azt az eredményt kaptuk, hogy a háziállatokból származó *Coxiella* minták nagyobb eséllyel (OR: Inf, 95% CI: 3,6-Inf, $p < 0,001$) tartalmaznak ARG-t, mint az egyéb forrásból származók. Egy pozitív minta az *Ehrlichia* nemzetségből közvetlenül egy juhból gyűjtött kullancsból származott, amit elemzésünkben vektor-eredetűnek minősítettünk. Az *Anaplasma* nemzetség mintái közül 9 volt haszonállat-eredetű, amelyek közül 6 szarvasmarha-, 1 ló-, és 2 juh-eredetű, de ezek közül egyik sem bizonyult ARG-pozitívnak. Az *Anaplasma* és a *Coxiella* nemzetségek közötti különbség az életciklusukból adódhat, mivel az *Coxiella* extracelluláris formája is fontos szerepet játszik a terjedésében, következésképpen nem teljesen a vektorokhoz kötött.¹⁴⁸ Természetesen kérdéses, hogy a *C. burnetii* környezeti formája képes-e géneket felvenni a környezetből, és hogy ezek mennyire lehetnek szükségesek a túléléshez antibiotikumok jelenlétében.

A *Francisella tularensis* néhány tekintetben hasonlít a *C. burnetii*-re. Terjedésében nem kizárólagos a vektorok szerepe,¹⁴⁹ és a fertőzött gazdáknak extracellulárisan is előfordulhat.^{151,152} Számos ARG-pozitív *F. tularensis* mintát találtunk, de ezek szinte kizárólag olyan kísérletből származnak, amelyben mesterséges antibiotikumos nyomást gyakoroltak a baktériumokra.

Ezenkívül természetesen lehetséges, hogy a vizsgált baktériumok szerezhettek rezisztenciagéneket, de azért nem találtuk meg azokat, mert ha fel is vettek, már eltávolították azokat magukból. Ennek oka lehet az, hogy a kullancs bélrendszere alapvetően más mikro-környezetet biztosít a baktériumoknak, mint a gazdaállat. A rezisztencia mechanizmusok folyamatos aktiválása anyag- és energiaigényes folyamat, így AB-hatás nélkül ezek a költségek meghaladják az előnyöket.^{135,153,154}

Összefoglalás

Eredményeink azt mutatják, hogy a vizsgált kullancsok-terjesztette kórokozó baktériumtörzsekből gyakran hiányoznak az ARG-k. Ugyanakkor a *Coxiella burnetii*-ben több ARG-t is azonosítottunk, amelyek haszonállatokból izolált törzsekben gyakrabban fordultak elő.

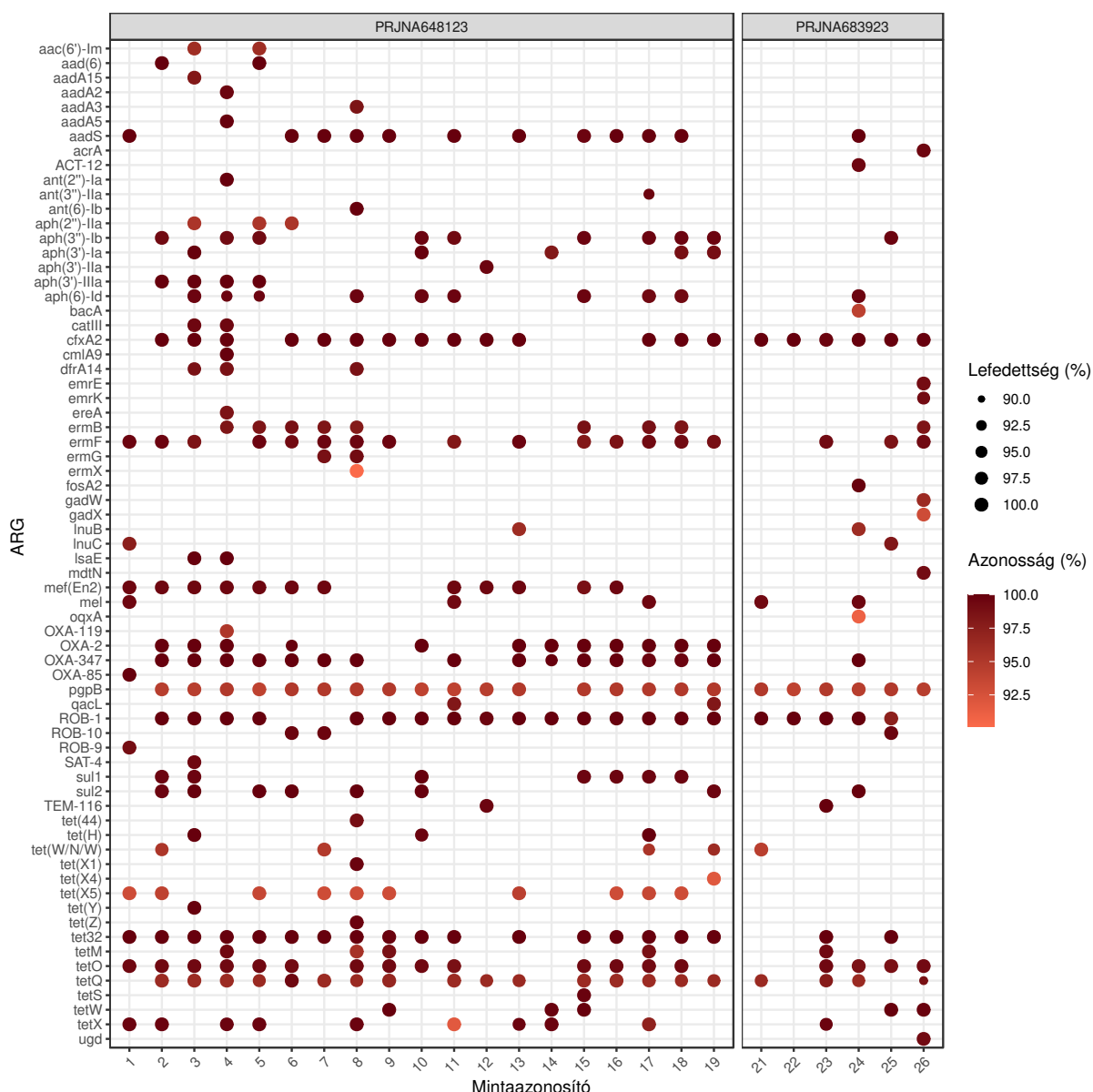
5.7. Kutyanál-rezisztom (XII.)

Napjainkban a kutyák mint társállatok jellemzően szorosabb kapcsolatban élnek gazdáikkal mint korábban. Ezt azt is jelenti, hogy a gyakoribb fizikai érintkezés nagyobb eséllyel juthatnak baktériumok az állatból az emberbe. Ebben a folyamatban a kutyanál kiemelkedő jelentőségű lehet, különösen harapásos sebzéseken esetében. Vizsgálatunk célja az volt, hogy betekintést nyerjünk kutyanálminták ARG-tartalmára, az azt hordozó baktériumfajokra és az ARG-k mobilitási potenciáljára vonatkozóan. Ebből a célból 26 shotgun metagenomszekvenált kutyanálmintát elemeztünk.

A magbakterioma nemzetségei az átlagos prevalenciájuk csökkenő sorrendjében a *Porphyromonas* (49%), *Prevotella* (15%), *Pasteurella* (12%), *Neisseria* (10%), *Capnocytophaga* (9%), *Conchiformibius* (7%), *Frederiksenia* (7%), *Cutibacterium* (6%), *Actinomyces* (5%), *Campylobacter* (4%), *Desulfomicrobium* (4%), *Bacteroides* (3%), *Fusobacterium* (3%), *Mycoplasma* (3%), *Treponema* (3%) és *Streptococcus* (2%). Az azonosított baktériumnemzetségek közül többet gyakran izolálnak fertőzött kutyaharapási sebekből. A kutyaharapásos fertőzések általában polimikrobiálisak, a harapásos seb baktériumai az állatok szájüregéből, a sérülést elszenvedő alany bőréről és a környezetből kerülnek oda. A kutyaharapások leggyakoribb kórokozói *Bacteroides*, *Capnocytophaga*, *Corynebacterium*, *Enterococcus*, *Fusobacterium*, *Moraxella*, *Neisseria*, *Pasteurella*, *Porphyromonas*, *Prevotella*, *Pseudomonas*, *Staphylococcus*, *Streptococcus* fajok,^{155,156} amelyek mindegyike megjelent az elemzett nyálmintákban. A mintákban azonosított egyéb nemzetségek túlnyomó többségével kapcsolatban vannak szakirodalmi adatok a kutyanyálban való előfordulásukról.^{155,157}

A vizsgált mintákból 25-ben azonosítottunk ARG-t, a 20. számú mintában egyetlen bakteriális readet sem találtunk, így abban ARG-k sem fordultak elő. A mintákban talált 69 féle ARG-nek mintánkénti maximális lefedettségét és a szekvenciaazonosságát foglalja össze az 5.10. ábra. Az ARG-k előfordulási gyakorisága viszonylag magas volt a nyálbakteriomban és baktériumfajhoz klasszifikálható részük jelentős számban plazmidhoz (n=83), profághoz (n=10) vagy iMGE-hoz (n=40) köthető.

Nyolc, a kutyaharapásos fertőzésekben gyakran előforduló nemzetség (*Bacteroides*, *Capnocytophaga*, *Corynebacterium*, *Fusobacterium*, *Pasteurella*, *Porphyromonas*, *Staphylococcus* és *Streptococcus*) tagjaihoz köthetően azonosítottunk ARG-eket. Állatok és kutyaharapásos betegek kezelésében a leggyakrabban használt amoxicillin–klavulánsav (AMC) és cefalosporinok mellett a linkozamidok, szulfonamidok és a fluorokinolonok is szerepet kapnak.^{158–164} Eredményeinket megerősítette egy 1830 kutyanyál-minta elemzésével végzett munkánk is.¹⁶⁵ Összesen hat ARG-típust mutattunk ki a kutyák nyálmintáiban, amelyek szerepet játszhatnak AMC-rezisztenciában, ezek mind a *TEM* vagy *OXA* család tagjai.^{166,167} A *TEM-116* gént az *Escherichia coli*-ban, míg a különböző *OXA*-k *Acinetobacter baumannii*, *Bacteroides*, *Capnocytophaga*, *Fusobacterium ulcerans* és *Pseudomonas* fajokban találtuk, amelyeknek klinikai jelentősége lehet a kutyaharapásos fertőzésekben. A linkozamidokat befolyásoló ARG-eket 17 baktériumfajban azonosítottunk, amelyek közül több lehetséges kórokozó: *Bacteroides fragilis* (*ErmF*), a *Capnocytophaga stomatis* (*ErmF*), *Porphyromonas* fajok (*ErmF*) *Streptococcus* fajok (*ErmB*, *lnuB* és *lnuC*). A szulfonamid-rezisztenciagének nyolc baktériumban jelentek meg, köztük a *Pasteurella multocida* (*sul2*) és a *Pseudomonas aeruginosa* (*sul1*) fajokban. Fluorokinolonok hatását befolyásoló ARG-t csak az *E. coli* hordozott (*acrA*, *gadW*, *gadX*).



5.10. ábra. Kutyanyálban mintánként azonosított ARG-k. Minden minta-ARG kombináció esetében csak a legjobb eredményt (lefedettség, szekvenciaazonosság) ábrázoljuk. A pontok mérete és színe a lefedettséget és a szekvenciaazonosságot szemlélteti. A 20. számú mintában nem volt azonosítható ARG. A túl hosszú génneveket rövidítettük (*acrA*: *E. coli acrA*; *emrE*: *E. coli emrE*).

Összefoglalás

A vizsgált kutyanyál-mintákban nagy számú és gyakran mobil ARG-t azonosítottunk. Ezek egy része olyan baktériumokhoz volt köthető, amelyek a kutyaharapásos sebek elterjedésében szerepet játszik, így azok AB-terápiájának hatékonyságát befolyásolhatják.

5.8. Antimikrobiális rezisztencia-potenciál (XIII.)

Egyes bakteriális eredetű kórfolyamatokban a rezisztom értelmezése különös figyelmet érdemelhet klinikai szempontból. Az ARG-k jelenlétének vagy hiányának klinikai felhasznál-

nálhatóságát alapvetően befolyásolja, hogy a fenotípusos rezisztencia milyen mértékben becsülhető az ARG-k alapján. Ennek vizsgálata céljából öt BioProjekt *Escherichia coli* színtenyészetek (n=574) AST eredményeit (vizsgált AB-k száma: 33) vetettük össze ugyanazon törzsek ARG-tartalmával.

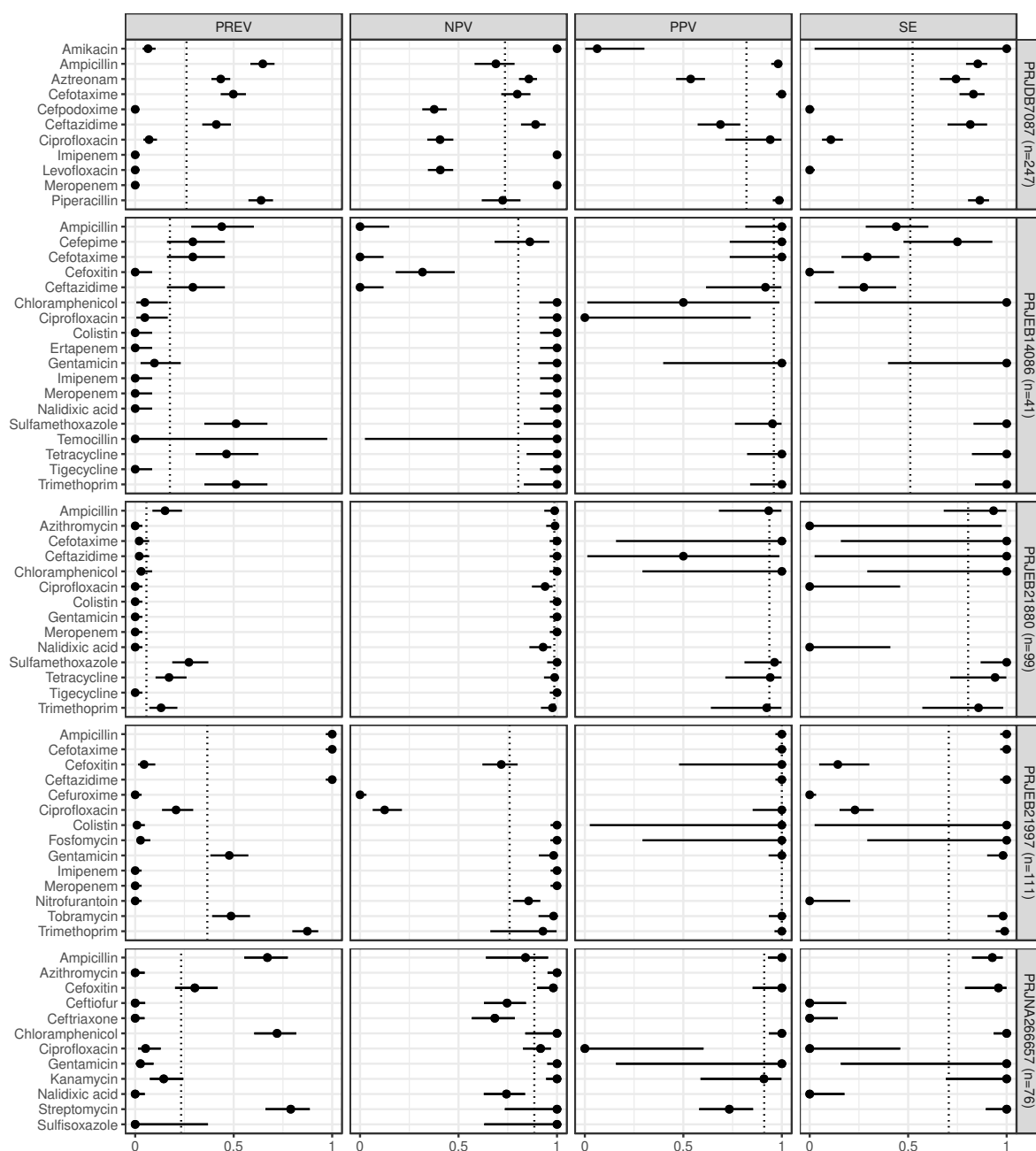
A törzsek fenotípusos és genotípusos rezisztenciavizsgálati eredményeinek összevetését (5.11-5.12. ábra) az alábbiak szerint foglalhatjuk össze. Az AB-k 39%-ánál a szenzitivitás meghaladta a 90%-ot. Más szavakkal, ezeknél az AB-oknál a fenotípusosan rezisztens törzsek legalább 90%-ban genotípusosan is rezisztensnek adódtak. A pozitív prediktív értékek alapján a genotípusosan rezisztens törzsek legalább 90% valószínűséggel fenotípusosan is rezisztensek az AB-k 45%-ában. Az AB-k 45%-ában kevesebb, mint 5% annak a valószínűsége, hogy egy fenotípusosan rezisztens törzs genotípusosan érzékenyként legyen azonosítva. Az antibiotikumok 88%-ánál a fenotípusosan érzékeny törzsek legalább 90%-os valószínűséggel genotípusuk alapján is érzékenyek. A genotípusosan érzékenyek azonosított törzsek legalább 90%-os valószínűséggel fenotípusosan is érzékenyek a vizsgált antibiotikumok 64%-ánál. A fenotípusosan érzékeny törzs genotípus alapján rezisztensként való azonosításának valószínűsége az antibiotikumok 85%-ánál kevesebb, mint 5%.

Az eredmények értelmezéséhez figyelembe kell vennünk az AST-vel kapcsolatban, hogy habár gold standardként tekintünk rá, nem hibátlan. Még az általunk használt BioProjektekben alkalmazott, EUCAST javasolta AST-módszer is vitatható eredményeket ad, akárcsak más klasszikus, fenotípuson alapuló módszerek. A laboratóriumi körülmények, technikák közötti eltérések különböző eredményekhez vezethetnek.¹⁶⁸

Egy gépi tanulásos, ARG-alapú fenotípusos AMR predikciót bemutató közlemény¹⁶⁹ szerint az egyes AB-k esetén eltérő pontosság érhető el. Így a tesztelt hatóanyagcsoportokkal szemben a fenotípusos AMR legpontosabban a makrolidok és a szulfonamidok esetében becsülhető. A legnagyobb bizonytalanságot, amelyeket kombinációkban is vizsgáltak, a β -laktámok esetében találták.¹⁶⁹

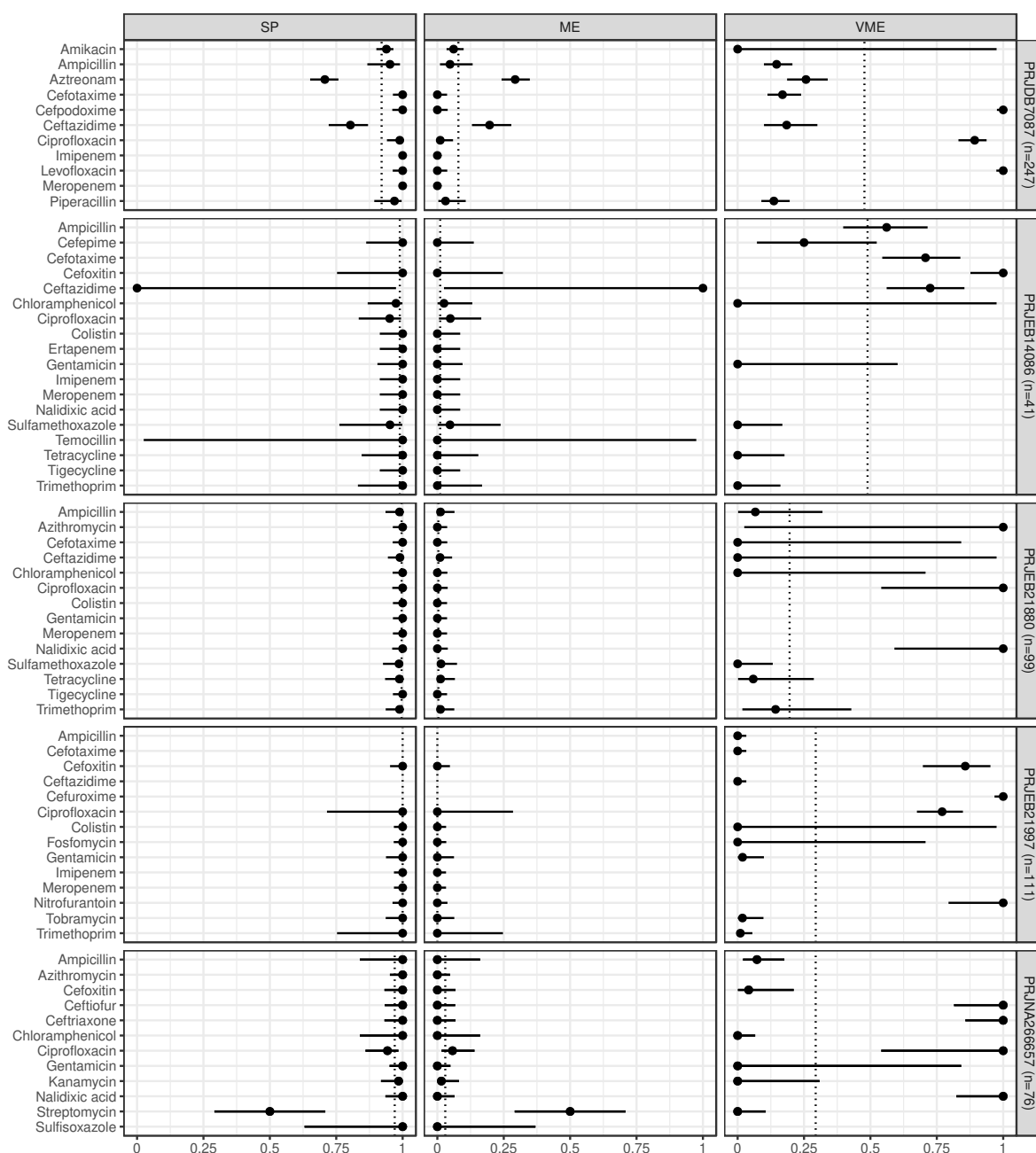
Az eredmények értékelésekor figyelembe veendő az is, hogy a vizsgálatot *E. coli* törzsek adatain végeztük. Hu et al. szerint az *E. coli* törzsekre vonatkozó predikciók kevésbé robusztusak és megbízhatók, mint más fajok, például a *Campylobacter jejuni* és az *Enterococcus faecium* esetében.¹⁶⁹ Ezek a fenotípusos AMR-profil előrejelzésben mutatkozó különbségek a komplex bakterióták vonatkozásában is relevánsak lehetnek. Ezenkívül érdemes az egyedi sejtek szintjén jelentkező AMR mellett az ún. kooperatív rezisztenciát is figyelembe venni. A mikrobiális közösségek dinamikája (függetlenül attól, hogy egy vagy több fajból álló populációról van szó) befolyásolhatja az antimikrobiális terápiára adott választ.¹⁷⁰ Exoenzimmel az antibiotikumokat inaktiváló baktériumsejt immunitást biztosíthat az enzim nélküli sejteknek.¹⁷¹ Ez a kooperatív rezisztencia minden mikroba-gazdag környezetben jelen lehet.¹⁷¹

A színtenyészetekkel ellentétben, komplex mikrobiológiai minták esetében elméletileg sem lehet hasonló fenotípus-genotípus összehasonlítást végezni. Ennek egyik oka, hogy a



5.11. ábra. Antimikrobiális rezisztencia-potenciál értékelési mértékek. A PREV oszlop a BioProjektben vizsgált antibiotikumokkal szembeni fenotípusos antimikrobiális rezisztencia prevalenciáját (95% CI-vel) mutatja. A negatív és pozitív prediktív értékek, valamint az érzékenység pontbecslései (95% CI-vel) az *NPV*, *PPV* és *SE* oszlopokban olvashatók. A pontozott függőleges egyenes a becslés BioProjektben belüli átlagát jelzi.

baktériumoknak csak kis része tenyészthető és tesztelhető könnyen fenotípusos rezisztencia szempontjából.¹⁷² Mikrobiológiai kulturomikai módszerek, mivel többféle tenyésztési környezet és táptalaj egyidejű alkalmazásával nagyobb áteresztőképességet, így számos nehezen tenyészthető baktérium azonosítását teszik lehetővé, talán részben megoldhatják ezt a problémát.¹⁷³ Ugyanakkor ennek a megközelítésnek az alkalmazása mellett is rejtve maradhatnak komplex mikrobiális közösségek által okozott kórképekben – akár kritikus –



5.12. ábra. Antimikrobiális rezisztencia-potenciál értékelési mértékek. A specifikus, a jelentős és a nagyon jelentős hiba pontbecslései (95% CI-vel) az *SP*, *ME* és *VME* oszlopokban olvashatók. A pontozott függőleges egyenes a becslés BioProjekten belüli átlagát jelzi.

szerepet játszó baktériumok. A shotgun metagenomikai vizsgálatok azonban lehetőséget nyújtanak arra, hogy a taxonok relatív gyakoriságának leírása mellett – ami különösen klinikai szempontból fontos – a mikrobiom génjeinek (pl. ARG) jelenlétére vonatkozóan is ismereteket szerezhessünk. Az így összesített ARG-prevalenciák felhasználhatók egy adott minta antimikrobiális rezisztencia-potenciáljának (AMRP) becslésében. Sajnos a bakteriológiai tenyésztés korlátai miatt az AMRP és a komplex baktériumközösség fenotípusos rezisztenciája közötti kapcsolatot jelenleg nem tudjuk számszerűen értékelni. Azonban ha a komplex mikrobiológiai mintákat baktériumok egyedi törzseinek halmazaként tekint-

jük, feltételezhetjük, hogy a törzsek fenotípusos és genotípusos rezisztencia/érzékenység megegyezőségi becslései rájuk is kiterjeszthetők. Klinikai szempontból a fő kérdés az, hogy mely antibiotikumok hatékonyak a betegséget kiváltó vagy fenntartó komplex mikrobiális közösségben jelen lévő baktériumok ellen.

Az *E. coli* törzsekre vonatkozó eredményeket figyelembe véve a genotípus alapján a fenotípusos érzékenység elfogadható megbízhatósággal prediktálható a vizsgált antibiotikumok többségére. Ha ezt a megközelítést kiterjesztjük a metagenomikus ARG-elemzésekre is, akkor klinikai alkalmazhatóságát a terápiás szerek kiválasztásában is fontolóra lehet venni. A metagenom-elemzéssel komplex mikrobiális közösségek által okozott fertőzéseknél azonosíthatók olyan AB-k, amelyek hatását befolyásoló ARG-k nem találhatók a mintában. Feltételezhetjük, hogy ezek közül azok hatékonyabbak lehetnek, amelyekhez köthető ARG-k nagyobb számban fordulnak elő az adatbázisban, mint amelyekhez kevesebb. Ugyanakkor fontos azt is látni, hogy a különböző antibiotikumok és hatóanyagcsoportok ARG-inek száma az egyes adatbázisokban jelentősen eltérhetnek,¹⁷⁴ és hogy egyik ARG-adatbázis sem teljes.

Összefoglalás

E. coli törzsek fenotípusos AMR-je antibiotikumtól függően becsülhető ARG-ik alapján. Amennyiben a szintenyészetekben tapasztalt fenotípus-genotípus megfeleltethetőséget komplex bakteriótákra is feltételezhetőként fogadjuk el, akkor azok rezisztomját értelmezhetjük az adott közösség antimikrobiális rezisztencia-potenciáljaként.

...Avagy mi tesz nemesbbé tégedet?
Egy szikra az, mely bennetek dereng,
Egy végtelen erőnek mozzanása;
S mint a pataknak egyes habjai,
Egy percre felcsillogva visszahullnak
Közös medröknek szürke mélyibe. -
Igen, tán volna egy, a gondolat,...

Madách Imre: Az ember tragédiája

6. Kitekintés

Az új- és harmadik generációs szekvenálási technológia számos orvosbiológiai kutási és alkalmazási területen nyitott meg új lehetőségeket. Bemutatott eredményeink e technológiának mikrobiális genomikában való alkalmazásából jöttek létre. A hagyományos mikrobiológiai eszközökkel nem leírható mikrobióta-komplexekre vonatkozó ismeretek számos vonatkozásban fontosak.

A méhek bélbakterióm eredményei rámutatnak arra, hogy az egészséges bakterióta – mint viszonyítási alap – nagy természetes változatosságot mutathat. Ez fontos szempontként szolgálhat emberi és háziállatok egészséges mikrobiótájának értékelésében. Mind a mézelőméh-, mind a kullancs-metagenomvizsgálatok során azonosítottunk kórokozókat. Ezek egy részére számíthattunk, másokra nem. Ez utóbbi eredmények mutathatnak rá a metagenomika egyik klinikai alkalmazási lehetőségére. Amikor is a klinikai mintában nem várt, hagyományos módszerekkel nem, vagy nagyon nehezen detektálható kórokozók azonosítására ad lehetőséget.

Az *Ixodes ricinus* bakterióm összetételében megfigyelt fejlődési alakok közötti eltérések alapján sikerült azonosítanunk az *Ixodiphagus hookeri*-t. Ez egy újabb detekciós irány példája. Mivel a végül azonosított parazitoid genomrészletei csak kis mennyiségben fordultak elő, azok célzott keresését a faj szimbiótáinak, az *Arsenophonus* és *Wolbachia* nemzetségek jelentős abundancia-eltérései implikálták. Ez a más taxonokra alapozott hipotézis-generálás, majd -vizsgálat különösen nagy jelentőségű lehet eukarióta patogének (pl. *Balamuthia mandrillaris*) klinikai metagenomikai vizsgálatában, amelyek genomjának egy jelentős része homológiát mutathat a gazdafajával.

A metagenomikai vizsgálatok lehetőségeinek értékeléséhez ugyanakkor fontos figyelembe venni, hogy azokat alapvetően befolyásolja az elérhető genomadatbázisok teljessége vagy, hiányossága. Ugyan ezek folyamatosan bővülnek, a mikroorganizmus-genomok területtől függően nagyon különböző mértékben tekinthetők teljesnek. Az adatbázisok növekedése szükségessé teszi a taxonklasszifikációban használat eszközök folyamatos fejlesztését, azoknak az elérhető számítógépes kapacitások melletti felhasználhatósága céljából.

Az antimikrobiális rezisztencia, mint napjaink egyik legjelentősebb, globális egészségügyi kihívása, kialakulásának, gyakoribbá válásának teljes komplexitásában közelítendő. Ebben az „egy egészség” koncepció fontos megközelítés. Az élő szervezetek létének alapvető mozzanata, hogy genetikai anyagáramlásban vesznek részt. Ennek során felvesznek és leadnak genomokat, genomrészleteket, mely jelentős részben táplálkozáson keresztül

valósul meg. A rezisztomvizsgálati eredményeink egy része arra mutat rá, hogy a takarmánnyal, élelmiszerrel nem elhanyagolható mennyiségű ARG juthat az állati vagy emberi szervezetbe. Az eredmények értelmezésében fontos szempont, hogy az azonosított ARG-ket túlnyomórészt szaprofita baktériumokból származóként klasszifikáltuk. Így a fogyasztó szervezetébe való bejutásuk nem jelent közvetlen egészségügyi következményeket. Ugyanakkor mivel az azonosított ARG-k egy része potenciálisan mobilis volt, megfelelő körülmények között HGT útján kórokozókba is kerülhetnek. A táplálkozáson túl egyéb forrásokból is juthatnak ARG-k állatokba vagy emberbe. A kullancs-terjesztette kórokozó baktériumok ARG-szegénysége evolúciós folyamatok további vizsgálati lehetőségeire irányítja a figyelmet. Azzal együtt, hogy a haszonállatokkal kapcsolatba hozható magasabb ARG-jelenlét az állategészségügyi gyakorlatokra is rámutat. A kutanyálban előforduló ARG-k harapással közvetlenül juthatnak emberi szövetekbe, és az azokat hordozó baktériumoknak ellenállóképességet biztosíthatnak a kezelésben felhasznált antibiotikumokkal szemben.

A bakteriális kórfolyamatok kezelésének sikere attól függ, hogy az alkalmazott antibakteriális szerre érzékenyek-e a kórokozók. A hatékony szer kiválasztásában felvethető, hogy a fenotípusos rezisztenciavizsgálatokat genomalapúakkal egészítsük ki. Utóbbi megközelítésben a kórfolyamatban azonosított rezisztom értelmezhető úgy, mint antimikrobiális rezisztencia-potenciál. Függetlenül attól, hogy az ARG-k szaprofita vagy patogén baktériumban vannak, jelenlétük miatt nem zárható ki, hogy fenotípusosan is kifejeződnek.

Az ARG-terhelés értékelésében számos további szervezetbe irányuló forrás vizsgálata szükséges a teljesebb kép céljából. Ezek közül a környezet, ezen belül a belélegzett levegő¹⁷⁵ fontos ARG-forrás lehet. Az „egy egészség” megközelítés alapján fontos lenne adatokat gyűjteni a zártan tartott haszonállatok környezetének levegőrezisztomjával kapcsolatban, mivel az ott dolgozó emberek számára folyamatos terhelést jelenthet, amit az állatokkal nem érintkező emberi közösségekbe (pl. otthon, kórház) továbbíthatnak. A rezisztom-, és mobilomkutatások esetén is látható az adatbázisok, eszközök folyamatos bővülése, fejlesztése, ami remélhetőleg pontosabb kép alkotását tesz lehetővé ezen a területen.

Fontosnak tartom kiemelni, hogy az általunk a rezisztomvizsgálatokban felhasznált adatok túlnyomó többségét nem mi generáltuk, hanem más kutatócsoportok. Mindegyik esetében elmondható, hogy az eredeti vizsgálati célok között nem szerepelt az ARG-k vizsgálata. Azonban mivel baktériumokat, illetve azokat tartalmazó összetett organizmusegyütteseket szekvenáltak, az ARG-k vizsgálatát is lehetővé tették. A szekvenálási adatok közzététele a nyitott tudomány rendkívüli forrását jelentheti, mivel az adatgeneráló kutatók által fel nem tett kérdések megválaszolására is lehetőséget nyújthatnak – más kutatóknak.. Így új gondolatokból eredő kérdések válaszait kevésbé korlátozhatják az anyagi lehetőségek.

Az elmúlt évtizedekben a tudományos kutatás alapvető átalakuláson ment keresztül, amelynek egyik legmeghatározóbb eleme a digitális adatok mennyiségének exponenciális növekedése. A modern biológiai kutatások során keletkező hatalmas adatmennyiség fel-

dolgozása és értelmezése egyre nagyobb számítástechnikai és programozási felkészültséget igényel, amellyel a különböző biológiai diszciplínák kutatói nem minden esetben rendelkeznek kellő mélységben. Ennek következtében a sikeres tudományos munka ma már szükségszerűen multidiszciplináris együttműködésekben alapul, ahol a biológiai szakértelem, a bioinformatikai módszertan és a biostatisztikai elemzés egymást kiegészítve járulnak hozzá az eredményekhez. Fontos hangsúlyozni, hogy az adatelemzési feladatok döntő többsége nem új szoftverek fejlesztését igényli, viszont elengedhetetlen a meglévő eszközök és programcsomagok mélyreható ismerete, valamint azok kreatív, az adott kutatási kérdéshez igazított kombinációjának alkalmazása, hiszen a biológiai problémák túlnyomó része nem szabványosítható megoldásokat kíván meg. Dolgozatomban első- és utolsó szerzőként jegyzett, fontosabb mikrobiális genomikai eredményeimet foglaltam össze. Ugyanakkor tudományos pályám során számos olyan kollaboratív kutatásban vettem részt, ahol nem első vagy utolsó szerzőként szerepelek, de az adatok bioinformatikai és biostatisztikai feldolgozásával érdemben hozzájárultam az eredmények megszületéséhez (lásd 47. lap). Bár ezek a munkák nem képezik a dolgozatom részét, örülök, hogy e projektek során kiváló kutatókkal, kutatócsoportokkal dolgozhattam együtt, és érdekes, esetenként úttörő tudományos kérdések megválaszolásában vehettem részt, és érdekes témakörökbe nyertem bepillantást. Ezekben az együttműködésekben szerzett tapasztalataim tették lehetővé, hogy az itt bemutatott genomikai kutatásokat elindítsam, vezessem, melyek segítségével – felidézve Cusanus-t – a mikrobiológiai modellünk sokszögéhez újabb szögeket adhattunk, talán kicsivel jobban megközelítve a kört...

7. Új tudományos eredmények

1. Mézelő méhek bélmikrobiótájának vizsgálatával:

- a) Bemutattuk, hogy klinikailag egészséges mézelőméh-dolgozók bélbakteriom-alkotó fajainak abundanciája szezonálisan, illetve klimatikus környezetük szerint is jelentős eltérést mutathat (I.).
- b) Metagenomikai vizsgálatok alapján a *Vairimorpha (Nosema) ceranae* hazai előfordulásával kapcsolatban mutattunk be adatokat. A kórokozó előfordulási gyakoriságában évszakos és klimatikus eltéréseket azonosítottunk. Filogenetikai elemzés alapján egy magyar szekvencia thaiföldi, spanyolországi és hawaii mintákkal mutatta a legnagyobb szekvenciális hasonlóságot (II.).
- c) Elsőként állítottuk össze mézelő méhek fonalas vírusának teljes genomjait, hazai állományokból, melyek filogenetikai vizsgálatunk szerint svájci szekvenciához hasonlítanak legjobban (III.).

2. *Ixodes ricinus* mikrobiom-vizsgálatával:

- a) Kórokozó baktériumok azonosítása mellett, fejlődési stádiumok (nimfa, felnőtt nőstény) és a mintavétel földrajzi helyének klimatikus viszonyai szerinti bakteriom-eltéréseket mutattunk be.
- b) Magyarországon elsőként azonosítottuk az *Ixodiphagus hookeri* genomszekvenciáit. A öt helyről származó parazitoid-pozitív minta közül négy földrajzi elhelyezkedése alapján egy nyugat-magyarországi klaszterre mutattunk rá.

3. Tej, fermentált tejtermékek, probiotikus termékek és baktériumtörzsek vizsgálatával:

- a) Emberi fogyasztásra szánt, piaci nyers tejből ARG-ket mutattunk ki metagenomikai vizsgálatokkal (VI.).
- b) Bemutattuk, hogy a joghurt-, és kefirtermékek, az azok előállításához használt oltókultúrák, baktériumtörzsek antimikrobiális rezisztenciagének (ARG) változatos spektrumát tartalmazzák. Rámutattunk arra, hogy az erjesztési folyamat során az ARG-ket hordozó baktériumok szaporodásával az ARG-k mennyisége is változik (VII.).
- c) Bemutattuk, hogy emberi fogyasztásra szánt probiotikumokban előforduló ARG-k nem elhanyagolható mértékben potenciálisan mobilisak (VIII.).
- d) Gyakran használt probiotikus baktériumfajok rezisztom-, és mobilom-változatosságára vonatkozóan mutattunk be adatokat (IX.).

4. Metagenomikai elemzés alapján elsőként mutattunk be adatokat a lucernaszilázs-rezisztomra, és -mobilomra vonatkozóan (X.).
5. Kullancs-terjesztette patogén baktériumok teljesgenom-szekvenálásainak felhasználásával végzett felmérésünk alapján rámutattunk, hogy többségükben nem várt alacsony gyakorisággal fordultak elő ARG-k, míg a *Coxiella burnetii* izolátumokban az ARG-k előfordulási gyakorisága kiemelkedő a szarvasmarha-eredetű mintákban (XI.).
6. Bemutattuk, hogy a kutyanyálban számos baktérium, köztük kórokozók is, nagy számú, az antibiotikumok széles spektrumának hatékonyságát negatívan befolyásoló ARG-t hordoz (XII.).
7. Fenotípusos és genotípusos antimikrobiális rezisztencia összevetése alapján felvetettük a komplex mikrobiológiai háttérű kórfolyamatokból származó minták rezisztomjának antimikrobiális rezisztencia-potenciálként való értelmezési lehetőségét (XIII.).

8. A dolgozat alapjául szolgáló közlemények

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- VIII. Tóth, A. G., Csabai, I., Judge, M. F., Maróti, G., Becsei, Á., Spisák, S., **Solymosi, N.**, Mobile antimicrobial resistance genes in probiotics. *Antibiotics* 10:11, 1287, 2021
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- X. Nagy, S. Á., Tóth, A. G., Papp, M., Kaplan, S., **Solymosi, N.**, Antimicrobial resistance determinants in silage. *Scientific Reports* 12: 5243, 2022
- XI. Papp, M., Tóth, A. G., Valcz, G., Makrai, L., Nagy, S. Á., Farkas, R., **Solymosi, N.**, Antimicrobial resistance gene lack in tick-borne pathogenic bacteria. *Scientific Reports* 13: 8167, 2023
- XII. Tóth, A. G., Tóth, I., Rózsa, B., Dubecz, A., Patai, Á. V., Németh, T., Kaplan, S., Kovács, E. G., Makrai, L., **Solymosi, N.**, Canine saliva as a possible source of antimicrobial resistance genes. *Antibiotics* 11:11, 1490, 2022
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9. A dolgozatban nem szereplő további közleményeim

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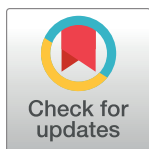
RESEARCH ARTICLE

Natural diversity of the honey bee (*Apis mellifera*) gut bacteriome in various climatic and seasonal states

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Abstract

As pollinators and producers of numerous human-consumed products, honey bees have great ecological, economic and health importance. The composition of their bacteriota, for which the available knowledge is limited, is essential for their body's functioning. Based on our survey, we performed a metagenomic analysis of samples collected by repeated sampling. We used geolocations that represent the climatic types of the study area over two nutritionally extreme periods (March and May) of the collection season. Regarding bacteriome composition, a significant difference was found between the samples from March and May. The samples' bacteriome from March showed a significant composition difference between cooler and warmer regions. However, there were no significant bacteriome composition differences among the climatic classes of samples taken in May. Based on our results, one may conclude that the composition of healthy core bacteriomes in honey bees varies depending on the climatic and seasonal conditions. This is likely due to climatic factors and vegetation states determining the availability and nutrient content of flowering plants. The results of our study prove that in order to gain a thorough understanding of a microbiome's natural diversity, we need to obtain the necessary information from extreme ranges within the host's healthy state.

Introduction

Honey bees are important pollinators with high economic value and ecosystem importance [1–3]. Their economic significance is based on their role in crop pollination and the different bee products they make [2, 3]. Honey, their most well-known product, is an important component of the human diet. Some evidence suggests that honey consumption can improve human health and might have a role in disease management [4]. However, honey bees are subjected to confined environments, and several factors threaten their health, including different

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pathogens, parasites and chemicals used as pesticides in agriculture [5–7]. The global decline of this key pollinator poses a threat to food security and to the maintenance of biodiversity [8]. The composition of honey bee bacteriota, for which the available knowledge is limited, is essential for their body's functioning. While there is increasing attention on the effects of different herbicides and pathogens on the bee gut microbiota [9–11], only a few data are available on the natural variability of the microbiota. Nevertheless, this could form the basis of studies exploring the effects of different harmful agents on honey bees' gut bacteriota. Without this knowledge, one cannot decide if any suspected factor places the bacteriota composition into an adverse state. Although there are studies on honey bee gut microbiota and microbiomes [12–14], there is little evidence on the environmental factors affecting it. It is assumed that seasonal and environmental factors can have an influence on the gut bacteriome composition in honey bees, for example through feeding habits. During the collection season, various flowering plants provide diverse feed for the bees. The vegetation cycles, flowering and pollen quantity and quality of plants are mostly influenced by meteorological conditions, especially precipitation and temperature. Our study aimed to get more detailed knowledge about the natural variation of gut bacteriomes in healthy worker honey bees, based on seasonal and different environmental conditions, in a country-wide repeated measure survey. We have assumed that environmental factors will be associated with changes in the bacteriome as such changes were observed in other vertebrate [15, 16] and arthropod [17, 18] species. However, season showed contradictory results in honey bees, when observed in the honey producing season [19–21], although Kešnerová and colleagues [22] have found differences between the bacteriome of winter bees and foragers. To achieve our research goal, we were guided by the consideration that extreme states of the bee gut microbiome (environmentally and seasonally) should be sampled. Hence, samples were taken during the two most distinctive periods of the honey collection season and sampling sites were selected from markedly distinct areas based on their climatic characteristics.

Materials and methods

Sampling design and sample collection

The study's main goal was to understand the natural variability in the gut bacteriome of healthy honey bees (*Apis mellifera*). To measure seasonal variation, two sampling occasions were planned, one at the onset and one at the peak of the honey producing season. However, as season is not the only variable that could be considered when one is interested in the factors that could affect the bacteriome (climate could be an important environmental factor as well), we have determined our samples to be representative of Hungary on the climate level. To obtain such samples, we conducted a stratified spatial random sampling [23] as detailed below.

We gathered the 10 year average of the yearly growing degree days (GDD) with base 10 [24, 25] and the yearly total precipitation data for all the 175 local administrative units (level 1, hereafter referred to as LAU) in Hungary. Meteorological data for the period 2008–2017 was gathered from the ERA-Interim reanalysis data repository [26] by the spatial resolution of 0.125°. We defined the two categories for our environmental variables as cooler-warmer and less-more for GDD and precipitation respectively.

Regarding GDD, the lower two quartiles were classified as cooler and the upper two quartiles as warmer. For precipitation, the yearly mean below the country-wide median was assumed as less and above the median as more. Each LAU was categorised by its own climatic variables (Fig 1). We created separate strata for each combinations of our two environmental variables.

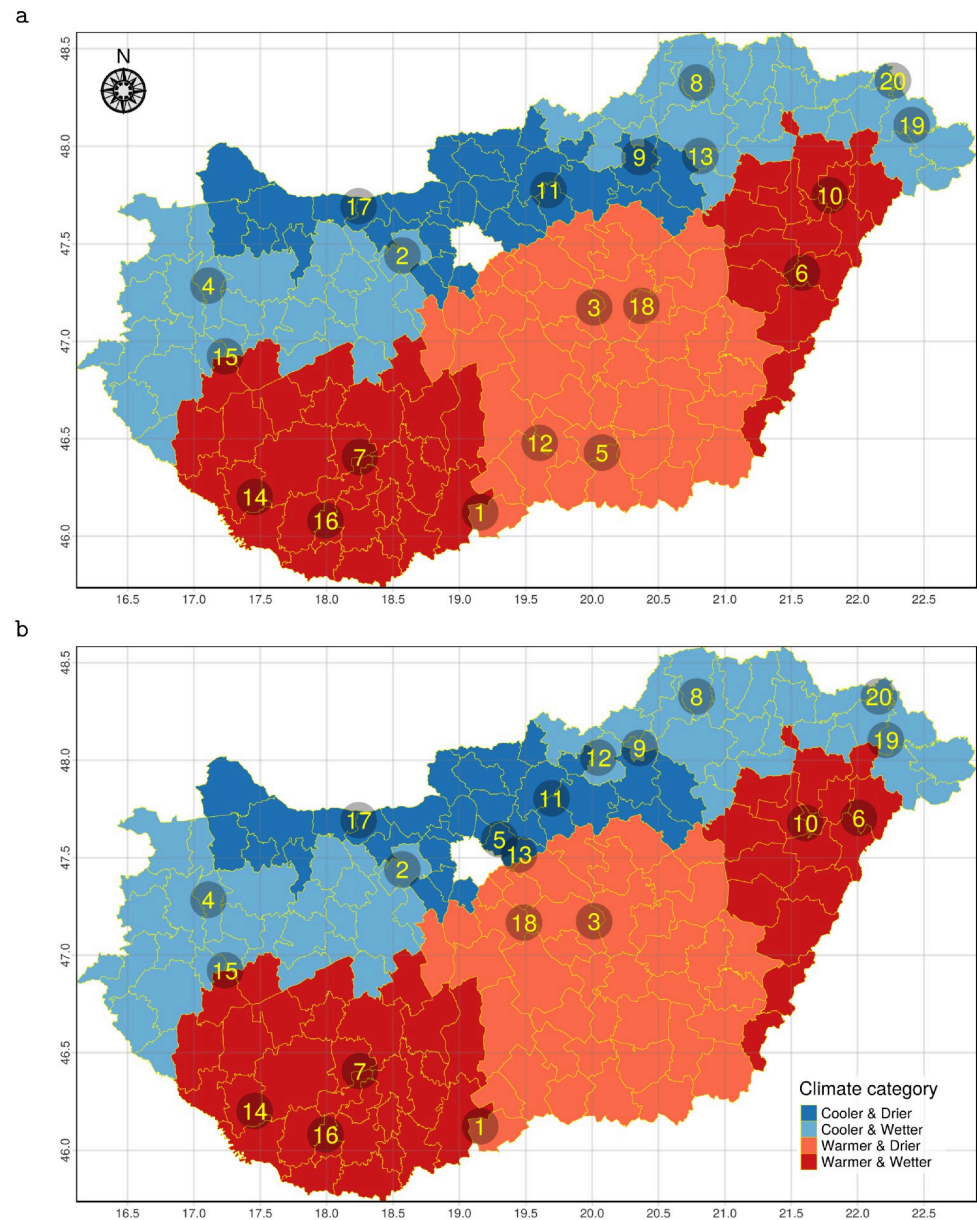


Fig 1. Climate category spatial pattern and sampling points. The Hungarian local administrative units (LAU) coloured by climatic categories based on growing degree days (GDD) and precipitation of the period 2008–2017. The numbers represent the identification numbers of the sampled apiaries in March (a) and May (b).

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To ensure that our samples are representative of Hungary at the climatic condition level, we have selected 20 LAUs so that the sample size of each previously defined strata was proportional to the stratifying GDD and precipitation categories' country-wide frequency. The R [27] package *spsurvey* [23, 28] was used for the stratified spatial random sampling of the LAUs as described above. One apiary was selected from each appointed LAU (making the total number of selected apiaries 20). To minimise the effect of the keeping conditions on our results, each apiary was selected based on personal conversations. Since in Hungary mainly Carniolan honey bees (*Apis mellifera carnica*) are in operation, the samples were drawn from colonies of that subspecies.

Sample collection was performed twice during the honey producing season (at the onset and at the peak). The first was done between 20/03/2019 and 25/03/2019 (Fig 1a) and the second during the period of 23/05/2019 to 01/06/2019 (Fig 1b), hereafter referred to as the sampling periods of March and May respectively. To obtain a representative sample of workers, two-level pooling was performed by apiaries to reduce the effects of possible biasing factors (e.g. age heterogeneity).

Three colonies were selected for sampling (in each apiary) at the first sample collection (sampling period March) and these same colonies were sampled at the second sample collection (sampling period May). Each time 20 workers were collected and frozen immediately by dry ice from each of the three selected colonies from every apiary.

During the sampling in May, in two apiaries (ID: 6, 13) only two of the three colonies from the March sampling period were accessible so only these two were sampled. Migration of the colonies occurred in eight of the apiaries (ID: 5, 6, 9, 10, 12, 13, 18, 20) between the two sampling periods. In the case of four apiaries of the above eight (ID: 5, 9, 12, 13), the environmental classification has changed between the two samplings. Two apiaries moved from warmer to cooler regions (ID 5, 12) and ID 12 apiary along with ID 9 moved from a LAU with less precipitation to one with more. One apiary (ID 13) has migrated from a region with more precipitation to a region with less. The sample sizes used by apiaries and sampling period are summarized in S1 Table.

Data on animal health history was collected in each of the sampled herds at both sampling times by questionnaire. We asked if the beekeeper had experienced significant mortality in the previous season or overwintering by the March sampling. By the May sampling, we asked if there was significant mortality between the two sampling times. In each of the apiaries at both sampling times, we received the answer that no such events were experienced.

Sample preparation

The collected samples were prepared for next-generation sequencing (NGS) in the Department of Parasitology and Zoology, University of Veterinary Medicine Budapest. From the deep-frozen workers, 10 by colonies were chosen. The bees' entire gastrointestinal tracts were removed and pooled on the apiary-sampling level. The gut preparation forceps was never used before and one forceps was applied only for one pool (3x10 guts) processing.

DNA extraction and metagenomics library preparation

The Quick-DNA Fecal/Soil Microbe Kit from Zymo Research was used for the simple and rapid isolation of inhibitor-free, high-quality host cell and microbial DNA from the bee gut samples. Isolated total metagenome DNA was used for library preparation. In vitro fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

Bioinformatic analysis

After merging the paired-end reads by PEAR [29], quality-based filtering and trimming was performed by Adapterremoval [30] using 15 as the quality threshold and only retaining reads longer than 50 bp. The *Apis mellifera* genome (Amel_HAV3.1) sequences host contaminants were filtered out by Bowtie2 [31] with the very-sensitive-local setting minimizing the false positive match level [32] in further metagenome classification. The remaining reads, after deduplication by VSEARCH [33], were taxonomically classified using Kraken2 (k = 35) [34] with the

NCBI non-redundant nucleotide database [35]. The core bacteria was defined as the relative abundance of agglomerated counts on species-level above 0.1% in at least half of the samples. The taxon classification data was managed in R [27] using functions of package phyloseq [36] and microbiome [37].

Statistical analysis

The within-subject diversity (α -diversity) was assessed using the numbers of observed species (richness) and the Inverse Simpson's Index (evenness). These indices were calculated in 1,000 iterations of rarefied OTU tables with a sequencing depth of 6,129. The average over the iterations was taken for each apiary. The α -diversity expressed by Inverse Simpson's Index was compared between the conditions using linear models. Comparing the samples collected in March and May, a mixed-effect model was applied to handle the repeated measure by apiary as a random factor. The between-subject diversity (β -diversity) was assessed by Bray-Curtis distance [38] based on the relative abundances of bacterial species. Using this measure, non-metric multidimensional scaling (NMDS) ordination was applied to visualise the samples' dissimilarity. To examine statistically whether the bacterial species composition differed by climatic or seasonal conditions, PERMANOVA (Permutational Multivariate Analysis of Variance [39]) and PERMDISP2 [40] procedures were performed using vegan package [41] in R [27]. The abundance differences in the core bacteriome according to the seasonal and climatic conditions were analysed by a negative binomial generalised model of DESeq2 package [42] in R [27]. This approach was applied following the recommendation of Weiss et al. [43]. None of the compared groups had more than 20 samples and their average library size ratio was less than 10. Since the apiaries were sampled repeatedly for capturing the seasonal effect, the samples were paired in the model. Regarding the multiple comparisons, an FDR-adjusted p-value (q-value) less than 0.10 was considered significant. The statistical tests were two-sided.

Results

In the results of our study, we first summarize the most relevant indicators of sequencing and taxon classification. After the within- and between-subject diversity of the whole bacteriome, we present the differentiating species of the core bacteriome.

Sequencing and taxon classification

The shotgun sequencing generated paired-end read counts of samples ranged between 311,931 and 546,924 with a mean of 413,629. The OTU table, created by Kraken2 taxonomic classification, contained counts of samples ranging between 11,646 and 114,573 with a mean of 44,280. The minimum, maximum and median read counts of the samples assigned as bacterial species were 6,129, 62,836 and 270,774 respectively.

Within-subject diversity

The numbers of observed species and the Inverse Simpson's Index α -diversity metrics by environmental and seasonal strata are shown in Fig 2. The Inverse Simpson's Index outliers in the samples collected in March from districts with less and more precipitation are the apiary ID 9 and ID 13 respectively. The apiary ID 12 sampled in March had an outlying high number of observed species too. From the same sampling period among the samples gathered from districts with more precipitation, apiary ID 8 appears to be an outlier.

In samples from the cooler environment collected in March, the α -diversity was significantly ($p = 0.0215$) higher than in samples from warmer districts. There was no significant

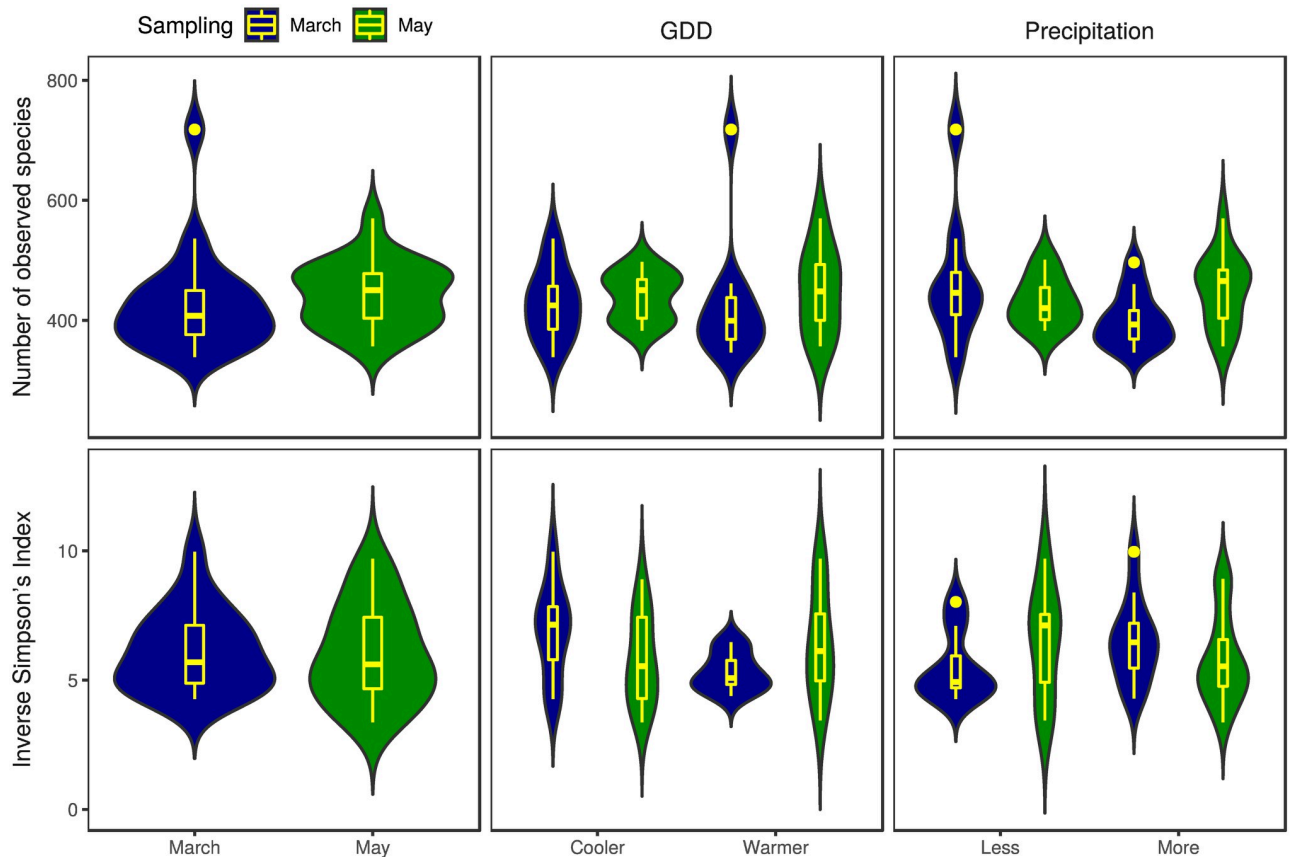


Fig 2. Richness and evenness of honey bee gut bacteriome by sample groups. The numbers of observed species (richness) and the Inverse Simpson's Index (evenness) as α -diversity metrics are presented as a violin and box plot combination. These indices were calculated in 1,000 iterations of rarefied OTU tables with a sequencing depth of 6,129. The average over the iterations was taken for each apiary. The violin plot shows the probability density, while the box plot marks the outliers, median and the IQR. For Inverse Simpson's Index, the comparison of samples from cooler and warmer districts collected in March showed significant ($p = 0.0215$) differences.

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difference in α -diversity between the precipitation categories of samples collected in March ($p = 0.178$). In samples collected in May, there was no significant difference between GDD or precipitation categories ($p = 0.463$ and $p = 0.456$ respectively).

Between-subject diversity

The dissimilarity of the samples' bacterial species profiles (β -diversity) is visualised by NMDS ordination (Figs 3 and 4) based on Bray-Curtis distance. The ordination stress was 0.144, 0.062 and 0.116 for all samples, samples of March and samples of May respectively. By PERMANOVA analysis of bacterial species composition, a significant ($p = 0.002$) difference was found between the samples from March and May. The samples' bacteriome from March showed a similar significant ($p = 0.02$) distance between the cooler and the warmer districts. From the same period, the precipitation levels did not differ significantly ($p = 0.155$). In the samples gathered in May, there was no significant distance between GDD and precipitation categories ($p = 0.277$ and $p = 0.849$ respectively). Both significant PERMANOVA results were confirmed by the PERMDISP2 distance-based tests for homogeneity of multivariate dispersions ($p = 0.033$ and $p = 0.003$ respectively).

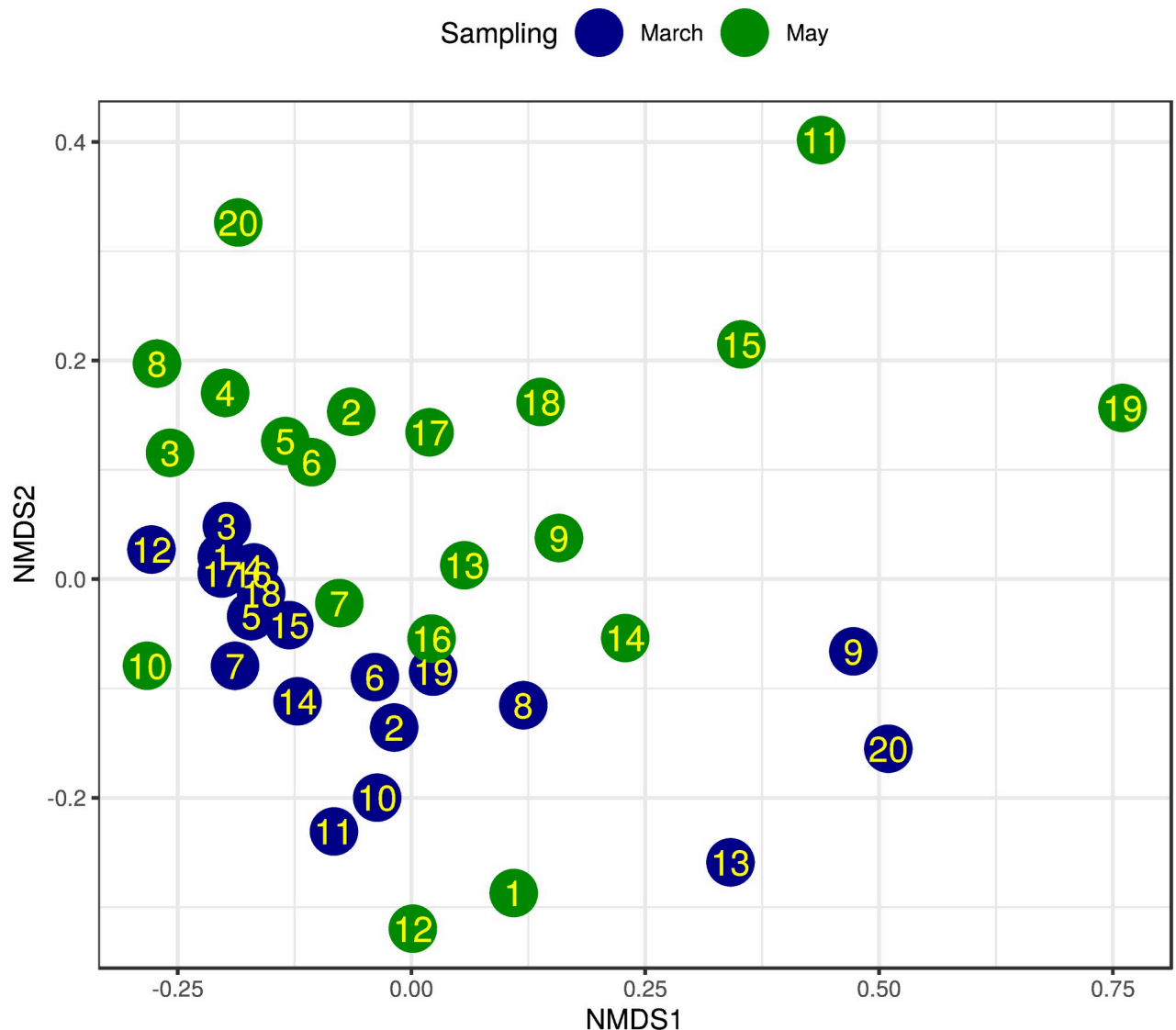


Fig 3. NMDS ordination of bacteriome for sampling March and May. Bray-Curtis dissimilarity was calculated using the species-level abundance of core bacteria. The samples from apiaries (IDs in dots) collected in March (blue) and May (green) are plotted using these dissimilarities. Based on the same measures, PERMANOVA analysis showed significant differences between the sampling time periods ($p = 0.002$, stress = 0.144).

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Core bacteriome and differentiating species

The core bacteriome members having relative abundance above 0.1% in at least half of the samples are *Bartonella apis*, *Bifidobacterium asteroides*, *Bifidobacterium coryneforme*, *Bifidobacterium indicum*, *Commensalibacter* sp. AMU001, *Frischella perrara*, *Gilliamella apicola*, *Lactobacillus apis*, *Lactobacillus bombi*, *Lactobacillus helsingborgensis*, *Lactobacillus kullabergensis*, *Lactobacillus kunkei*, *Lactobacillus mellis*, *Lactobacillus* sp. wkB8 and *Snodgrassella alvi*. The relative abundances of each apiary's core bacteriome species are plotted by sampling periods and environmental strata in Fig 5. Table 1 shows the overall and grouped mean and standard deviation of core bacteriome species' relative abundances.

Associations between seasonal conditions, climatic condition levels and the abundance of core bacteriome species were examined using negative binomial generalized linear models

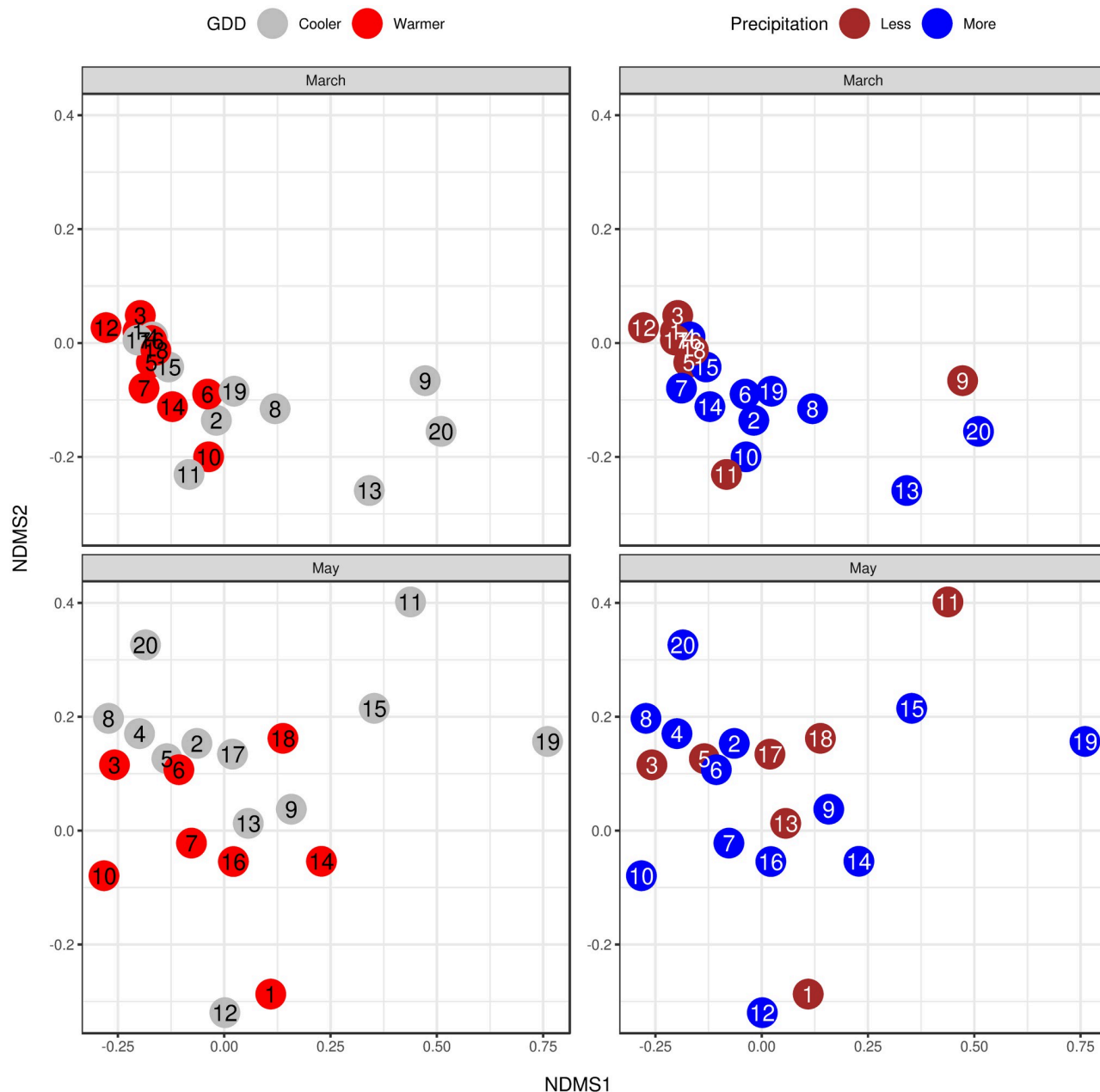


Fig 4. NMDS ordination of bacteriome for environmental condition categories by sampling period. The colours represent the environmental condition categories and the numbers correspond to the apiary IDs. The stress was 0.062 and 0.116 for March and May respectively. The samples' bacteriome from March showed significant ($p = 0.02$) distance between the cooler and warmer districts. From the same period, the precipitation levels did not differ significantly ($p = 0.155$). In the samples gathered in May, there was no significant distance neither between GDD nor precipitation categories ($p = 0.277$ and $p = 0.849$, respectively).

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[42] (Table 2). The abundance of *B. apis* (FC: 15.41, $q < 0.00001$), *B. asteroides* (FC: 1.61, $q = 0.0084$), *C. sp. AMU001* (FC: 2.46, $q = 0.00001$), *L. helsingborgensis* (FC: 1.7, $q = 0.008$) and *S. alvi* (FC: 1.49, $q = 0.011$) significantly increased from March to May. In the same comparison, the abundance of *L. apis* (FC: 0.64, $q = 0.0066$), *L. bombi* (FC: 0.64, $q = 0.0052$), *L. kullabergensis* (FC: 0.57, $q = 0.00056$) and *L. mellis* (FC: 0.64, $q = 0.0052$) was significantly decreased. In the samples collected in March, the abundance of *L. kunkei* (FC: 3.86, $q = 0.094$)

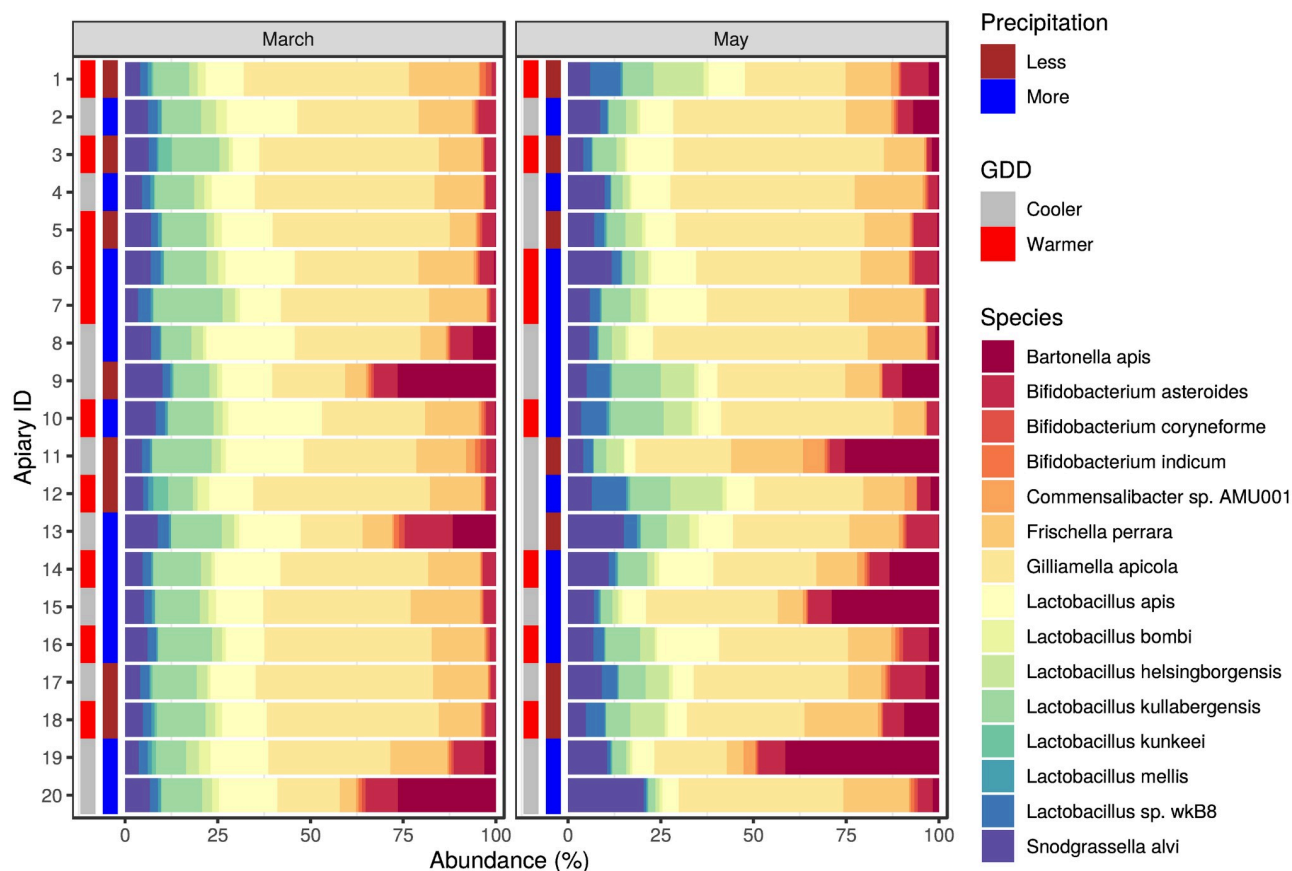


Fig 5. Core bacteriome composition of honey bee gut samples. The relative abundance is plotted for the first (March) and second (May) sampling. Besides the bacterial species of the core bacteriome, the environmental condition (growing degree-day (GDD) and precipitation) categories of sampling places are also marked.

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was significantly higher in warmer regions than in cooler ones. In the same period, the abundance of *B. apis* (FC: 0.02, $q < 0.00001$) and *B. asteroides* (FC: 0.47, $q = 0.0027$) was significantly lower in warmer LMUs than in cooler ones. In March samples, the abundance of *L. kunkeei* (FC: 0.13, $q = 0.011$) was significantly lower in districts with more precipitation than in LMUs with less precipitation. In samples collected in May, none of the core bacteriome species showed significant alterations in abundance neither by GDD categories nor by the precipitation levels. The relative abundance distribution of significantly different species per group is summarized in [S1 Fig](#).

Discussion

Bees as pollinators are essential for both ecology and agriculture. Several results have been reported on the composition of their gut bacteria. However, repeated measurement results from climatic stratified spatial sampling on a country-wide basis are not known in the literature. Our work aimed to gain data regarding the natural diversity of gut bacteriome in healthy worker bees. For this objective, we classified the local administrative units of Hungary into climatic strata based on the longer time series commonly used in climatology. In climate science, this is used to filter out weather fluctuations, thus determining the climate of an area for a given period. We compared warmer to colder and drier to wetter areas within the March

Table 1. Relative abundances by environmental and seasonal categories.

	Species	All samples Mean (SD)	GDD Cooler	Warmer	Precipitation Less	More
March						
	<i>Bartonella apis</i>	3.78 (8.25)	7.41 (10.7)	0.14 (0.14)	3.41 (9.32)	4.02 (7.89)
	<i>Bifidobacterium asteroides</i>	4.08 (3.02)	5.64 (3.62)	2.52 (0.92)	2.83 (1.64)	4.92 (3.49)
	<i>Bifidobacterium coryneforme</i>	0.64 (0.43)	0.72 (0.48)	0.56 (0.38)	0.73 (0.54)	0.58 (0.35)
	<i>Bifidobacterium indicum</i>	0.62 (0.41)	0.69 (0.44)	0.55 (0.39)	0.71 (0.52)	0.56 (0.33)
	<i>Commensalibacter</i> sp. AMU001	0.56 (0.49)	0.66 (0.65)	0.45 (0.24)	0.7 (0.73)	0.46 (0.22)
	<i>Frischella perrara</i>	12.49 (4.11)	11.46 (4.85)	13.52 (3.13)	12 (4.25)	12.82 (4.17)
	<i>Gilliamella apicola</i>	37 (10.63)	31.92 (11.56)	42.08 (6.9)	41.6 (10.67)	33.93 (9.85)
	<i>Lactobacillus apis</i>	15.05 (4.73)	16.23 (4.03)	13.87 (5.29)	12.76 (3.93)	16.57 (4.75)
	<i>Lactobacillus bombi</i>	1.73 (0.71)	1.75 (0.78)	1.71 (0.68)	1.72 (0.75)	1.74 (0.72)
	<i>Lactobacillus helsingborgensis</i>	2.71 (0.61)	2.91 (0.57)	2.52 (0.61)	2.26 (0.49)	3.01 (0.49)
	<i>Lactobacillus kullabergensis</i>	11.78 (2.77)	11.17 (2.42)	12.4 (3.08)	11.49 (2.82)	11.98 (2.84)
	<i>Lactobacillus kunkeei</i>	0.6 (1.13)	0.27 (0.35)	0.94 (1.52)	1.22 (1.61)	0.2 (0.3)
	<i>Lactobacillus mellis</i>	0.75 (0.29)	0.72 (0.29)	0.77 (0.31)	0.77 (0.36)	0.73 (0.26)
	<i>Lactobacillus</i> sp. wkB8	2.34 (0.41)	2.37 (0.28)	2.3 (0.53)	2.07 (0.34)	2.52 (0.37)
	<i>Snodgrassella alvi</i>	5.88 (1.8)	6.09 (2.11)	5.66 (1.5)	5.74 (2.06)	5.97 (1.69)
May						
	<i>Bartonella apis</i>	7.6 (11.43)	10.13 (13.9)	3.81 (4.87)	6.19 (8.99)	8.36 (12.84)
	<i>Bifidobacterium asteroides</i>	5.12 (2.24)	5.29 (2.37)	4.86 (2.16)	6.11 (2.83)	4.59 (1.75)
	<i>Bifidobacterium coryneforme</i>	0.43 (0.29)	0.42 (0.28)	0.44 (0.33)	0.42 (0.2)	0.43 (0.33)
	<i>Bifidobacterium indicum</i>	0.44 (0.28)	0.43 (0.25)	0.45 (0.34)	0.41 (0.19)	0.45 (0.33)
	<i>Commensalibacter</i> sp. AMU001	1.33 (1.37)	1.58 (1.66)	0.96 (0.69)	1.72 (1.89)	1.13 (1.02)
	<i>Frischella perrara</i>	12.79 (4.43)	12.42 (4.67)	13.35 (4.28)	13.79 (4.18)	12.25 (4.62)
	<i>Gilliamella apicola</i>	38.71 (10.71)	38.92 (11.45)	38.4 (10.25)	37.91 (12.12)	39.15 (10.38)
	<i>Lactobacillus apis</i>	8.74 (3.92)	6.82 (2.18)	11.62 (4.29)	7.67 (3.26)	9.32 (4.24)
	<i>Lactobacillus bombi</i>	0.97 (0.5)	0.95 (0.56)	1 (0.43)	1.07 (0.73)	0.92 (0.34)
	<i>Lactobacillus helsingborgensis</i>	5.15 (3.81)	4.74 (3.79)	5.77 (4)	6.64 (3.69)	4.35 (3.76)
	<i>Lactobacillus kullabergensis</i>	6.52 (3.43)	5.56 (3.42)	7.96 (3.08)	6.26 (1.61)	6.66 (4.15)
	<i>Lactobacillus kunkeei</i>	0.18 (0.17)	0.18 (0.2)	0.18 (0.14)	0.12 (0.15)	0.21 (0.18)
	<i>Lactobacillus mellis</i>	0.42 (0.19)	0.41 (0.2)	0.42 (0.19)	0.43 (0.27)	0.4 (0.15)
	<i>Lactobacillus</i> sp. wkB8	3.44 (2.44)	3.07 (2.5)	4.01 (2.4)	4.06 (2.07)	3.11 (2.63)
	<i>Snodgrassella alvi</i>	8.15 (4.16)	9.09 (4.64)	6.74 (3.07)	7.18 (3.91)	8.67 (4.35)

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samples. Samples taken in May were analyzed in the same way. March and May samples from the same apiaries were also compared. Thus, our results provide new data on the effects of the climatic environment and the seasons. As some stocks changed their location during the two samplings, the question may arise as to how this may bias the results. In our opinion, this effect is negligible, as the climatic strata were compared within the March samples and within the May samples. Most can be suggested that the May samples are not climatically representative since, in the case of four apiaries, the environment in May was different from that in March.

We have evaluated differences between environmental conditions, namely temperature and precipitation. It is well known that honey bees possess a relatively simple bacteriome in their gut, constituted of only a small number of species. There are members of the bacteriome that are always present, these are often referred to as core members. Core bacteria of the honey bee gut bacteriome are *S. alvi*, *G. apicola*, and a few *Lactobacillus* and *Bifidobacterium* species. Besides the core members, there are frequent but not essential species as well, e.g. *B. apis* and *F. perrara* [17, 19, 44–46]. Our results are in agreement with these previous findings, as the

Table 2. Abundance alterations of core bacteriome by seasonal and climatic conditions. A negative binomial model estimated the association between species abundance of core bacteriome and sampling seasons, GDD- and precipitation level.

Samples	Species	Mean counts [†]	Fold change (95% CI)	q [§]	Mean counts	Fold change (95% CI)	q
		GDD Warmer vs. cooler			Precipitation More vs. less		
May	<i>Bartonella apis</i>	1068.87	0.02 (0, 0.08)	<0.00001	1068.87	0.99 (0.15, 6.53)	*
	<i>Bifidobacterium asteroides</i>	1144.10	0.47 (0.31, 0.71)	0.00274	1144.10	1.52 (0.94, 2.47)	0.34335
	<i>Bifidobacterium coryneforme</i>	180.26	0.78 (0.47, 1.3)	0.55291	180.26	0.72 (0.43, 1.2)	0.44555
	<i>Bifidobacterium indicum</i>	176.76	0.81 (0.49, 1.33)	0.55291	176.76	0.72 (0.43, 1.19)	0.44555
	<i>Commensalibacter</i> sp. AMU001	162.07	0.68 (0.37, 1.25)	0.46158	162.07	0.58 (0.32, 1.05)	0.34335
	<i>Frischella perrara</i>	3662.30	1.18 (0.84, 1.65)	0.55291	3662.30	0.97 (0.68, 1.37)	0.85067
	<i>Gilliamella apicola</i>	11004.31	1.29 (0.94, 1.78)	0.42005	11004.31	0.75 (0.55, 1.04)	0.34335
	<i>Lactobacillus apis</i>	4366.20	0.84 (0.65, 1.09)	0.46158	4366.20	1.17 (0.89, 1.54)	0.47268
	<i>Lactobacillus bombi</i>	495.19	1.03 (0.73, 1.44)	0.86802	495.19	0.89 (0.63, 1.26)	0.67884
	<i>Lactobacillus helsingborgensis</i>	786.53	0.87 (0.71, 1.07)	0.46158	786.53	1.2 (0.96, 1.5)	0.34335
	<i>Lactobacillus kullabergensis</i>	3454.42	1.11 (0.87, 1.41)	0.55291	3454.42	0.96 (0.74, 1.24)	0.80541
	<i>Lactobacillus kunkeei</i>	187.49	3.86 (1.25, 11.89)	0.09368	187.49	0.13 (0.05, 0.36)	0.01120
	<i>Lactobacillus mellis</i>	213.99	1.13 (0.79, 1.61)	0.62292	213.99	0.86 (0.59, 1.25)	0.59928
	<i>Lactobacillus</i> sp. wkB8	681.21	0.97 (0.8, 1.18)	0.84023	681.21	1.1 (0.89, 1.36)	0.58331
	<i>Snodgrassella alvi</i>	1706.23	0.92 (0.71, 1.2)	0.62292	1706.23	0.94 (0.71, 1.24)	0.78937
May	<i>Bartonella apis</i>	1553.31	0.28 (0.08, 1.01)	0.22572	1553.31	1.46 (0.36, 5.85)	0.86672
	<i>Bifidobacterium asteroides</i>	942.33	0.83 (0.56, 1.22)	0.74756	942.33	0.9 (0.6, 1.37)	0.86672
	<i>Bifidobacterium coryneforme</i>	79.75	0.92 (0.49, 1.73)	0.98269	79.75	1.13 (0.59, 2.18)	0.86672
	<i>Bifidobacterium indicum</i>	81.39	0.9 (0.49, 1.63)	0.98269	81.39	1.23 (0.66, 2.28)	0.86672
	<i>Commensalibacter</i> sp. AMU001	255.76	0.51 (0.26, 1)	0.22572	255.76	0.72 (0.35, 1.49)	0.86672
	<i>Frischella perrara</i>	2522.61	1.02 (0.66, 1.56)	0.98269	2522.61	0.98 (0.62, 1.54)	0.92494
	<i>Gilliamella apicola</i>	7869.43	1.01 (0.65, 1.57)	0.98269	7869.43	1.09 (0.69, 1.73)	0.86672
	<i>Lactobacillus apis</i>	1698.58	1.62 (1.07, 2.45)	0.22572	1698.58	1.23 (0.77, 1.97)	0.86672
	<i>Lactobacillus bombi</i>	183.30	1.06 (0.68, 1.66)	0.98269	183.30	1.03 (0.65, 1.65)	0.92494
	<i>Lactobacillus helsingborgensis</i>	952.28	1.2 (0.66, 2.17)	0.98269	952.28	0.79 (0.43, 1.45)	0.86672
	<i>Lactobacillus kullabergensis</i>	1258.90	1.5 (0.93, 2.41)	0.29310	1258.90	1.17 (0.7, 1.97)	0.86672
	<i>Lactobacillus kunkeei</i>	36.06	1.08 (0.39, 2.97)	0.98269	36.06	1.65 (0.59, 4.57)	0.86672
	<i>Lactobacillus mellis</i>	78.82	1 (0.65, 1.56)	0.98269	78.82	1.12 (0.7, 1.77)	0.86672
	<i>Lactobacillus</i> sp. wkB8	648.44	1.33 (0.75, 2.36)	0.74756	648.44	0.91 (0.49, 1.66)	0.86672
	<i>Snodgrassella alvi</i>	1567.32	0.66 (0.43, 1.02)	0.22572	1567.32	1.43 (0.9, 2.25)	0.86672
		May vs. March					
All	<i>Bartonella apis</i>	1538.45	15.41 (6.07, 39.17)	<0.00001			
	<i>Bifidobacterium asteroides</i>	1074.14	1.61 (1.16, 2.24)	0.00837			
	<i>Bifidobacterium coryneforme</i>	118.29	0.74 (0.52, 1.05)	0.12117			
	<i>Bifidobacterium indicum</i>	118.69	0.78 (0.55, 1.11)	0.18588			
	<i>Commensalibacter</i> sp. AMU001	232.29	2.46 (1.7, 3.57)	0.00001			
	<i>Frischella perrara</i>	3046.15	1.17 (0.85, 1.61)	0.34279			
	<i>Gilliamella apicola</i>	9304.64	1.22 (0.92, 1.62)	0.18588			
	<i>Lactobacillus apis</i>	2740.93	0.64 (0.48, 0.86)	0.00656			
	<i>Lactobacillus bombi</i>	309.86	0.64 (0.48, 0.84)	0.00519			
	<i>Lactobacillus helsingborgensis</i>	928.22	1.7 (1.19, 2.43)	0.00800			
	<i>Lactobacillus kullabergensis</i>	2115.13	0.57 (0.43, 0.76)	0.00056			
	<i>Lactobacillus kunkeei</i>	99.88	0.64 (0.36, 1.14)	0.16276			
	<i>Lactobacillus mellis</i>	133.69	0.64 (0.49, 0.85)	0.00519			

(Continued)

Table 2. (Continued)

Samples	Species	Mean counts [†]	Fold change (95% CI)	q [§]	Mean counts	Fold change (95% CI)	q
		GDD Warmer vs. cooler			Precipitation More vs. less		
	<i>Lactobacillus</i> sp. wkB8	680.91	1.35 (0.98, 1.87)	0.10342			
	<i>Snodgrassella alvi</i>	1700.90	1.49 (1.12, 1.98)	0.01099			

[†]Sequence read counts were normalized by dividing raw counts by DESeq size factors

[§]FDR-adjusted p-value. FDR adjustment was conducted in each pairwise comparison separately

*DESeq method can't estimate p-values without outlier replacement

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core bacteriome found in this study contained *Snodgrassella*, *Gilliamella* and different *Lactobacillus* and *Bifidobacterium* species (*B. asteroides*, *B. coryneforme*, *B. indicum*, *L. apis*, *L. helsingborgensis*, *L. kullabergensis*, *L. mellis*) which were all previously described as core members. Also, the frequently observed but non-core members *B. apicola* and *F. perrara* were also present. Surprisingly, we found *L. kunkei* to be present in every sample in our experiment, which, although being a regular member in the honey crop, rarely presents in the gut [21]. The presence of *L. kunkei* in the core bacteriome of our study could be the result of our gut preparation protocol, namely that the whole gastrointestinal tract was extracted during sample processing.

We assumed that a seasonal shift in gut bacteriome would be identified as such differences have been observed in many other invertebrate and vertebrate species. For instance, seasonal variation of the gut bacteriome was found in humans [47, 48], non-human primates [49, 50], other mammalian species [51, 52], fishes [15], birds [53] and arthropods as well [54, 55]. However, seasonal changes are most likely linked to other factors, such as changes in the feeding habit of the animal or its lifestyle. Thus other potential factors, such as the effect of environmental conditions, should also be accounted for when the natural variation of the microbiome is considered. Although there is a lot of information on the honey bee gut bacteriome composition, little is known about its seasonal and environmental variation. Kešnerová and colleagues [22] examined the variation of the honey bee gut bacteriome throughout a year and found marked differences between winter bees and foragers. However, other studies which mainly focused on the variation during the honey producing season observed little to no differences [19, 20]. To our knowledge, however, there is no large-scale study to evaluate the effect of environment on the honey bee gut bacteriome. Although previous studies have found differences between honey bees kept in two different locations [12, 17], detailed understanding of environmental conditions is still missing. In keeping with our initial expectations, we observed significant differences between seasonal states and environmental conditions. The β -diversity was significantly different between March and May based on NMDS ordination (Fig 3) and March samples between warmer and cooler regions differed either in their α - and β -diversity (Figs 2 and 4). Besides, several bacterial species of the core bacteriome of our study have shown significant differences between seasonal and environmental states. Based on the NMDS ordination, less variability in β -diversity was found between apiaries in March than in May (Fig 4). Although warmer and cooler regions separated either in March or May samples as well, the observed higher variability could be a reason why this difference was found to be significant only in March (Fig 4). However, between precipitation levels, β -diversity didn't show such clear differences neither in March nor in May (Fig 4). We could explain these differences as a transition from early after winter, when bees still need feed supplementation and their

bacteriome is not fully transitioned to the summer state, to a bacteriome characteristic to summer, where apiary level and regional differences can shape its composition. Bacteriomes in the winter can show substantial differences in different insect species [56, 57]. However, during the honey-producing season, a more even distribution of species can be observed in foragers, with *Gilliamella* being one of the most abundant members. Although the bacterial composition of our March samples is more similar to the summer state of the honey bee gut bacteriome (indicating that it is almost completely transformed from the winter state), *Lactobacilli* still occupy a large proportion of the core bacteriome, which is significantly reduced in May (Fig 5). Besides, in contrast to the findings of Kešnerová and colleagues [22], *B. apis* shows a notable increase from March to May in our results (Fig 5). We could reason that the observed smaller variability of β -diversity in March samples (Fig 4) could be the consequence of the lack of flowering plants and the fact that beekeepers use similar feed supplements to complete the nutritional needs of the colonies. However, as the β -diversity of warmer and cooler LAUs significantly differed in March, it is straightforward to assume that flowering is initiated earlier in warmer regions. The elevated abundance of *L. kunkeei* could also indicate the onset of nectar collection as this is a highly specialised bacterial inhabitant of the honey crop. It was shown that *L. kunkeei* is nearly absent in winter, however its abundance gradually increases from spring to summer [58, 59]. In May, however, no significant difference in β -diversity was found between environmental conditions, although this could be the reason for the higher variability found between apiaries. During the peak of the honey producing season, many factors could affect the microbial composition of the gut and thus account for the higher variation. It is well known that diet can shape the microbiome composition of humans and other species [49, 60–65], including honey bees and bumblebees [66, 67]. Although owners of the apiaries were consistent in keeping their bees in regions covered in acacia, slight differences might occur between individual regions. Pesticides can also influence the gut microbial communities of honey bees [68]. Even though the owners of the apiaries sampled in this study didn't observe any sign of poisoning on their farms, subclinical pesticide exposure can affect the gut microbiome of honey bees even without any visible impact on the colony [69]. Furthermore, it is possible that intrinsic effects, such as genotype, can also affect the microbial composition of the honey bee gut, as was found in the case of *Drosophyla melanogaster* [70].

The honey bee is an important pollinator species worldwide [71, 72]. Its high economic value resides in its role in crop pollination and the wide variety of products they make [1–3]. They were also found to be a useful model animal for several biological research areas, including microbiome research [73, 74]. The gut bacteriome of honey bees is an essential determinant of their health. It possesses a myriad of functions that benefit its host, for example, it enables the degradation of different polysaccharides originating from the bees diet, such as pollen walls [75, 76]. It might also have a role in recycling the nitrogen waste materials of honey bee nitrogen metabolism [76] and can metabolise potentially toxic sugars for bees [75]. Besides these metabolic functions, the gut microbiome has a positive impact on the host immune system [45] and it protects the bees from different pathogens [45, 77]. Understanding the normal composition and natural variation of the gut bacteriome of honey bees is an important foundation for future research. It is necessary for understanding how different pathologic conditions can alter its composition and to work out protocols to return it to a healthy state [78]. Our results provide data on the association of the honey bee bacteriome with season, precipitation and temperature in temperate climatic conditions. Due to the two-level pooling, we can assume that the effect of other untreated factors (e.g. age heterogeneity) besides the environmental factors under investigation can be neglected. Including such strata in the sampling design of further studies would be valuable. The results presented, together with potential

future studies, can increase our understanding of the natural fluctuation of the healthy bacteriome of honey bees and could help in the preservation of their health.

Conclusion

Based on our results, one may conclude that the composition of healthy core bacteriomes in honey bees varies depending on the climatic and seasonal conditions. This is probably since climatic characteristics and vegetation states determine the availability and nutrient content of flowering plants. The results of our study prove that in order to gain a thorough understanding of a microbiome's natural diversity, we need to obtain the necessary information from extreme ranges of the host's healthy state.

Supporting information

S1 Fig. Relative abundance distributions. Boxplots denoting the actual point distribution for the differential species in comparisons were significant.
(PDF)

S1 Table. Sample sizes by apiaries and families for the both sampling periods. During the sampling in May, in two apiaries (ID: 6, 13) only two of the three colonies from the March sampling period were accessible so only these two were sampled.
(PDF)

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Author Contributions

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***Vairimorpha (Nosema) ceranae* distribution based on a honey bee gut microbiome survey in Hungary**

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***Vairimorpha (Nosema) ceranae* előfordulása Magyarországon, mézelő méhek (*Apis mellifera*) bélmikrobiom-felmérése alapján**

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ÖSSZEFOGLALÁS

Az európai mézelő méh (*Apis mellifera*), világszerte jelentős rovarfaj a mezőgazdaság számára. A méhcsaládokat számos kórokozó fenyegeti, köztük a *Vairimorpha (Nosema) apis* és *Vairimorpha (Nosema) ceranae* egysejtű gomba. Sokáig csupán a *V. apis* okozta kórkép (*nosemosis apium* – gyomorvész) volt ismert, azonban a közelmúltban egyre inkább az újonnan behurcolt *V. ceranae* előfordulása és kórtani szerepe vált jelentőssé. Egy korábbi vizsgálatukban a szerzők klímátikusan reprezentatív mintavételt végeztek a hazai méhcsaládokból márciusban és májusban, a méhek bélmikrobiomjának shotgun metagenomikai vizsgálata céljából. Minden mintában azonosítottak *V. ceranae* eredetű szekvenciákat. A kórokozó szignifikánsan gyakoribb volt májusban, valamint ugyanebben a mintavételi időpontban a csapadékosabb területeken. A *V. apis* fajnak nem találták a nyomát.

SUMMARY

Background: *Apis mellifera* is one of the most important arthropod species for agriculture. Several factors are known to compromise their health, posing a serious economic risk. Among these factors, pathogens and mainly parasites are especially important, including the single-celled fungi *Vairimorpha apis* and *Vairimorpha ceranae*. Historically, *V. ceranae* was not considered to infect *A. mellifera*; however, recently, it was found in this species as well and has become more prominent worldwide.

Objective: During a shotgun metagenomic survey on honey bees' gut bacteriome, we found sequences originating from *V. ceranae* in all of our samples. Our findings of this species in the Hungarian *A. mellifera* gut metagenome is presented in this study.

Materials and Methods: We have performed a climatically representative sampling on the Hungarian honey bee population in 2019. Twenty apiaries were selected for the analysis and sampled twice in the honey-producing season, at its onset (March) and peak (May). Shotgun metagenomic sequencing was performed to determine the microbiome composition of the samples. We have statistically analyzed the differences in *V. ceranae* relative abundance between the two sampling times and determined the effect of temperature and precipitation.

Results and Discussion: Reads classified as *V. ceranae* were found in all twenty samples at both sampling times, which indicates that this species is widely distributed in Hungary. Interestingly, *V. ceranae* was significantly more abundant in May, which could support the observations that the epidemiology of this species indeed shows a seasonal pattern. Despite the previous importance of *V. apis*, we were unable to identify this species in our samples. Considering the environmental factors, *V. ceranae* was significantly more abundant in samples from regions with higher precipitation in May.

HÁZI MÉH

A növények beporzásán és a méhészeti termékeken keresztül az európai mézelő méh (*Apis mellifera*) jelentősége az ember számára felbecsülhetetlen [1–3]. Az utóbbi évtizedekben a méhek életben maradását és termelőképességét számos tényező, így pl. a különféle kémiai anyagok széleskörű használata a mezőgazdaságban, valamint számos kórokozó veszélyezteti [4–6]. Az *A. mellifera* fertőző kórképei között kiemelkedő jelentőségűek a parazitózisok, közöttük is a *Varroa destructor* okozta atkakór (varroosis), valamint a nosemosis [5, 7, 8]. Ez utóbbi kórképben korábban a *Vairimorpha* (*Nosema*) *apis* (Microsporidia: Nosematidae) és újabban a *Vairimorpha* (*Nosema*) *ceranae* (Microsporidia: Nosematidae) fajoknak tulajdonítanak oktatni szerepet [7–9]. Ezek obligát intracelluláris parazitikus gombák [10, 11], amelyeket 2020-ban a *Nosema* nemzetségből a *Vairimorpha* nemzetségbe sorolták át [11].

Az utóbbi évtizedekben a méhek életben maradását és termelőképességét számos tényező veszélyezteti

Hazánkban korábban a gyomorvész okozó *V. apis* fordult elő

Az *A. mellifera* esetében korábban kizárólagosan a *V. apis* okozta fertőzés fordult elő [9, 12–14], amelyet hazánkban nosemosis, vagy gyomorvész néven régóta ismertek [15]. Az eredetileg az indiai méh (*Apis cerana*) saját kórokozójának tartott a *V. ceranae*-t 1996-ban írták le [16], amely gazdaspektrum-növekedés következtében az *A. mellifera* fertőzésére is képessé vált [9, 12–14]. Európában és Ázsiában egyaránt 2005-ben azonosították először *A. mellifera*-ban [17, 18], ezután több európai országban, köztük 2006-ban hazánkban is megfigyelték [12, 13, 15, 19]. Napjainkra a nosemosis kórokozói közül a *V. ceranae* vált dominánssá *A. mellifera*-ban is, ennek ellenére *V. apis* okozta fertőzöttség is előfordul [14, 15, 19–21]. Érdekesség, hogy többször leírtak vegyes fertőzéseket mind *A. mellifera* [12], mind *A. cerana* esetében [9]. A két legfontosabb fajon, vagyis a *V. apis* és *V. ceranae*-n kívül egyéb *Nosema* fajokat is megfigyeltek már *A. mellifera*-ban, amelyek kóroktani jelentősége még kérdéses [21].

A *Vairimorpha* fajok elsősorban a méhek középbelének hámsejtjeiben szaporodva fejtik ki kórokozó hatásukat

A *Vairimorpha* fajok elsősorban a méhek középbelének hámsejtjeiben szaporodva fejtik ki kórokozó hatásukat [14, 19, 21–23]. Miután kifejezetten a bélhez kötött patogénekről van szó, a bélsárnak jelentős szerepe van a kórokozó terjesztésében, akár a méhcsaládon belül, akár a külvilágban [14, 21, 22]. Kórlefolys tekintetében különbséget tapasztaltak a *V. apis* és *V. ceranae* által okozott kórképek között. Az előbbi esetében inkább heveny, hasmenéses tünetekkel járó kórlefolys jellemző, míg az utóbbi gyakran alakít ki idült megbetegedést [14, 19]. Ennek kialakulásában szerepe lehet ez utóbbi kórokozó és a méhek bélbakteriómja között tapasztalt pozitív kapcsolatnak [23]. Az első kísérletes tanulmányok a *V. ceranae* fertőzöttséggel összefüggésben a méhek életkorának lerövidülését tapasztalták, így a kórokozót összefüggésbe hozták a kaptárelhagyás (Colony Collapse Disorder, CCD) tünetegyüttesével is [24]. Annak ellenére, hogy későbbi vizsgálatok során egymásnak ellentmondó eredményeket találtak [7, 13, 14, 20, 25, 26], úgy tűnik, hogy az utóbbi időben beigazolódni látszik ez a feltevés [27]. Járványtani szempontból fontos különbség, hogy a *V. apis* esetében jól ismert a fertőzöttség, a megbetegedések szezonálisága, míg a *V. ceranae* szezonálisan változó megjelenése [27–31] mellett az egész évben való előfordulást is lehet tapasztalni [27, 32].

Az újgenerációs szekvenálás elterjedésével megszorodott a méhek mikrobiomját célzó vizsgálatok száma

Az újgenerációs szekvenálás (new generation sequencing, NGS) elterjedésével megszorodott a méhek mikrobiomját célzó vizsgálatok száma [33–36]. Ezek jelentős része azonban a mintát alkotó baktériumközösség 16S rRNS-génjének szekvenálásán keresztül vizsgálja a bakterióm összetételét. Megemlítendő azonban, hogy ez a megközelítés csupán korlátozott lehetőséget biztosít a mikrobióta egészének elemzésére. Az ún. shotgun metagenomika során azonban a minta teljes DNS-tartalmának feldarabolásával és az így kapott fragmentumok szekvenciájának véletlenszerű meghatározásával egy sokkal részletesebb képet nyerhetünk a mintát alkotó mikrobiális közösség összetételéről [37].

2019-ben országos mintagyűjtést végeztünk a mézelő méhek bélmikrobiom-variabilitásának szezonális és klimatikus (hőmérséklet és csapadékmennyiség sze-

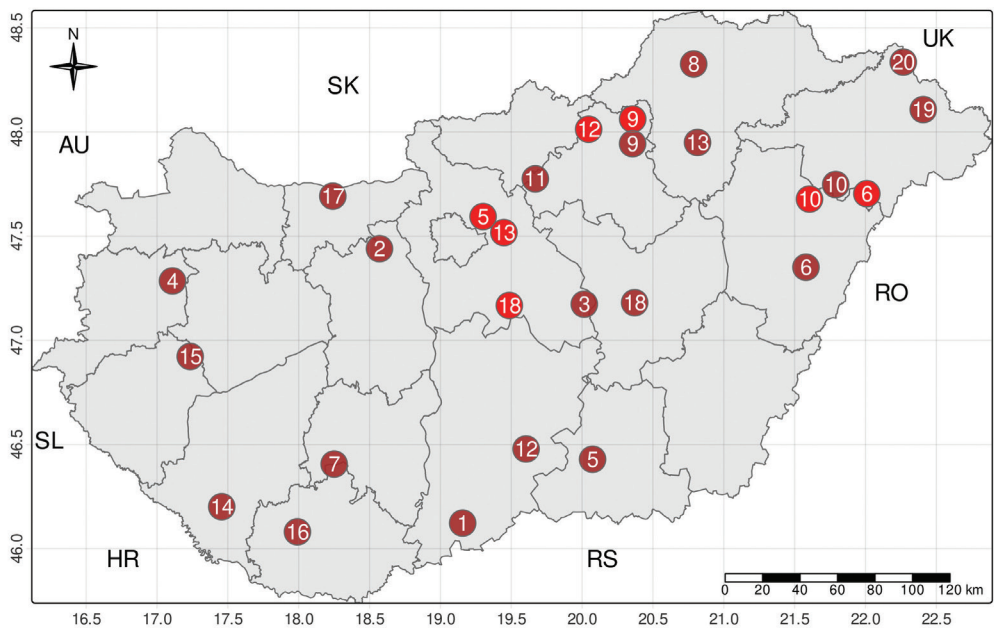
rinti) megismerése céljából. Mintáinkat shotgun metagenom szekvenálással szekvenáltuk, ahol a bélbakteriom vizsgálatán túl [36] lehetőségünk volt a mikrobióta egyéb alkotóinak, így a pl. a vírusok, úgymint az *Apis mellifera* filamentous virus [38], valamint a *V. ceranae* jelenlétének, továbbá szezonális és klimatikus variabilitásának meghatározására. Ez utóbbi kórokozó faj vizsgálata során kapott eredményeinket jelen tanulmányunkban mutatjuk be.

ANYAG, MÓDSZER

MINTAGYŰJTÉS ÉS ELŐKÉSZÍTÉS

A szerzők éghajlati környezetük alapján országosan reprezentatív módon összesen 20 méhészetből vettek mintákat

A mintavétel tervezésének és lebonyolításának részletei megtalálhatók a PAPP és mtsai (2022) közleményének anyag és módszer című fejezetében [36]. E helyen csak az eredmények értelmezéséhez szükséges részleteket foglaljuk össze. 2019-ben egy az országra térben kiegyensúlyozott mintavételre került sor, amely során két alkalommal, a méztermelési időszak kezdetén (március) és csúcspontján (május) gyűjtöttünk méheket. Összesen 20 méhészetet (1. ábra) választottunk ki, úgy, hogy éghajlati környezetük alapján országosan reprezentatív mintát kapjunk. Az éghajlati környezetet az éves akkumulált növekedési foknappal (growing degree days, GDD) és az éves csapadékkal jellemeztük. A méhészetek kiválasztásának alapját a járások adták, amelyeket a környezeti változók szerint két-két



1. ÁBRA. Mintavételi pontok Magyarországon, vármegyehatárokkal

A barna pontok a márciusi, ill. a nem vándorló állományok májusi mintavételi helyét, míg a piros pontok a májusban vándorló állományokat (5., 6., 9., 10., 12., 13., 18. azonosítójú) jelzik. A 20. méhészet esetében a két mintavétel közti földrajzi eltérés olyan kicsi, hogy a térképen nem ábrázoltuk külön. A szomszédos országok ISO 2 jelölése: Ausztria (AU), Horvátország (HR), Románia (RO), Szerbia (RS), Szlovákia (SK), Szlovénia (SL), Ukrajna (UK)

FIGURE 1. Sampling points in Hungary with county boundaries

The brown dots indicate the March sampling sites and the May sampling sites of non-migratory apiaries, while the red dots indicate the May migratory apiaries (ID: 5, 6, 9, 10, 12, 13, 18). For apiary 20, the geographical difference between the two sampling dates is so small that it is not shown separately on the map. Neighbouring countries are presented by ISO 2 character codes: Austria (AU), Croatia (HR), Romania (RO), Serbia (RS), Slovakia (SK), Slovenia (SL), Ukraine (UK)

Méhészetenként
három-három
családból gyűjtött 10-10
egészséges dolgozó
gyomor-bélrendszerét
távolították el
szekvenálás céljából

Az Illumina Nextseq
szekvenálóval kapott
rövid szekvenciákat
bioinformatikai
módszerekkel elemezték

Az adatok elemzése
során azonosították
a *V. ceranae* genetikai
állományát
a mintákban

osztályba soroltuk. Így a GDD alapján hűvösebb és melegebb, a csapadék alapján szárazabb és csapadékosabb járásokat azonosítottunk. A mintavételhez, éghajlati tulajdonságaik alapján, 20 reprezentatív járást választottunk ki. Ezekből a járásokból egy-egy méhészetet választottunk, ahol elvégeztük a mintavételt. A méhcsaládok vándoroltatása miatt 8 méhészet (5., 6., 9., 10., 12., 13., 18. és 20. azonosítójú) állománya a márciusi mintavételtől eltérő földrajzi helyre került a májusi mintavételre, a részleteket az 1. ábra mutatja. A 20. méhészet esetében ez az eltérés olyan kicsi volt, hogy a térképen nem különül el. Méhészetenként három-három családból gyűjtött egyedekből családonként 10 egészséges dolgozó gyomor-bélrendszerét távolítottuk el szekvenálás céljából. Ennek során mindkét mintavételi időponthoz méhészetenként egy-egy kevert mintát, ún. pool-t hoztunk létre. Ezekből a pool-ozott mintákból Illumina NextSeq szekvenálóval párosított végű rövid leolvasási szekvenciákat, ún. paired-end short read-eket generáltunk.

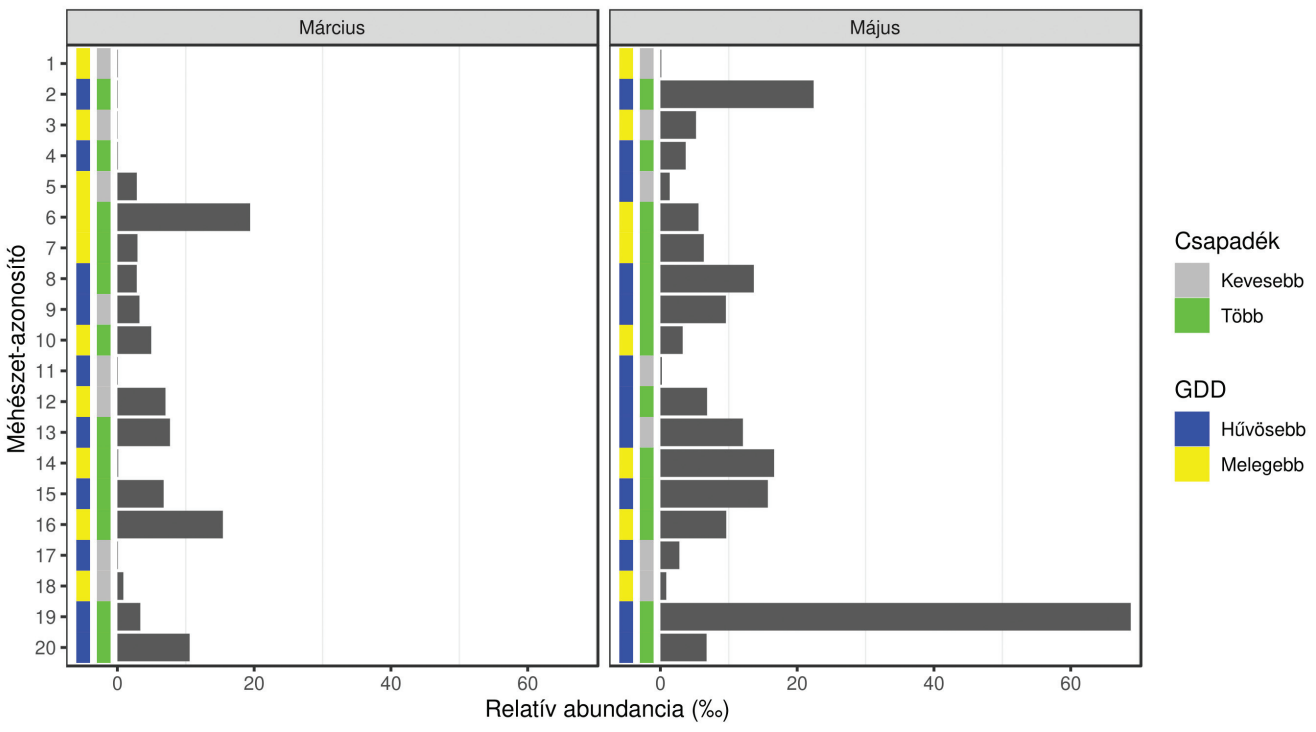
BIOINFORMATIKAI ÉS STATISZTIKAI ELEMZÉSEK

A minőség alapú szűrést, valamint a nyers leolvasási szekvenciák (short read-ek) nem megfelelő minőségű szakaszainak eltávolítását (ún. trimmelését) az Adapterremoval [39] szoftverrel végeztük, 20-as minőségi küszöbértéket használva, és csak az 50 bp-nél hosszabb szekvenciákat megtartva. Az így megmaradt read-eket a Kraken2 [40] szoftver segítségével osztályoztuk taxonómiaiilag (35 bázispár hosszúságú k-mereket használva), az EuPathDB46-Clean adatbázis felhasználásával [41]. A taxon-osztályozási eredményeket a phyloseq [42] és a microbiome R-csomag függvényeivel dolgoztuk fel [43]. A szezonális és időjárási csoportokban kapott abundanciák közötti eltéréseket a DESeq2 csomaggal elemeztük [44]. A szezonális hatás elemzése során ún. kevert modellt alkalmaztunk az ismételt mérések kezelésére, amelyben a méhészetek, mint random tényezők szerepeltek. A statisztikai tesztek eredményéhez kétoldali p-értéket adtunk meg, amelyeknél a 0,05-nél kisebb értékeket tekintettük szignifikánsnak. A rövid leolvasási szekvenciák hosszabb, összefüggő szakaszokká történő összeillesztését (az ún. de novo assembly-t) MEGAHIT (v1.2.9) [45] segítségével végeztük, a kontigok (összeillesztett szakaszok) pontosításához a POLCA (v4.1.0) szoftvert használtuk [46]. A filogenetikai elemzést a polar tube protein 1 (PTP1) gén nukleinsav-szekvenciái alapján végeztük el, melynek során a saját és a nyilvános adatbázisokban elérhető szekvenciákat páronként blastn [47] segítségével összehasonlítottuk, valamint feltételezett kapcsolatot egy genetikai törzsfán ábrázoltuk. A törzsfá [48] alapját a MAFFT [49] (v7.490) segítségével végzett többszörös szekvenciaillesztés (multiple sequence alignment, MSA) képezte. A legjobb szubsztitúciós modellt a phangorn [50] csomag függvényeivel választottuk ki, a bayes-i információs kritérium (Bayesian information criterion, BIC) alapján. A generált neighbor-joining fát maximum-likelihood módszerrel optimalizáltuk. A bootstrap-értékeket 100 iterációval állítottuk elő. Minden adatfeldolgozás és ábrázolás R-környezetben történt [51].

EREDMÉNYEK

A shotgun metagenomikai szekvenálás 311 931 és 546 924 közötti párosított végű short read-et generált a mintákból, az átlag 413 629 volt. Az összes mintában ($n = 40$) azonosítani tudtunk *V. ceranae* eredetű szekvenciákat, amelyek mintánkénti relatív gyakoriságát a 2. ábra foglalja össze. A *V. ceranae*-hoz rendelt read-ek minimális, maximális és medián mintánkénti száma 16, 31 373, ill. 1 758. Megemlítendő, hogy találtunk néhány read-et, amely egy másik *Nosematidae* családba sorolt microsporidium, a *Nosema bombycis* genomjára illeszkedett (minimum: 11, maximum: 60, medián: 29).

A *V. ceranae* szezonális és klimatikus viszonyok szerinti különbségének mértékét az ún. fold change segítségével jellemeztük, amely egy becslés az egyes vizsgált



2. ÁBRA. A *Vairimorpha ceranae*-hoz sorolt read-ek relatív abundanciája az első (márciusi) és második (májusi) mintavétel idején
Az ábrán színekkel jelöltük az éves akkumulált növekedési foknap (GDD) és a csapadékmennyiség, mint éghajlati változók szerinti kategóriákat

FIGURE 2. Relative abundance of *Vairimorpha ceranae* originated reads for the first (March) and second (May) sampling
The environmental condition, growing degree-day (GDD), and precipitation categories of sampling sites are also marked

A csapadékosabb területekről, ill. a májusban gyűjtött mintákban nagyobb mennyiségben azonosítottak *V. ceranae* eredetű szekvenciákat

kategóriák közötti gyakoriság arányok eltérésének mértékére. A statisztikai elemzéseink szerint a *V. ceranae*-ból származó read-ek nagyobb számban fordultak elő a májusi mintákban ($n = 20$), mint a márciusi mintákban ($n = 20$) (FC: 3,13, 95%CI: 1,61–6,11, $p < 0,001$). A márciusi minták esetében a hőmérséklet és csapadék tekintetében nem tudtunk szignifikáns különbségeket feltárni, azonban a melegebb területekről gyűjtött mintákban ($n = 10$) nagyobb mennyiségben azonosítottuk a *V. ceranae* eredetű szekvenciákat, mint a hűvösebb területekről gyűjtött minták ($n = 10$) esetében (FC: 1,66, 95%CI: 0,44–6,21, $p = 0,451$). Ugyanezen mintavétel esetén a csapadékosabb ($n = 12$) területekről gyűjtött mintákban nagyobb arányban azonosítottuk a *V. ceranae*-hoz sorolt read-eket, mint a szárazabb ($n = 8$) területekről gyűjtött mintákban (FC: 2,98, 95%CI: 0,81–10,97, $p = 0,101$).

Májusban a hőmérséklet esetében szintén nem figyeltünk meg szignifikáns különbséget, azonban a melegebb területekről gyűjtött mintákban ($n = 8$) kisebb mennyiségben tudtunk *V. ceranae* eredetű read-eket azonosítani (FC: 0,44, 95%CI: 0,16–1,20, $p = 0,107$). Csapadékosabb ($n = 13$) területek esetében azonban a májusi minták szignifikánsan nagyobb arányban tartalmaztak *V. ceranae* eredetű read-eket, mint a szárazabb ($n = 7$) területekről gyűjtöttek (FC: 3,77, 95%CI: 1,42–10,00, $p = 0,008$).

A májusi minták közül a 19. méhészet esetében, de novo assembly (vagyis a szekvenciák hosszabb, összefüggő szakaszokká történő összeillesztése) segítségével létre tudtuk hozni a *PTP1* gén egy 649 bp hosszúságú darabját. Ez a szakasz a blastn-el végzett páronkénti illesztés alapján a JF772107.1 "NCBI accession ID" azonosítójú *PTP1* génnel mutatta a legnagyobb egyezőséget (szekvenciális

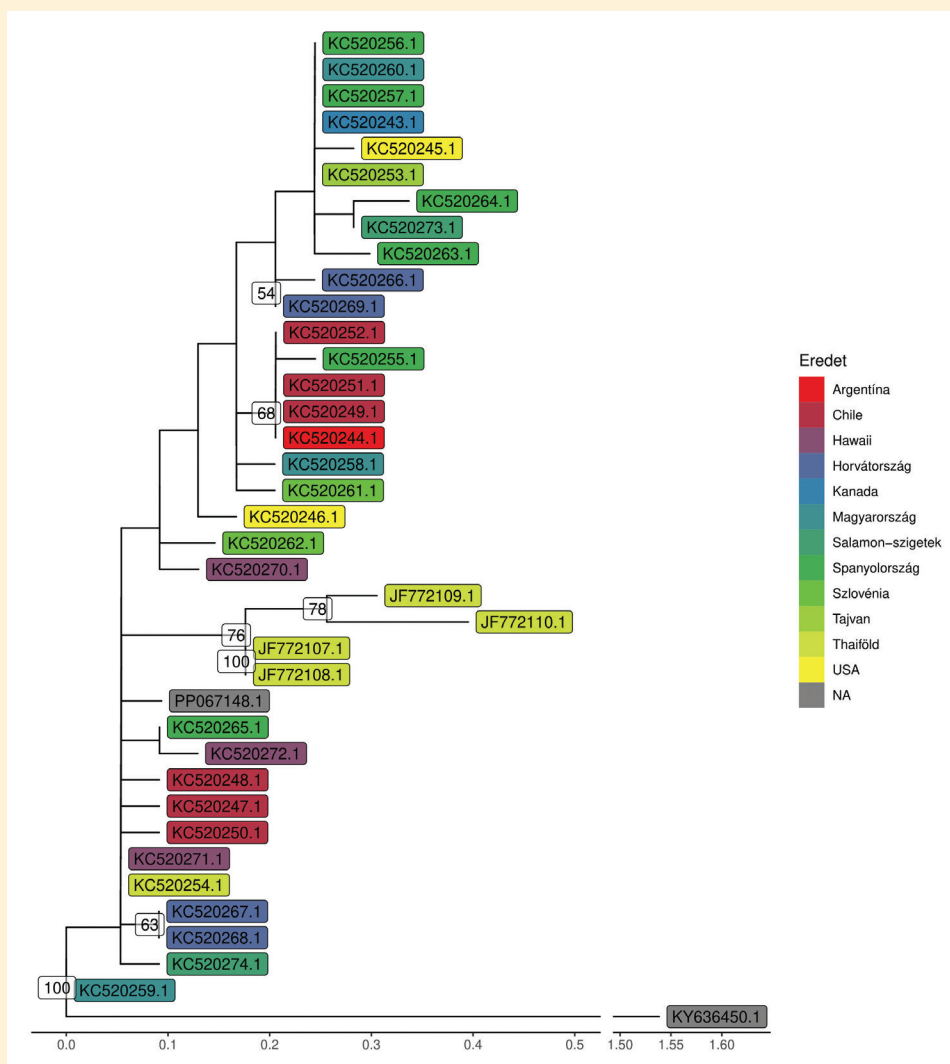
azonosság: 644/650; gap: 2/650). A 3. ábra a *PTP1* gén DNS-szekvenciáján alapuló génfát mutatja. A fa elkészítéséhez a BIC alapján legjobbként azonosított TPM1u szubsztitúciós modellt használtuk.

3. ÁBRA. A polar tube protein 1 (PTP1) DNS szekvenciája alapján készített törzsfa

A mintákat a hozzájuk tartozó NCBI accession ID azonosítók jelölik. A *Nosema bombycis* (KY636450.1) PTP1 génszekvenciáját, mint csoporton kívüli mintát használtuk a fa elkészítéséhez. A PP067148.1 azonosító jelöli azt a PTP1 szekvenciát, amelyet a 19-es méhészet májusi mintavételi eredményéből illesztettünk össze. Az 50%-nál nagyobb bootstrap-támogatottságú értékek a fa ágain jelölve vannak

FIGURE 3. Gene-tree based on the DNA sequence of the polar tube protein 1 (PTP1)

NCBI accession IDs are shown, and the *Nosema bombycis* (KY636450.1) was used as an outgroup. The PP067148.1 item represents the PTP1 sequence assembled from the sample collected in May at the apiary nr. 19. Bootstrap support levels above 50% are indicated by the numbers at the branches



MEGVITATÁS

Mintáink mindegyike tartalmazott a taxonómiai azonosítás során *V. ceranae*-hoz sorolt szekvenciákat. Miután eredményeink az ország területére térben kiegyensúlyozott mintából származtak, így megállapítható, hogy a faj rendkívül széleskörben elterjedt hazánkban, és a fertőzöttség valószínűleg a hazai méhcsaládok jelentős részét érinti. A parazita magyarországi elterjedésére vonatkozó megfigyelésünk összhangban van a korábban leírtakkal, ahol Csáki és mtsai (2015) [32] 90% feletti prevalenciával találták meg a fajt mintáikban. Annak ellenére azonban, hogy egyes mediterrán országokban [52], valamint az Egyesült Államok Texas államában [53] az általunk megfigyelthez hasonlóan nagy prevalenciáról számoltak be, a legtöbb vizsgálat során ezeknél kisebb értékeket tapasztaltak [54–58]. Fontos azonban megjegyezni, hogy az alkalmazott módszer jelentősen befolyásolhatja a becslések pontosságát, így pl. Csáki és mtsai (2015) [32] a PCR-alapú vizsgálatok esetén nagyobb érzékenységről számoltak be a fénymikroszkópos technikákkal szemben.

Valamennyi vizsgált mintában találtak *V. ceranae*-hoz sorolt szekvenciákat

A közelmúltig igen jelentős *V. apis* eredetű read-eket nem sikerült azonosítaniuk

A vizsgálatok során egy Európában eddig nem leírt faj, a *Nosema bombicis* genomját is azonosították

A *V. ceranae* okozta fertőzöttség mértéke általában a tavaszi vagy nyári hónapokban jelentősebb

Az általunk végzett felmérésben a méhcsaládok beválogatásánál feltételként szabtuk meg, hogy a méhészek nem tapasztaltak állományukban betegségekre utaló tüneteket. Ennek megfelelően eredményünk is alátámasztja, hogy a *V. ceranae* jelen lehet egészségesnek tűnő méhcsaládokban is. Ez nem meglepő, hiszen a kórokozóval kapcsolatban megállapították, hogy gyakran okoz krónikus, akár hosszabb ideig tünetmentes fertőződést [7, 14, 23, 25], ahol fontos szerepe lehet a különféle stresszoroknak a betegség kialakulásában [58]. Ez a jelenség különösen veszélyessé teszi a *V. ceranae*-t, hiszen bizonyos esetekben csupán olyan késői tünetekben nyilvánul meg a fertőződés, mint a kaptárelhagyás jelensége [27].

Az Európában rendkívül domináns *V. ceranae* mellett azonban a közelmúltig igen jelentős *V. apis* eredetű read-eket nem sikerült azonosítanunk mintáinkban. Ennek oka lehet a parazita teljes hiánya az általunk vizsgált méhcsaládokban, azonban az is elképzelhető, hogy olyan kis arányban volt jelen ez a faj a mikrobiótában, hogy a kimutatása az általunk alkalmazott szekvenálási mélységgel nem volt lehetséges. Csáki és mtsai (2015) [32] olyan kevés *V. apis*-szal fertőzött családott találtak a magyarországi felmérésükben, hogy esetünkben akár a faj teljes hiánya sem tűnik valószínűtlennek.

Mindamellet, hogy a *V. apis*-t nem sikerült azonosítani, találtunk olyan leolvadási szekvenciákat, amelyek egy másik microsporidium, a szintén *Nosematidae* családba tartozó *Nosema bombicis* [11] genomjára illeszkedtek. Ez a faj a selyemhernyók (*Bombyx mori*) *nosematosis*-át okozza [59], és legjobb tudomásunk szerint egyelőre még nem írták le európai mézelő méhek esetében. Annak az okát, hogy szekvenciáink mégis erre a genomra illeszkedtek, magyarázhatjuk az általunk alkalmazott módszer specifitásával, amelyet jelentősen befolyásolhat a genomok hasonlósága, valamint az adatbázisok minősége. Mindamellet azonban, hogy a megfigyelésünk legnagyobb valószínűség szerint téves pozitív találat, mindenképpen érdemesnek tartottuk megemlíteni, hiszen az *N. bombicis* egyben a *Nosema* nemzetség típusfaja is.

Felmérésünkben nem találtunk különbséget a fertőzöttséget mutató méhészek számában a márciusi és májusi mintavételi időpontok között, amely elsőre nem meglepő, hiszen a *V. ceranae* esetében többször megfigyelték már, hogy szezonális ingadozás nélkül, egész évben jelen van [27, 32]. Mindezek ellenére azonban a parazita részaránya a metagenom-mintáinkban szignifikánsan nagyobb (több, mint háromszoros) értéket mutatott a májusi mintáinkban. Ez a megfigyelésünk azonban alátámaszthatja azokat az eredményeket, miszerint a *V. ceranae* okozta fertőzöttség mértéke szezonálisan változó lehet, ahol általában a tavaszi vagy nyári hónapokban tapasztalható jelentősebb fertőzöttség [27–31].

A kórokozó gyakoriságának változásában, valamint ezzel együtt akár a szezonális kialakulásában több faktor is szerepet játszhat [27], ezek közül pedig fontos lehet a különféle környezeti és éghajlati tényezők hatása. Ismert, pl., hogy a *V. ceranae* elsősorban a melegebb hőmérsékleten tud optimálisan szaporodni [58], valamint a *V. apis*-hoz képest nagyobb érzékenységet mutat a hidegre [13, 60]. Ezzel összhangban saját megfigyeléseink is alátámaszthatják a hőmérséklet jelentőségét, hiszen a mintákban nagyobb arányban figyeltük meg a *V. ceranae*-t a melegebb régiókból származó családok esetében. Fontos megjegyezni azonban, hogy miután a hőmérsékleti adatok a 10 éves GDD-átlag alapján lettek meghatározva, így a valós hőmérséklet-különbségek nem álltak rendelkezésünkre, amely akár magyarázhatja, hogy nem sikerült szignifikáns eltéréseket azonosítanunk.

Törökországban megfigyelték, hogy a csapadékosabb területeknek fontos szerepe van a *V. apis* járványtanában [61]. Mindezen túl ez a faj a szezonális ingadozása során is a hideg és magas páratartalmú időszakban szaporodik el jelentősen [27]. Ennek oka lehet az is, hogy a méhek ilyenkor több időt töltenek a kaptárokon belül [20]. *V. ceranae* esetében azonban eddig nem tudtak összefüggést kimutatni a kórokozó és a csapadék viszonylatában [20, 53], csupán magasabb páratartalmú

területeken figyelték meg a fokozottabb jelenlétét [52, 62]. Ezekkel az eredményekkel szemben a mi felmérésünk szignifikáns kapcsolatot tárt fel a parazita mennyisége és a csapadék között a májusi mintavétel során nyert mintáink esetében. Megfigyelésünk így jelezheti ennek a környezeti tényezőnek a fontosságát a *V. ceranae* terjedésében.

Célunk volt az általunk *V. ceranae*-ként azonosított leolvasási szekvenciák alapján a hazai törzseket összehasonlítani a nyilvános adatbázisokban elérhetővel. Ehhez a szekvenciákat mintánként hosszabb szakaszokká illesztettük össze, ún. de novo assembly-t végeztünk. Ennek során a 19-es mintában azonosítani tudtuk a PTP1 gén szekvenciáját. Ez a gén a parazita poláris tubulusát (amely a sejtekbe történő bejutásában játszik fontos szerepet) alkotó öt fehérje közül kódolja az egyiket [63]. Az általunk azonosított PTP1 génszekvencián (ezt a szekvenciát PP067148.1 azonosítóval jelöltük 3. ábránkon) kívül az NCBI GenBank adatbázisában még három, Magyarországról származó PTP1-szekvencia érhető el, amelyeket GOMEZ-MORACHO és mtsai (2015) adtak közre [64]. Elvégeztük a saját szekvenciánk és a nyilvános adatbázisokban elérhető egyéb PTP1 szekvenciák összehasonlítását. Ennek során a páronkénti szekvencia-összehasonlításban egy thaiföldi szekvenciával (JF772107.1) mutatta a legnagyobb hasonlóságot. Mindezek ellenére azonban a mintánk a szekvenciaillesztések alapján készített törzsfán két klaszter közé esett. Ezek egyike több thaiföldi mintát tartalmaz, míg a másik egy spanyolországi és hawaii mintát.

A *V. ceranae* jelentőségét elsősorban a mézelő méhek megbetegítésén keresztül okozott gazdasági kár jelenti, azonban fontos megemlíteni a parazita terjedésének ökológiai kockázatát is. Ahogyan a *V. ceranae* az eredetinek gondolt gazdafajról, vagyis az *A. cerana*-ról átkerült az európai mézelő méhre [12], úgy akár más, ökológiai jelentőségű rovarfajok fertőzésére is képessé válhat. Ez a kockázat különösen jelentős, hiszen megfigyelték a jelenlétét *A. cerana*-n és *A. mellifera*-n túl más *Apis* nemzetségbe [65], valamint különféle *Bombus* nemzetségbe tartozó fajok esetében [66, 67], ill. kimutatták *Meliponini* fajokból és *Polybia scutellaris*-ból [68]. Mindezekon túl az *Osmia bicornis* kísérletes fertőzés során érzékenységet mutatott a parazitával szemben [69]. A vadméhek, mint szintén különös jelentőségű beporzók, ökológiailag és gazdaságilag is igen jelentős fajok [70], amelyek azonban gyakran mutatnak fokozottabb érzékenységet bizonyos károsító hatásokkal szemben, mint ahogyan azt pl. peszticidek esetében már leírták [71]. A *V. ceranae* jelentősége így az agráriumban tartott szociális méhfajok károsításán keresztül kifejtett gazdasági károkozó hatásán túl sokkal jelentősebb lehet [21].

Eredményeink tehát megerősítik a *Vairimorpha ceranae* hazai előfordulására vonatkozó korábbi adatokat, ezzel kiemelve a faj jelentőségét a magyarországi méhészetekben. Megfigyeléseink, az eddigi hazai vizsgálatokkal ellentétben a faj szezonális ingadozásának jelentőségére, valamint az éghajlati tényezők szerepére hívják fel a figyelmet. Ennek megfelelően, azért, hogy a *V. ceranae* epidemiológiáját jobban megértsük a hazai méhészetekben, szükség van a kórokozó és környezete kapcsolatrendszerének (stressz-tényezők) mélyebb feltárására. Ennek ismerete alapvető fontosságú lehet az ellene folytatott védekezésben, mivel specifikus gyógyszer jelenleg nem áll rendelkezésre.

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A *V. ceranae* a
vadméhekre is
veszélyt jelenthet

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Apis mellifera filamentous virus from a honey bee gut microbiome survey in Hungary

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In Hungary, as part of a nationwide, climatically balanced survey for a next-generation sequencing-based study of the honey bee (*Apis mellifera*) gut microbiome, repeated sampling was carried out during the honey production season (March and May 2019). Among other findings, the presence of *Apis mellifera* filamentous virus (AmFV) was detected in all samples, some at very high levels. AmFV-derived reads were more abundant in the March samples than in the May samples. In March, a higher abundance of AmFV-originated reads was identified in samples collected from warmer areas compared to those collected from cooler areas. A lower proportion of AmFV-derived reads were identified in samples collected in March from the wetter areas than those collected from the drier areas. AmFV-read abundance in samples collected in May showed no significant differences between groups based on either environmental temperature or precipitation. The AmFV abundance correlated negatively with *Bartonella apihabitans*, *Bartonella choladocola*, and positively with *Frischella perrara*, *Gilliamella apicola*, *Gilliamella* sp. ESL0443, *Lactobacillus apis*, *Lactobacillus kullabergensis*, *Lactobacillus* sp. IBH004. De novo metagenome assembly of four samples resulted in almost the complete AmFV genome. According to phylogenetic analysis based on DNA polymerase, the Hungarian strains are closest to the strain CH-05 isolated in Switzerland.

Honey bees (*Apis mellifera*) are important pollinators with high economic value and ecosystem importance,^{1–3} and are exposed to confined environments, and several factors threaten their health, including various pathogens, parasites, and chemicals used as pesticides in agriculture^{4–6}. The global decline of this important pollinator poses a threat to food security and biodiversity conservation⁷. The composition of the honey bee's normal or altered microbiota, for which the available knowledge is limited, may also affect their body function. Although there are studies on honey bee gut microbiota^{8–10}, there is little evidence on the environmental factors that influence it. Some results show that seasonal and environmental factors can influence the composition of the gut bacteriome in honeybees.^{11–15} The honey bee gut bacteriome has long been known to be composed of a few core bacterial species^{11,16,17}. However, beyond the bacteriome, the viral composition of the microbiome is increasingly gaining more attention in bees¹⁸, as in humans^{19,20}. Most of these studies understandably focus on bacteriophages as they play an essential role in shaping the composition of the bacteriome^{18–21}. However, the viruses of the honey bee itself might be just as important. Especially considering those found in the gut and feces, which can contribute to their spread^{22–24}. Many of these viruses are important pathogens^{22,23}, while others, such as the *Apis mellifera* filamentous virus (AmFV), are little to not pathogenic to bees^{25,26}. AmFV is the most significant DNA virus of the honey bee^{25,26} and for a long time was the only known one²⁷. This property of the virus is important because it allows us to investigate the relationship between the bacteriome and AmFV in metagenomic studies targeting bacteria. Despite the fact that the role of AmFV in various diseases is still uncertain, a better understanding of its ecology can bring us closer to understanding its role.

In 2019, a nationwide, climatically balanced survey was conducted in Hungary to investigate changes in the gut microbiome of honey bees based on next-generation sequencing (NGS). The bacteriome results of the study were published by Papp et al. (2022)¹⁵. In the course of the analyses, we found a large number of short reads originating from AmFV. In this paper, we present the results of a detailed analysis of the AmFV sequences found in the survey.

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Methods

Sample collection and preparation

Details of the design and conduct of the sampling can be found in the materials and methods section of Papp et al. (2022)¹⁵, here, we summarise only the methodological details necessary for interpreting the results. In 2019, a country-wide sample collection was performed twice during the honey-producing season, at its onset (March) and the peak (May). A total of 20 sampled apiaries (Fig 1) were selected to obtain a representative sample according to their climatic environment. The climatic environment was characterized by yearly growing degree days (GDD) and the yearly total precipitation. We defined the two categories for our environmental variables as cooler-warmer and less-more for GDD and precipitation, respectively. From individuals collected from three families per apiary, the gastrointestinal tracts of 10 healthy workers per family were removed for sequencing. Paired-end reads were generated from pooled samples per apiary using an Illumina NextSeq sequencer.

Bioinformatic and statistical analysis

Quality-based filtering and trimming were performed by Adapterremoval²⁸ using 20 as the quality threshold and only retaining reads longer than 50 bp. The remaining reads were taxonomically classified using Kraken2 (k=35)²⁹ with the NCBI non-redundant nucleotide database³⁰. The taxon classification data was managed using functions of package phyloseq³¹ and microbiome³². The abundance differences were analyzed by the DESeq2 package³³. Analyzing the seasonal effect, a mixed-effect model was applied to handle the repeated measures by apiary as a random factor. The SparCC correlation coefficient quantified the relationship between the relative abundances of core microbiome species and *AmFV*.^{34,35} The core microbiome was defined with a relative abundance on species-level above 0.5% in at least one of the samples. The statistical tests were two-sided, and p-values less than 0.05 were considered significant. The cleaned reads were aligned to the *AmFV* genome (KR819915.2) by Bowtie2³⁶ with the very-sensitive-local setting. De novo assembly was carried out using MEGAHIT (v1.2.9)³⁷, polishing of the contigs was performed with POLCA (v4.1.0)³⁸, and scaffolds were created by RagTag (v2.1.0)³⁹ using the *AmFV* genome (KR819915.2)²⁵. The average nucleotide identity (ANI) of scaffolds compared to the genome KR819915.2 was estimated by pyani (v0.2.12).⁴⁰ For the genome annotation Prokka (v1.14.6)⁴¹ was used guided by the genome KR819915.2. Predicted protein homology analysis was performed using the NCBI BLASTP (v2.14.0)⁴² algorithm with a minimum e-value of 1.0e-5 on two reference genomes (KR819915.2, OK392616.1). Phylogenetic analysis was performed based on the amino acid sequences of the DNA polymerase gene. The gene-tree was constructed⁴³ based on multiple sequence alignment by MAFFT (v7.490)⁴⁴. The best substitution model was selected by functions of phangorn package⁴⁵ based on the Bayesian information criterion. The generated neighbor-joining tree was optimized by the maximum likelihood method. Bootstrap values were produced by 100 iterations. All data processing and plotting were done in the R-environment⁴⁶.

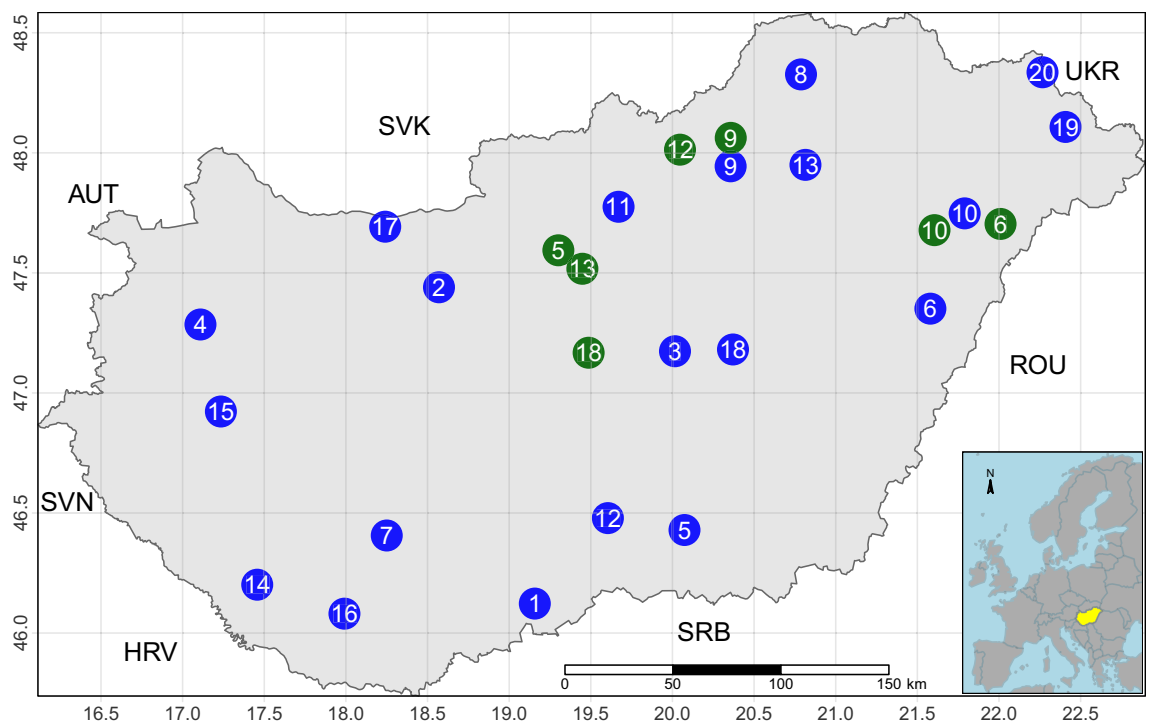


Figure 1. Sampling points in Hungary. The green dots indicate migrating apiaries in May. The blue dots indicate migratory apiaries at the time of sampling in March, as well as non-migratory apiaries. The inset map shows the study region, Hungary in Europe, colored yellow. Neighboring countries are presented by ISO 3 character codes: Austria (AUT), Croatia (HRV), Romania (ROU), Serbia (SRB), Slovakia (SVK), Slovenia (SVN), Ukraine (UKR).

Results

The shotgun sequencing generated paired-end read counts of samples are ranging between 311,931 and 546,924, with a mean of 413,629. The OTU table, created by Kraken2 taxonomic classification, contained counts of samples ranging between 175,576 and 314,586 with a median of 262,292. The minimum, maximum, and median read counts of the samples assigned as viral-originated were 443, 72,010, and 1,074, respectively.

The viral hits were dominated by reads matching the genome of *AmFV*. All of the samples contained reads from this species, and their relative abundance per sample is summarised in Fig 2. *AmFV*-derived reads were more abundant in the March samples than in the May samples (fold change (FC): 5.53, 95%CI: 2.38-12.84, $p<0.001$). In March, a higher abundance of *AmFV*-originated reads was identified in samples collected from warmer areas compared to those collected from cooler areas (FC: 26.05, 95%CI: 7.31-92.81, $p<0.001$). A lower proportion of *AmFV*-derived reads were identified in samples collected in March from the wetter areas than in those collected from the drier areas (FC: 0.33, 95%CI: 0.13-0.8, $p=0.014$). The level of *AmFV*-reads found in samples collected in May showed no significant differences between groups based on either environmental temperature or precipitation (FC: 1.44, 95%CI: 0.61-3.39, $p=0.40$; FC: 1.85, 95%CI: 0.78-4.37, $p=0.16$).

From the core microbiome species *Bartonella apihabitans* ($r=-0.384$, $p<0.001$), *Bartonella choladocola* ($r=-0.341$, $p<0.001$), *Frischella perrara* ($r=0.549$, $p<0.001$), *Gilliamella apicola* ($r=0.513$, $p<0.001$), *Gilliamella* sp. ESL0443 ($r=0.502$, $p=0.004$), *Lactobacillus apis* ($r=0.532$, $p<0.001$), *Lactobacillus kullabergensis* ($r=0.515$, $p=0.002$), *Lactobacillus* sp. IBH004 ($r=0.399$, $p<0.001$), *Snodgrassella alvi* ($r=0.485$, $p=0.028$) showed a significant correlation with *AmFV*.

De novo assembly of four samples (apiary ID 10, 16, 18 in March and 13 in May) resulted in almost the complete *AmFV* genome. For these samples, Table 1 shows the coverage and depth of the reads over the reference genome (KR819915.2) and statistics on the scaffolds and the ORFs identified within them.

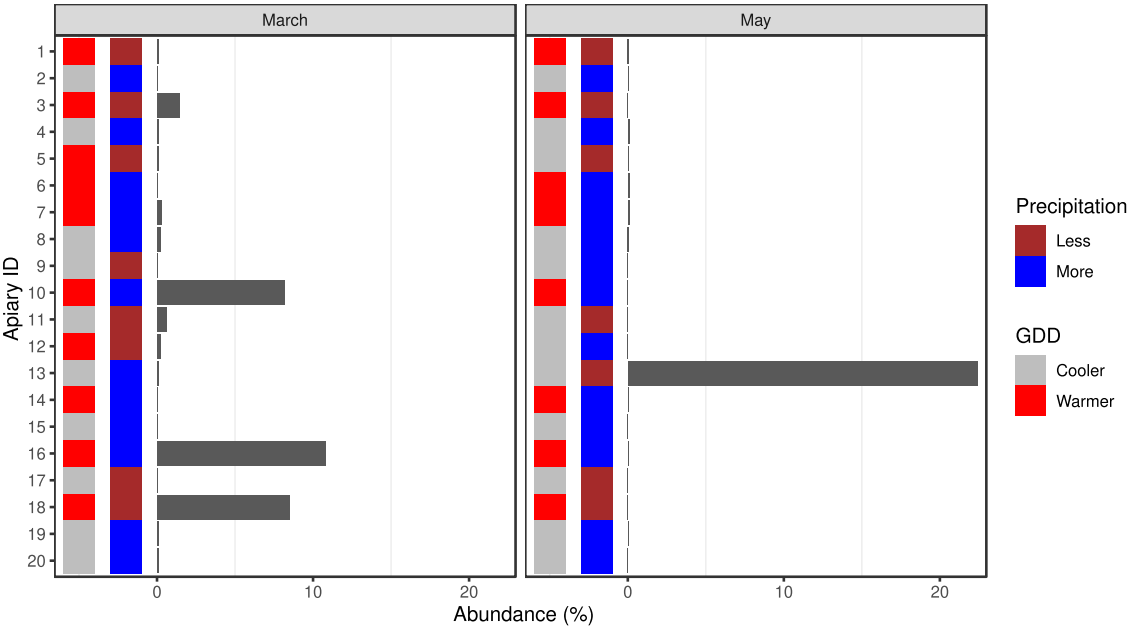


Figure 2. Relative abundance of *Apis mellifera* filamentous virus originated reads for the first (March) and second (May) sampling. The environmental condition, growing degree-day (GDD), and precipitation categories of sampling sites are also marked.

Apiary ID/month	Reads		MAG				ORF	
	Coverage (%)	Depth (x)	GenBank ID	Length (bp)	Gaps (%)	ANI (%)	Predicted	Matched
10/March	98.8	8	OR644611.1	504,639	2.09	97.78	153	70
16/March	99.1	13	OR644609.1	502,090	0.84	97.78	292	139
18/March	99.0	10	OR644610.1	501,579	0.74	97.78	231	119
13/May	99.6	31	OR270109.1	499,660	0.60	97.75	286	139

Table 1. Alignment and assembly statistics. Columns 2 and 3 describe the coverage of reads on the reference genome and average depth. The lengths of the scaffolds created by the de novo assembly and the proportion of gaps within them are shown in columns 5 and 6. Column 7 shows the average nucleotide identity (ANI) of scaffolds estimated for the reference genome (KR819915.2). The last two columns show how many ORFs were predicted in the scaffolds generated and how many of these ORFs were predicted to have a protein product that matched a CDS product of the reference genome.

Table 2 summarises the predicted proteins in the scaffolds generated from our samples that can be linked to products with predicted functions in the KR819915.2 and/or OK392616.1 genomes. Figure 3 shows the gene-tree based on the amino acid sequences of the DNA polymerase gene with the best substitution model JTT+I.

Discussion

Even though samples presented in this study were taken from healthy bee specimens, *AmFV* was detected in all samples. This is in line with our current knowledge of the virus, according to which it is only pathogenic in acute cases and/or if the bee colony is under stress^{26,47,48}. The virus is otherwise commonly prevalent, or even endemic in bee colonies, and besides its very likely oral-fecal transmission route, can possibly be spread transovarially from the queen to the workers²⁵.

KR819915.2		OK392616.1		ORF coverage%/identity% in scaffolds			
Accession ID	Product	Accession ID	Product	OR270109.1	OR644609.1	OR644610.1	OR644611.1
AKY03074.1	Zinc-dependent metalloprotease	UQL06506.1	Hypothetical protein	41.5/99.3	100.9/97.0	100.9/97.4	41.5/99.5
AKY03075.1	Hypothetical protein	UQL06507.1	PK	100.0/99.6	100.0/98.8	100.0/99.9	100.2/98.8
AKY03077.1	Hypothetical protein	UQL06509.1	BRO-1	92.8/99.4	99.4/99.4	99.4/99.4	99.4/99.4
AKY03078.1	Hypothetical protein	UQL06510.1	PARP	100.2/96.8	100.2/96.6	100.0/99.8	99.3/96.2
AKY03080.1	Kinesin motor domain	UQL06512.1	Hypothetical protein	100.0/99.1	100.0/99.1	100.0/99.3	100.0/99.0
AKY03085.1	BRO domain	UQL06516.1	BRO-2	100.4/90.3	100.0/91.0	100.2/90.7	99.8/90.4
AKY03088.1	Hypothetical protein	UQL06519.1	Putative BTB/POZ domain-containing				
AKY03092.1	AAA+-type ATPase	UQL06523.1	AAA family ATPase	100.0/99.5	100.0/99.5	100.0/99.5	100.0/99.5
AKY03096.1	Thymidylate synthase	UQL06526.1	Hypothetical protein	100.0/99.3	100.7/98.4	100.7/98.5	100.0/98.9
AKY03097.1	Hypothetical protein	UQL06527.1	Thymidylate synthase				
AKY03103.1	Hypothetical protein	UQL06531.1	Serine protease inhibitor	95.0/94.0	96.2/95.6	38.7/99.5	95.8/95.0
AKY03111.1	Tyrosine recombinase	UQL06534.1	Integrase, partial	100.0/99.5	100.0/99.6	100.0/98.5	100.0/98.9
AKY03112.1	Hypothetical protein	UQL06535.1	Myristoylated membrane	99.1/98.3	99.1/98.2	99.3/98.7	99.4/98.2
AKY03126.1	Hypothetical protein	UQL06546.1	PIF-5	100.6/99.1	100.6/99.1	100.6/99.1	100.6/98.8
AKY03129.2	Hypothetical protein	UQL06549.1	PIF-1	105.2/99.6	100.4/99.5	100.4/99.1	30.3/99.6
AKY03137.1	Hypothetical protein	UQL06555.1	Putative RING finger protein	100.0/100.0	100.0/100.0	100.0/99.7	100.0/100.0
AKY03138.1	Hypothetical protein	UQL06556.1	BRO-3		100.0/100.0	100.0/99.6	100.0/99.6
AKY03143.1	DNA polymerase B	UQL06561.1	DNA polymerase family B	99.5/98.5	99.5/99.0	7.4/99.3	99.4/99.0
AKY03144.1	Hypothetical protein	UQL06562.1	BRO-4	100.0/99.8	100.0/99.8	100.0/99.8	100.0/99.8
AKY03146.1	Hypothetical protein	UQL06564.1	PIF-0	99.7/99.7	99.7/99.4	99.7/99.6	99.7/99.7
AKY03149.1	Hypothetical protein	UQL06567.1	RING finger	98.6/97.7	100.0/100.0	99.3/98.9	100.0/99.3
AKY03151.1	Hypothetical protein	UQL06569.1	HZV 115-like protein	100.0/99.8	100.0/99.8	100.0/99.8	100.0/99.8
AKY03157.1	Pif domain	UQL06575.1	PIF-3	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0
AKY03164.1	Similar to DNA ligase I	UQL06580.1	DNA ligase 3	99.2/98.8	99.6/99.1	99.6/99.2	99.2/98.6
AKY03169.1	Hypothetical protein	UQL06585.1	PIF-2	100.0/99.8	100.0/100.0	100.0/100.0	100.0/100.0
AKY03170.1	Similar to bacterial gamma-glutamyl-transpeptidase	UQL06586.1	SEA domain-containing protein Gamma-glutamyltranspeptidase	99.1/97.8	99.1/98.5	99.1/98.6	98.2/97.6
AKY03175.1	BRO domain	UQL06591.1	BRO-5	96.5/93.7	96.5/94.1	53.5/52.3	
AKY03177.1	Hypothetical protein	UQL06593.1	BRO-6	102.1/61.1	102.1/60.3	56.5/66.3	
AKY03179.2	Hypothetical protein	UQL06595.1	BRO-7	185.1/98.1	184.0/96.7	138.7/96.7	
AKY03180.1	Hypothetical protein	UQL06596.1	BRO-8	100.0/98.9	100.0/97.0	100.0/98.6	
AKY03183.1	Ribonucleotide reductase, large subunit	UQL06599.1	Ribonucleotide reductase 1	100.0/99.9	56.6/100.0	100.0/99.9	
AKY03191.1	Similar to RecB exonuclease	UQL06606.1	Hypothetical protein	100.0/99.8	100.0/99.4	100.0/99.8	
AKY03202.1	BRO domain	UQL06616.1	BRO-9	100.0/100.0	100.0/99.4	100.0/100.0	
AKY03226.1	Hypothetical protein	UQL06638.1	PIF-4	40.9/100.0	100.0/100.0	100.0/100.0	
AKY03237.1	Hypothetical protein	UQL06646.1	MdSGHV070	99.7/95.3	99.8/94.8	98.7/94.6	
AKY03262.2	Hypothetical protein	UQL06666.1	Chitin-binding type-4 domain-con- taining protein	109.5/97.5	110.0/97.6		
AKY03304.1	Similar to bacterial phosphatidylinosi- tol-specific phospholipase C, catalytic domain	UQL06696.1	Phospholipase C	100.0/100.0	100.0/100.0		
WKY35419.1	Ribonucleotide reductase, small subunit	UQL06680.1	Ribonucleotide reductase 2	99.7/98.7	89.6/97.7		

Table 2. ORF homologs correspond to genes with predicted functions in the KR819915.2 and/or OK392616.1 genomes. The last four columns show the coverage and sequence identity of the product of the ORFs predicted in our scaffolds that match the product of the reference genome (KR819915.2) with the highest similarity.

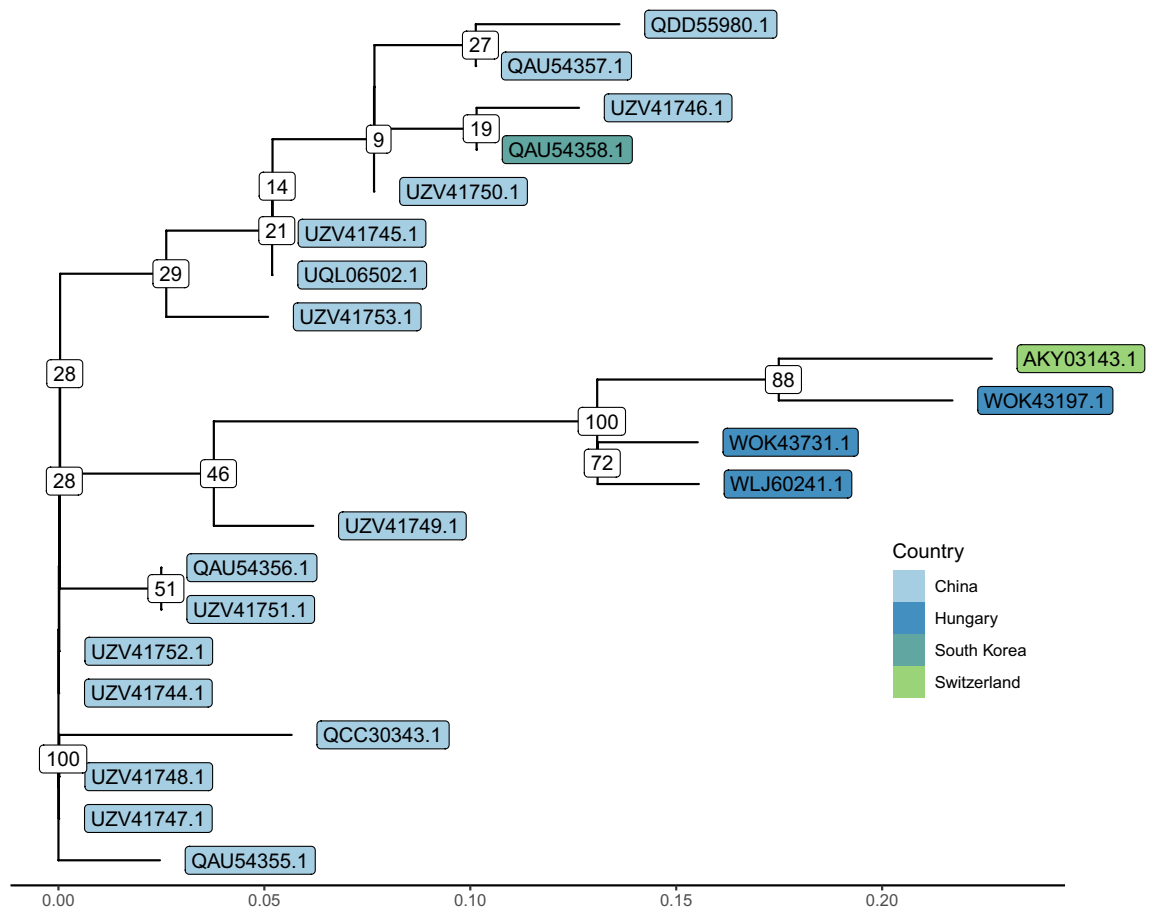


Figure 3. Gene-tree based on DNA-polymerase amino acid sequences. The genes WOK43731.1, WOK43197.1 and WLJ60241.1 were assembled from the samples of apiary 10 and 16 in March and apiary 13 in May, respectively. Most of the sequences derived from *Apis mellifera*, with some from other hosts: *Apis andreniformis* (QCC30343.1), *Apis cerana* (UZV41744.1), *Apis cerana cerana* (QAU54355.1), bumblebee (QAU54357.1), *Galleria mellonella* (QDD55980.1).

In contrast to other studies, we have observed a decline in *AmFV* abundances as the honey-producing season advanced. Bailey et al. observed an increasing prevalence of infected colonies from the beginning of autumn until the end of spring, which was followed by a steep decline⁴⁹. Similarly, Hartmann and colleagues observed a significant difference in *AmFV* loads between autumn and springtime²⁶. It is possible that regional climatic differences might influence the dynamics of viral loads. Further, large-scale surveys are necessary, however, to confirm such patterns.

Furthermore, the relationship of *AmFV* with the other components of the microbiome can provide us useful information. We found a significant positive correlation with *AmFV* for several bacteria. In some of these, the abundance of the bacterial species also decreased as the season progressed, as we have observed previously for *L. apis* and *L. kullabergensis*¹⁵. For other species, such as *F. perrara*, *G. apicola* and *S. alvi*, we have not observed a seasonal pattern in our previous analysis¹⁵. Among these, *F. perrara* is especially of interest, which is known to stimulate the immune system of the honey bees strongly⁵⁰. This species, as a potential opportunistic pathogen⁵¹, may act as a stressor on the bees, thus explaining the observed positive correlation with *AmFV*. Significant negative correlation was only found for two newly described *Bartonella* species, *B. apihabitans* and *B. cholidocola*⁵². Regarding the only previously known *Bartonella* species in bees, *B. apis*, our previous analysis of the same samples found an increase in abundance of this bacterial species with the progression of the season¹⁵. This might suggest that even these two newly described species may have shown similar changes, which may explain the negative correlation.

The reason for the seasonal peaks of *AmFV* or the association between the virus and other members of the honey bee microbiome is yet unknown. Furthermore, the effect of annually higher *AmFV* abundances on the immune system of bees needs to be determined. Moreover, other studies found correlations in the number of *AmFV* and other RNA viruses, such as Deformed Wing Virus (DWV) and Black Queen Cell Virus (BQCV)^{26,53}, indicating the benefit that could be achieved if the microbiome could be analyzed as a whole.

Our results confirm the high prevalence of the virus, as previous studies suggested^{26,49}. There were, however, 3 March samples (sample numbers 10, 16, and 18) in which we found an exceptionally high abundance of *AmFV*. This may be somewhat related to our observations on seasonality, as the virus was not present in such high

numbers in the corresponding May samples. Accordingly, the abundance of the virus decreased as the season progressed. In contrast, however, sample 13 was dominated by *AmFV* in May and, therefore, may be of further interest. The spike in virus abundance may indicate the presence of some stressor on the colony, which has not yet caused observable symptoms in the animals. Bee viruses have been described, for example, to increase in abundance in response to certain pesticides in a concentration-dependent manner^{54,55}, which is thought to be due to the immunomodulatory effect of pesticides⁵⁴. If indeed the emergence of stressors could be associated with such a fluctuation in viral abundance, it could also suggest the use of the virus as a health indicator and contribute to the diagnosis of various complex disease processes.

Besides their direct effect on honey bees, the spread of various microorganisms between *A. mellifera* or other bee species of economic importance and wild bees can be of particular economic and ecological significance⁵⁶. The spillover of pathogens between different arthropod species has been observed for several microorganisms related to *A. mellifera*⁵⁷, among which *AmFV* is no exception. It has been detected in several bee species and also in wasps, flies and beetles^{56–59}. However, it is important to note that according to our current knowledge, *AmFV* does not show significant pathogenic effect on *A. mellifera* nor at the colony nor the individual level²⁶, which does not necessarily mean that solitary wild bee species do not show susceptibility to the virus as well. Indeed, it is possible that these species may show increased sensitivity to certain adverse effects due to their solitary lifestyle, as it has already been shown for the effect of pesticides⁶⁰. Furthermore, the positive association of the virus with BQCV²⁶, which has also been detected in wild bee species⁵⁷, may contribute to the potential harm caused by *AmFV*. Therefore it might be assumed that the ecological and economic importance of *AmFV* can be significantly determined by the impact the virus may have on wild bee species and their importance in pollination services⁶¹.

Based on the phylogenetic analysis, the sample from apiary 16, collected in March from the southwest of Hungary, had the highest similarity to the Swiss reference genome. Both other samples (apiary ID 10 March and 13 May) that contained the DNA-polymerase gene in full length were derived from Eastern Hungary. Even though sample 13 from May is relatively closer to the west of the country due to colony migration, it is originally from Eastern Hungary, and the permanent beekeeping premises are 76 air kilometers away from March sampling point 10. Accordingly, it can be supposed that the viral strain from sample 13 collected in May has already been present at the overwintering location and migrated to the May sampling point. It can be assumed that the mediating effect of the imports of honey and propolis from Hungary to Switzerland is responsible for the close genetic relationship of the Hungarian strains of *AmFV*.

More than one hundred ORFs were identified in each of four of our scaffolds that did not show any similarity to the reference genome with the homology assumptions. In a previous study, Yang and colleagues (2022)⁶² predicted the functionality of newly detected ORFs from *AmFV* isolates. As our sequences were of metagenomic origins, the predicted products may derive from organisms other than *AmFV* despite our sequences' high similarity to the reference sequence. Accordingly, we have presented only those products that appeared in any of the two whole genomes.

In the present study, we report 4 new assemblies of the *AmFV* from Hungary. Our results may provide deeper insights into the genome organization of this virus. Furthermore, our results suggest a seasonal trend of the virus abundance, i.e., there is a decrease in it in the gastrointestinal tract of bees as the production season progresses. The high abundance of the virus in some bee colonies and its association with certain members of the microbiome, in particular *F. perrara*, may suggest a link between the virus and various stressors. It may, therefore, be important either as an agent of complex disease processes or as an indicator of the health status of the hive.

Data availability

The short read data of sample data are publicly available and can be accessed through the PRJNA685398 from the NCBI Sequence Read Archive (SRA).

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Author contributions

N.S. takes responsibility for the integrity of the data and the accuracy of the data analysis. L.B., L.M., N.S., and R.F. conceived the concept of the study. G.M., L.B., L.M., N.S., and R.F. performed sample collection and procedures. A.G.T., M.P., and N.S. participated in the bioinformatic and statistical analysis. A.G.T., M.P., and N.S. participated in the drafting of the manuscript. A.G.T., G.M., L.B., L.M., M.P., N.S., and R.F. carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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The authors declare no competing interests.

Additional information

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Ixodes ricinus tick bacteriome alterations based on a climatically representative survey in Hungary

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ABSTRACT The microbial communities of disease vectors may represent a key feature in several biological functions and thus deserve special attention in light of climate change and the consequent need to develop novel control strategies. Nevertheless, vector-borne microbial networks are still poorly understood. Assessing vectors' microbial interactions and climatic dependencies may contribute to better estimating pathogen transmission characteristics and public health risks. In a climatically representative country-wide survey, *Ixodes ricinus* ticks were collected from 17 locations in Hungary. Using shotgun metagenomic sequencing, the bacteriome composition was analyzed by investigating the relationship between the abundance of nymphs and females in various climatic environments. Bacterial composition on the genus level revealed a significant difference between the samples from females and nymphs. Within the core bacteriome, females and nymphs showed significant variation in the following genera: *Arsenophonus*, *Bacillus*, *Candidatus Midichloria*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*, and *Wolbachia*. Among females, according to temperature strata, the following were found differentiating: *Curtobacterium*, *Pseudomonas*, and *Sphingomonas*. There was no genus with a significant difference in precipitation categories for females. *Curtobacterium* showed significant variation between temperature and *Bacillus* and *Curtobacterium* for various precipitation levels in the nymphs. The composition of vector-borne bacteriome members showed significant alterations at sampling points with different climatic conditions and development stages of the tick hosts. Our findings not only pave the way toward understanding tick-borne bacterial networks and interdependencies but also shed light on the high potential for the presence of a possible biological tick control species, the tick parasitoid, *Ixodiphagus hookeri* based on related bacteriome patterns.

IMPORTANCE Climate-sensitive disease vectors, such as ticks, respond to the environment with changes in their microbiome. These changes can affect the emergence or re-emergence of various vector-borne pathogens, such as the causative agent of Lyme borreliosis (LB) or tick-borne encephalitis. This aspect is particularly emphasized in light of climate change. The climatically representative assessment of microbiome differences in various developmental stages of the most common Central European tick species, *Ixodes ricinus*, deepens our understanding of the potential climatic factors behind microbial relative abundance and interaction changes. This knowledge can support the development of novel disease vector control strategies.

KEYWORDS *Ixodes ricinus*, bacteriome, climate, female, nymph

Almost all aspects of human life are directly or indirectly affected by the Earth's climate system dynamics. Many organisms, such as arthropods being poikilotherm organisms, are particularly sensitive to climate factors (1). Recent estimates suggest that around 80% of the world's human population is at risk of one or more vector-borne

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disease(s) (2). The geographical distribution of arthropod vectors is changing due to climate change (3, 4). The response of ticks and dipteran vectors to the changing climate appears to differ (5). While bivalves respond to short-term weather and climate changes with rapid responses, ticks are affected by spatiotemporal averages of climate variation rather than short-term or localized climate variability. This suggests that changes in the risk of disease spread by dipterans can be expected in the short-term, while the prevalence of tick-borne diseases (TBDs) may be expected over a more extended period of time (5). *Ixodes ricinus* is the primary vector of the most prevalent TBDs in Europe, tick-borne encephalitis, and Lyme borreliosis (LB). Although reports on the prevalence of these TBDs do not show consistent global trends (6, 7), the European *I. ricinus* populations do. As a consequence of climate change, *I. ricinus* has been found at extreme altitudes and latitudes other than its former range and its population has shifted further north within the European continent (4, 8, 9). Similarly, due to some recently studied adaptation processes (10), the geographic range of LB can expand to non-endemic areas, and also to higher altitudes and latitudes (6, 7). In consideration of climatic factors, a shift toward a more thermophilic tick fauna has also been described in Hungary (11).

The eukaryotic, and as such, tick-borne microbiota impacts numerous biological functions of the host causing a variety of detrimental, neutral, or beneficial effects. In addition, certain bacterial assemblages can affect tick-borne pathogens of public health importance. Thus, understanding the changes in tick microbiota associated with the climatic environment could aid in the deeper understanding of tick biology (12, 13). Because the tick bacteriome contains a number of climate-dependent, environment-dependent components, bacterial interaction patterns may also be determined by climatic factors (14). In our work, we investigated the diversity of the bacteriome of *I. ricinus* samples from nymphs and females from wetter or drier and cooler or warmer environments based on a climatically representative survey in Hungary.

MATERIALS AND METHODS

Sampling design and sample collection

As the study's main goal was to understand the natural bacteriome differences in *I. ricinus*, we designed the sampling to be representative of Hungary. In order to achieve this, we identified sampling points representative of climatic conditions (15). In Hungary, there are 175 local administrative units (LAU 1), for each of these units (geographical areas), we calculated the 10-year average of the yearly growing degree days (GDD) with base 10°C and the total yearly precipitation for each of the years. Meteorology data for the period of 2008 to 2017 was gathered from the ERA-Interim reanalysis data repository (16) with a spatial resolution of 0.125°. For each of the two environmental variables, binary categories were defined: GDD with classes "cooler" and "warmer" and precipitation with classes "less" and "more". Regarding GDD, the lower two quartiles were classified as "cooler" and the upper two quartiles as "warmer". For precipitation, the yearly means below the country-wide median were defined as "less" and scores above the median as "more". Each LAU 1 was categorized with its own climatic variables. By stratified spatial random sampling (17, 18), 20 LAUs were chosen as sampling areas. The strata's sample size was proportional to the stratifying GDD and precipitation categories' country-wide frequency to be representative. All data management and analysis were performed in the R environment (19). A forest edge to sample from was identified within each of the 20 selected LAUs. Between 23/3/2019 and 20/5/2019, questing ticks were collected by flagging and dragging. In the laboratory (BSL2), ticks from the frozen samples were classified taxonomically. Ten nymphs and ten females of *I. ricinus* were selected randomly per sampling site. Since we could not collect the minimum of ten females and ten nymphs at three sampling sites, only the samples collected at the remaining 17 sites were included in the sequencing and downstream analyses (Fig. 1). Before DNA extraction, the ticks were washed twice with 99.8% alcohol.

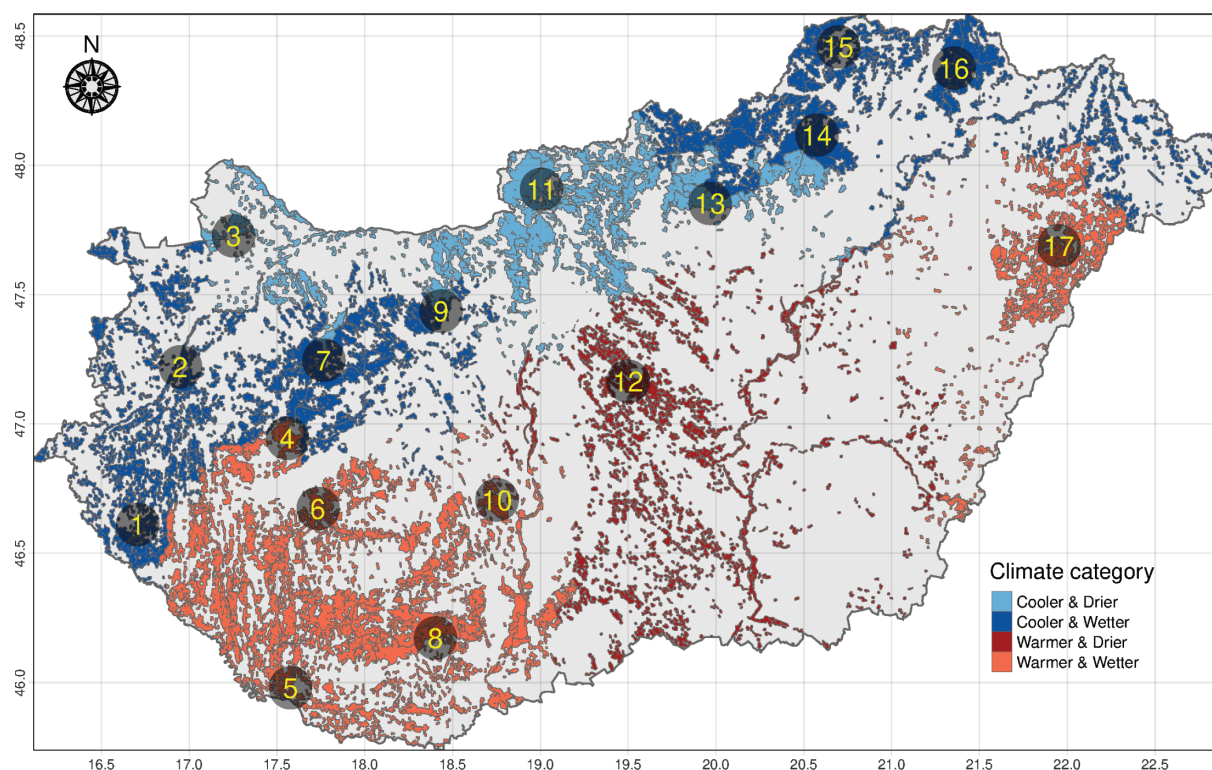


FIG 1 Climate category spatial pattern and sampling points. The color of the sylvan areas is defined by the climatic categories based on GDD and precipitation over the period 2008–2017. The points are relatively homogeneous within Hungary geographically.

DNA extraction and metagenomics library preparation

For sequencing, we formed pools of nymphs per sampling site and did the same for females. Ticks were subjected to DNA extraction using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and the supplementary protocol designated for the purification of total DNA from ticks for the detection of *Borrelia* DNA according to the manufacturer's instructions. The blackPREP Tick DNA/RNA Kit (Analytik Jena GmbH) was used for the DNA isolation. Isolated total metagenome DNA was used for library preparation. *In vitro*, fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

Bioinformatic analysis

After merging the paired-end reads by PEAR (20), quality-based filtering and trimming were performed by TrimGalore (v.0.6.6, <https://github.com/FelixKrueger/TrimGalore>), with a setting of 20 as a quality threshold, retaining reads longer than 50 bp only. The remaining reads after deduplication by VSEARCH (21) were taxonomically classified using Kraken2 (k = 35) (22) with a database created (26/03/2022) from the NCBI RefSeq complete archaeal, bacterial, and viral genomes. For taxon assignment, the confidence 0.5 parameter was used to obtain more precise hits. Core bacteria was defined as the relative abundance of agglomerated counts on genus levels above 1% in at least one of the samples. The taxon classification data were managed in R (19) using functions of package phyloseq (23), microbiome (24), and metacoder (25). The pre-processed reads were assembled to contigs by MEGAHIT (v1.2.9) (26) using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. The assembly-generated contigs that were classified to a pathogen bacteria genus

by Kraken2 we reclassified by BLAST (27) on the representative prokaryotic genomes (downloaded on 16/6/2022). For each contig, the longest and the smallest e-value hit were kept and reported.

Statistical analysis

The within-subject (α) diversity was assessed using the numbers of observed species (richness) and the Inverse Simpson's Index (evenness). These indices were calculated in 1,000 iterations of rarefied operational taxonomic unit (OTU) tables with a sequencing depth of 158. The average over the iterations was taken for each sample. The α -diversity expressed by Inverse Simpson's Index was compared between the conditions using linear models. Comparing the female and nymph samples collected, a mixed-effect model was applied to manage the repeated measurements by sampling site as a random factor.

The between-subject (β) diversity was assessed by UniFrac distance (28) based on the relative abundances of bacteria species. Using this measure, principal coordinate analysis (PCoA) ordination was applied to visualize the samples' variance. To examine statistically whether the bacterial species composition differed by strata Permutational Multivariate Analysis of Variance (PERMANOVA (29)) was performed using the package vegan (30) in R (19).

The abundance differences in core bacteriome between groups were analyzed by a negative binomial generalized model of DESeq2 package (31) in R (19). This approach was applied following the recommendation of Weiss et al. (32). None of the compared groups had more than 20 samples, and their average library size ratio was less than 10. According to the multiple comparisons, the FDR-adjusted P -value less than 0.05 was considered significant. The SparCC correlation coefficient quantified the relationship among the relative abundances of bacterial species (33, 34). The statistical tests were two-sided.

RESULTS

After the basic demography of the samples, we present the α -diversity of the full bacteriome. From the analysis of the core bacteriome, we report the species that are part of it, the β -diversity, and the differences of the genera level expressed relative abundances. The Supplementary Material summarizes species of core bacteriome, the detected pathogen bacteria, and the correlations of bacteria genera (Fig. S1).

Among the ticks collected at the sampling sites, the median proportion of nymphs was 76.52% (IQR: 19.33), females 15.32% (IQR: 8.58), and males 7.91% (IQR: 11.47).

The numbers of observed species and the Inverse Simpson's Index α -diversity metrics by strata are shown in Fig. 2. Alpha diversity showed no significant difference between groups in either metric. For the comparison of females and nymphs, the P -value was 0.138. Within females, the P -values obtained by comparing groups from colder and warmer environments and drier and wetter environments were $P = 0.562$ and $P = 0.577$, respectively. Within nymphs, the comparative metrics were $P = 0.174$ and $P = 0.309$, respectively.

Core bacteriome

The core bacteriome is composed of the genera shown in Fig. 3. For the list of species identified within genera see Supplementary Material.

The variability of the samples' core bacteriome genus profiles (β -diversity) is visualized by PCoA ordination (Fig. 4) based on weighted UniFrac distance. The PERMANOVA analysis of the bacterial composition on the genus level revealed a significant ($P = 0.005$) difference between the samples originating from females and nymphs. The core bacteriome of female samples showed no significant distance between either GDD ($P = 0.444$) or precipitation ($P = 0.244$) categories. Similarly, there was no significant difference between groups within nymphs (GDD $P = 0.108$, precipitation $P = 0.722$).

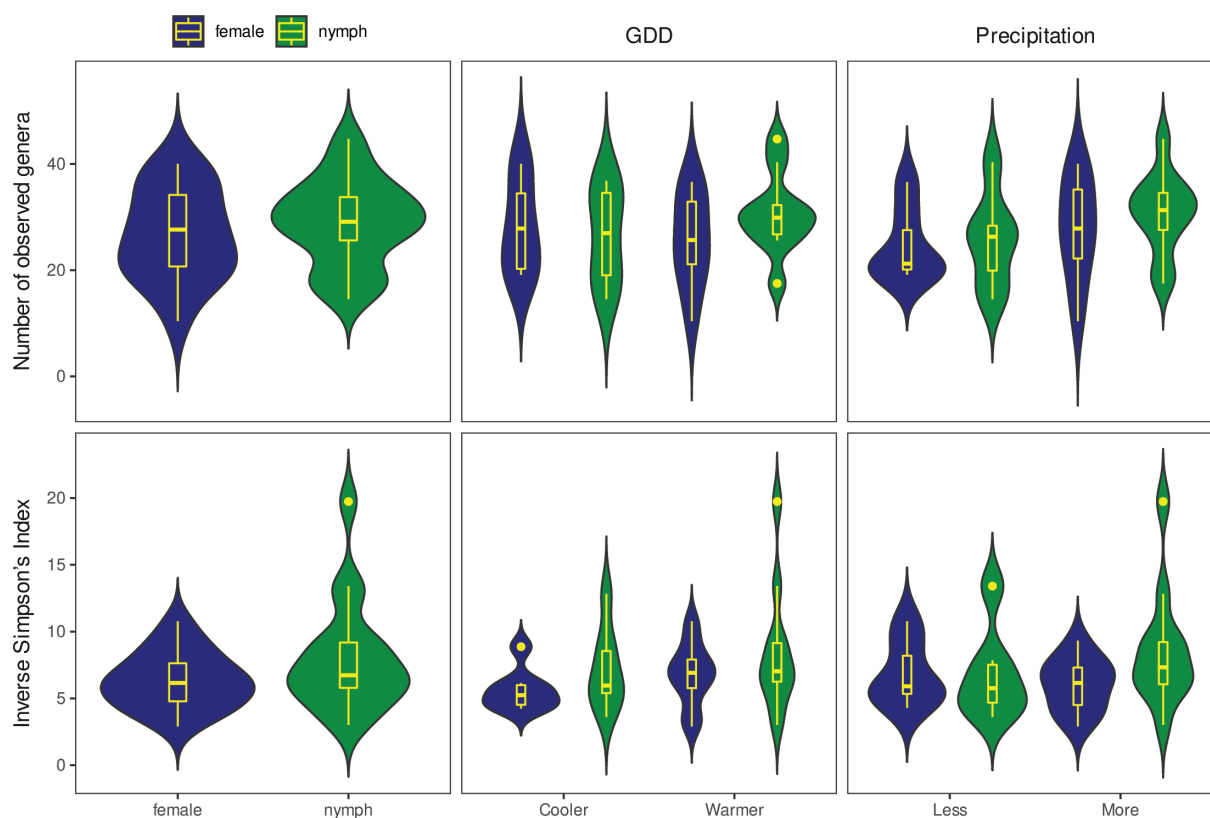


FIG 2 Richness and evenness of *Ixodes ricinus* bacteriome by sample groups. The number of observed species (richness) and the Inverse Simpson's Index (evenness) as α -diversity metrics are presented in the form of a violin and box plot combination. These indices were calculated from 1,000 iterations of rarefied OTU tables with a sequencing depth of 158. The average over the iterations was taken for each sampling site. The violin plot shows the probability density of metrics, while the box plot marks the outliers, median, and IQR.

Abundance differences

The abundance differences (log₂ median fold change, Log₂FC) of groups per taxon comparison are summarized in Fig. 5. Comparing females and nymphs, the following genera showed significant (adjusted $P = 0.05$) differences in abundance: *Arsenophonus*, *Bacillus*, *Candidatus Midichloria*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*, *Wolbachia*. In the female samples, according to GDD, the following were found differentiating: *Curtobacterium*, *Pseudomonas*, and *Sphingomonas*. There was no genus with a significant difference in precipitation categories in females. In the nymphs, *Curtobacterium* showed a significant variation between GDD groups and *Bacillus* and *Curtobacterium* between precipitation levels.

DISCUSSION

Our shotgun sequencing-based microbiome analysis assesses an in-depth characterization of *I. ricinus* nymphs and adult females collected from a climatically representative set of sampling points in Hungary. Our study revealed a comprehensive picture of the bacterial diversity and associations in various host categories in *I. ricinus* ticks over the various climatic regions of Hungary.

While *I. ricinus* ticks had previously been linked with relatively higher alpha diversity scores than other common tick species (35), no significant difference was observed among the nymph and adult female stages or warmer-colder and drier-wetter areas of origin. Carpi and colleagues found that the bacterial communities of geographically distant ticks of the same developmental stage differ more from those from the same regions (36). Moreover, Batool and colleagues found that in Ukraine, a neighboring

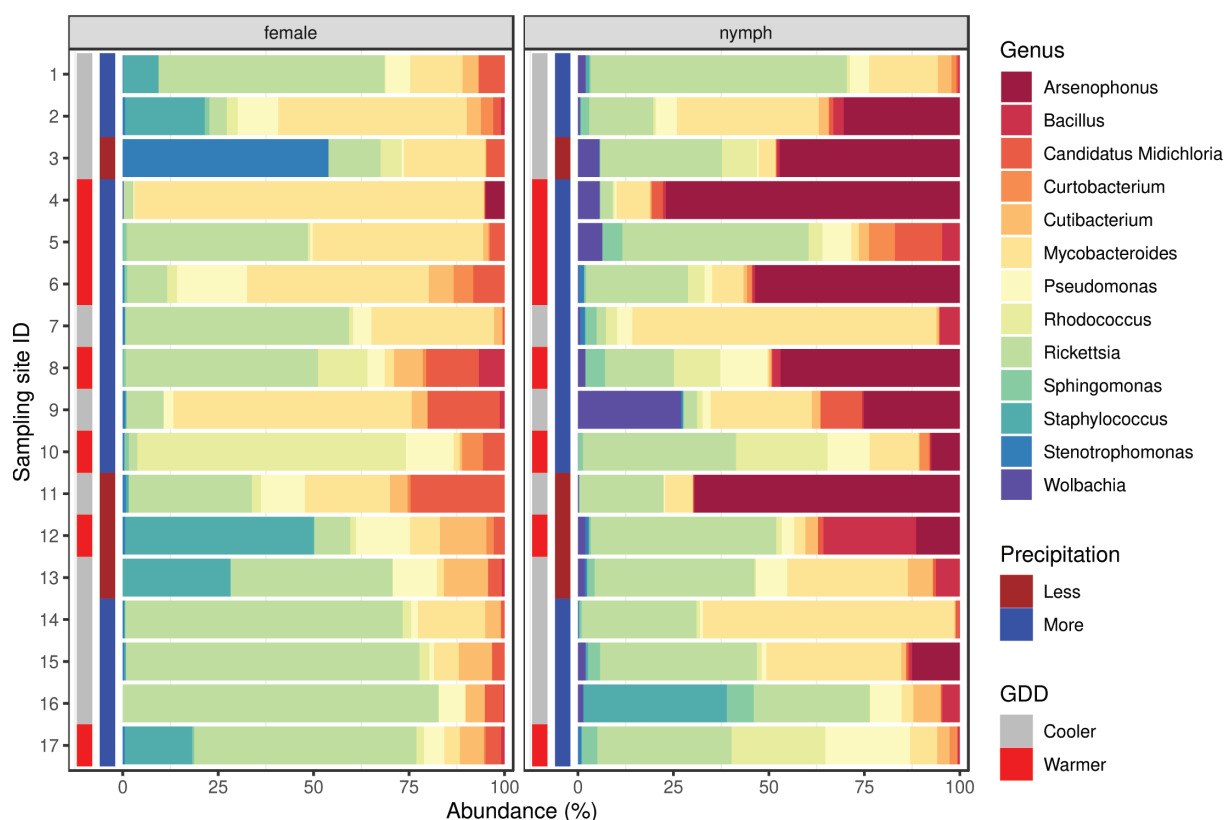


FIG 3 Core bacteriome composition of *Ixodes ricinus* samples. The relative abundance is plotted for the females and nymphs. Besides the bacterial genera of the core bacteriome, the environmental condition (GDD and precipitation) categories of sampling locations are also marked.

country to Hungary with an area around 6.5 times as big, the alpha diversity analyses demonstrated differences in tick microbiota patterns of various administrative regions (37). For the dissimilarity of the samples' core bacteriome genus profiles (beta diversity), inter-regional comparison of developmental stages (females or nymphs) produced significant differences, while developmental stage-wise testing of the climatic condition-associated localization did not. Similarly, Batool and colleagues described that tick sex comparisons resulted in significant differences on various beta-diversity tests regardless of the area of origin (37). While these results are interesting to compare, the different study models and testing methods should be considered.

During the tick-collection phase of our study, three categories of ticks were evaluated for metagenome sequencing: nymphs, adult females, and adult males. Nevertheless, the sequencing of adult males was rejected as the number of males was lower than nymphs and adult females at all sampling points. Sex ratio shifts by ticks are not uncommon (38, 39). The elucidation of the reason for the observed shift toward females in the adult life stage in our study would require further investigation. Nonetheless, the presence of certain maternally inherited genera, namely *Arsenophonus*, *Rickettsia*, *Spiroplasma*, and *Wolbachia* in the metagenomes is noteworthy. These genera are described to induct parthenogenesis, feminize or kill males, and, thus, manipulate the reproduction of their host species toward the production of daughters (40, 41). Sex ratio skewness may be adaptive from the perspective of upper-generation ticks since it diminishes the competition of related males in tick-dense localities by reducing their numbers while increasing the number of related females that can be fertilized by the smaller male population (42, 43).

Besides the members of the core bacteriome of nymphs and adult females, reads deriving from further bacterial genera with relatively lower abundance rates but high clinical relevance (pathogens), such as *Anaplasma*, *Bartonella*, *Borrelia*, *Borrelia*, and

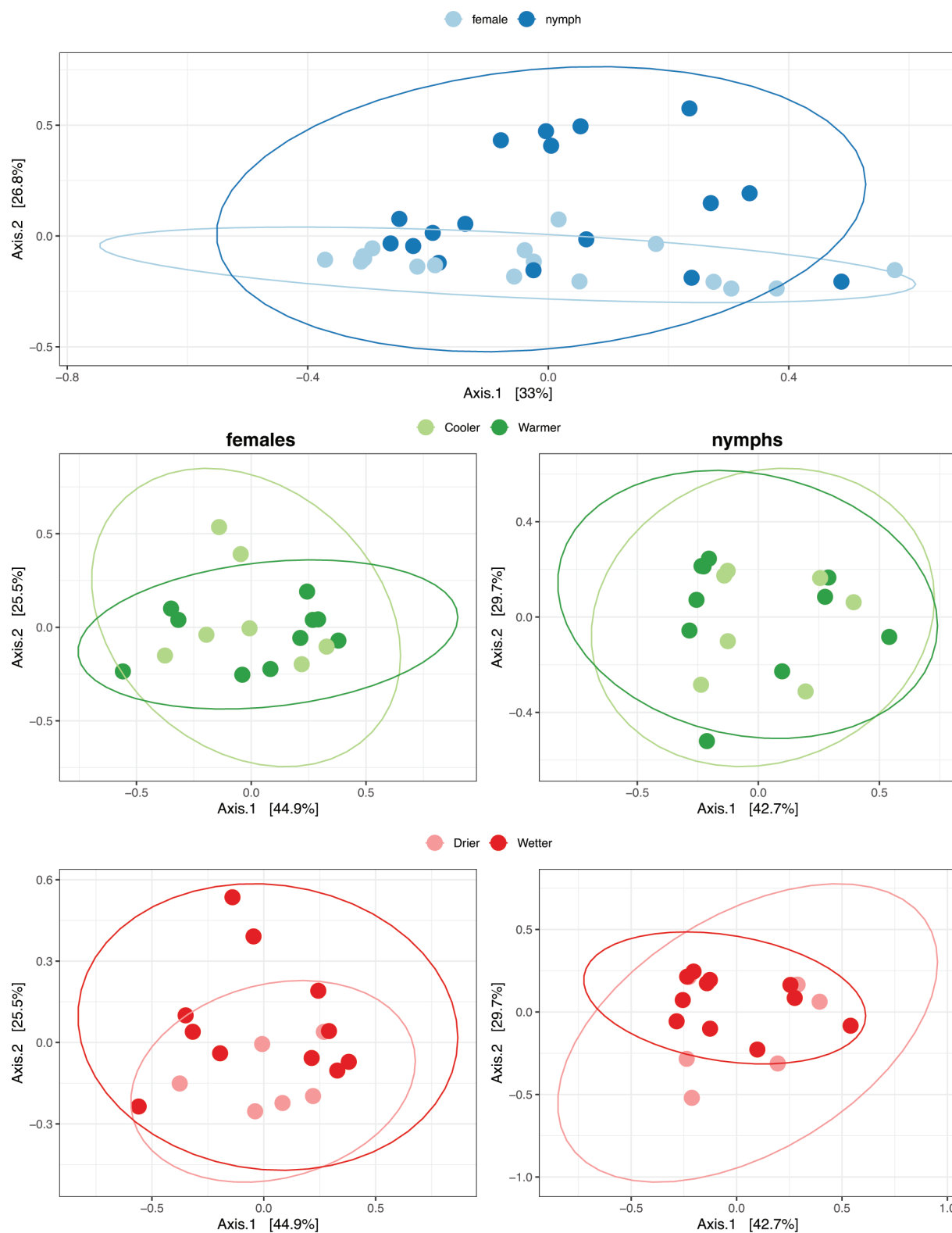


FIG 4 PCoA plots of β -diversity estimated based on the core bacteriome of *Ixodes ricinus* samples.

Ehrlichia have also been detected. While *Anaplasma phagocytophilum*, the cause of *Anaplasmosis* has previously been associated with the presence of a tick parasitoid, *Ixodiphagus hookeri*, based on its lifestyle and its mode of hunting (44), its positive

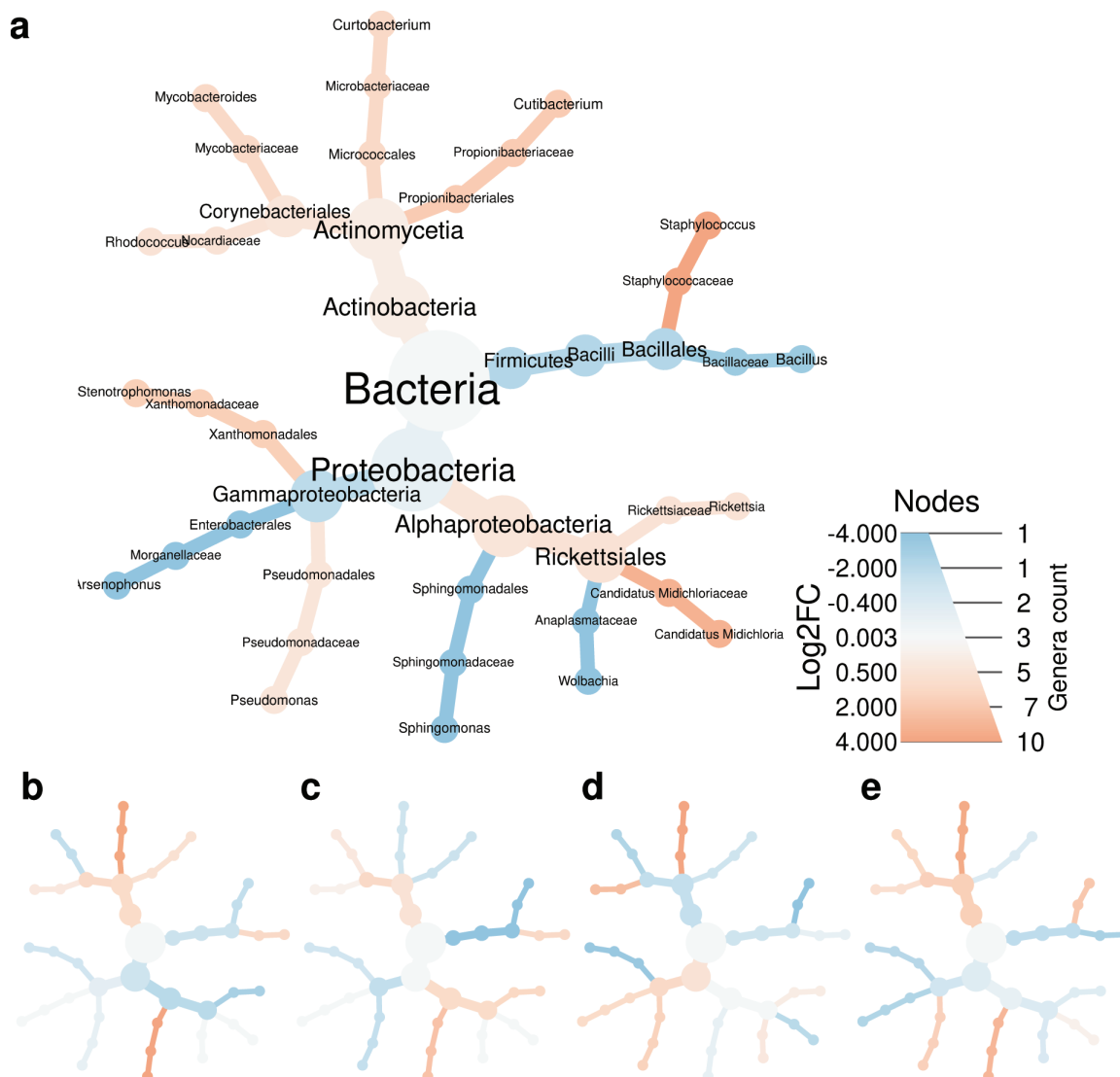


FIG 5 Core bacteriome abundance fold changes by taxonomic ranks. The colors represent the Log2FC of the median abundances of the compared groups. Subfigure (a) shows the ratio of the abundances in females comparing nymphs as a reference. Figure (b) compares female samples from warmer areas to cooler ones. Figure (c) compares samples from drier areas to those from wetter areas among females. Comparisons of GDD groups in nymphs are shown in figure (d), while comparisons of precipitation groups are shown in figure (e).

correlation with the genera of *Arsenophonus* and *Wolbachia*, endosymbionts of *I. hookeri*, was not affirmed (13). Within our study, the geolocations where *Anaplasma* sp. occurred did not match the detection points *I. hookeri* in Hungary described in a recent study (45). *I. ricinus* ticks are considered to be the vector of the *Borrelia burgdorferi sensu lato* complex, which is responsible for LB (46–48). Despite the high incidence rates of Lyme disease in Europe (49), *Borrelia burgdorferi* itself was not detected in our samples, while other species of *Borrelia* and *Borrelia* were identified. Since the causative agent of Lyme disease is normally mentioned as *B. burgdorferi sensu lato* (50, 51), referring to this species broadly, involving other members of the genera as well, the presence of *Borrelia garinii*, *Borrelia valaisiana*, *Borrelia afzelii*, *Borrelia coriaceae* and *Borrelia miyamotoi* can also be associated with this common disease. *Bartonella* spp. and *Ehrlichia* spp. are pathogens that are also often isolated in European settings (52–54). The presence of pathogens may influence the composition of tick microbiota (55).

Some genera constituting the core bacteriome, namely *Arsenophonus*, *Candidatus Midichloria*, *Rickettsia*, and *Wolbachia* are believed to be maternally inherited or strongly tick-associated due to direct or indirect reasons, while the members of *Bacillus*, *Curtobacterium*, *Cutibacterium*, *Mycobacteroides*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*, and *Stenotrophomonas* are related to soil, water, plants, skin or mucosa of vertebrates, thus may rather be acquired from the environment of the ticks (13, 13, 37, 56, 57). The representatives of the environmental genera may either participate in ticks' transient or long-term microbiota. Several environmental, tick gut, or surface bacteria cause opportunistic infections in humans, especially in patients with an immunocompromised history (58, 59). Even though certain bacteria, such as cutibacteria and staphylococci, may be considered bacterial contaminants from the sample processing steps (13), according to a recent study, the effects of a possible, minor level contamination are not noticeable in the overall relative bacterial abundance (37). Nevertheless, it is possible that some environmental genera were only present at the cuticle of the collected ticks regardless of the repeated laboratory washing steps with 99.8% alcohol.

Except for *Cutibacterium*, *Mycobacteroides*, *Rickettsia*, and *Stenotrophomonas*, genera constituting the core bacteriome showed statistically significant alterations in the examined tick groups differing in life stage and climatic condition-associated geographical origins. This finding is plausible considering the fact that both the life stage and the season-associated climatic conditions are very important in the composition of the tick bacteriome (60, 61).

The members of *Rickettsia* are maternally inherited or transstadially passed symbionts (62, 63) that may cause tick-borne infections (62) and have been described to be dominant in tick microbiomes in several studies (36, 64–68). However, high rickettsial abundance rates are not necessarily present in the ticks. Certain studies presented relatively low rickettsial genome fragment counts (37). The reason for these alterations is that the appearance rates of *Rickettsia* spp. vary between the geographical populations of ticks. According to a recent study, the members of *Rickettsia* are more abundant in ticks from forests (57, 69), where ticks included in the present study are also derived. Considering that nymphal rickettsial counts were high in other studies as well (64), while male ticks are normally associated with relatively fewer members of the genus *Rickettsia* (64, 65), the number of rickettsias seems to decrease throughout the life of male ticks gradually. This theory is in line with a study on microbiome changes during tick ontogeny (70). On the other hand, certain rickettsial species are also associated with male-killing, which may also cause the decreasing numbers associated with male adult ticks (71). *Rickettsia helvetica* and *Rickettsia monacensis*, two species identified in our samples as well, have been connected with the presence of adult *I. hookeri* wasps which are the parasitoids of ticks. The reason for the association may either be related to the role of the parasitoid wasps in the circulation of rickettsias among ticks or with digested bacterial DNA in the wasp body lumen (72).

Even though the members of *Mycobacteroides* are considered as either long-term or transient environmentally derived residents of the tick microbiota, recent studies revealed that certain species might be able to multiply inside the host and be transovarially inherited (56, 73). Nevertheless, the relative abundance of the species seems dependent on the geographic region (66). In our study, a statistically non-significant shift could have been observed in the direction of cooler and dryer regions. Considering climatic tendencies, Thapa and colleagues reported higher mycobacterial genome fragment counts in ticks from Texas than from Massachusetts (66), which shows a contrary mycobacterial temperature preference. The observed differences may be caused by the different species-level *Mycobacterium* composition in the U.S. and Europe. Since several *Mycobacterium* species may be pathogenic to humans or animals, this finding may have a public health significance, especially in the light of current climate change trajectories.

Cutibacterium and *Stenotrophomonas* are common environmental bacteria that have been associated with ticks in several studies (13, 35, 37). *Cutibacterium* is strongly associated with ticks in forests (69), where our *I. ricinus* ticks are derived from. *Stenotrophomonas maltophilia*, a species identified in our samples, is an opportunistic pathogen often isolated from the infections of immunocompromised individuals (59).

The majority of the genera constituting the core bacteriome were found to demonstrate significant differences among various life stages and climatic conditions associated with tick host groups. With the abundance rates of specifically tick-associated genera, *Arsenophonus*, *Candidatus Midichloria*, and *Wolbachia*, the significant differences among adult female ticks and nymphs were clearly explainable.

Arsenophonus, which showed significantly lower abundance rates in females than in nymphs, is a widespread, mainly insect-associated bacterial genus with a wide spectrum of either parasitic or symbiotic host relations (57, 74). While the high number of *Arsenophonus*, more precisely *Arsenophonus nasoniae* associated reads could suggest the dominance of this genus in the nymph microbiota; its presence is associated with *I. hookeri*, a parasitoid wasp of ticks that is supposed to have a wide geographical range worldwide. *I. hookeri* oviposits to larval and nymphal hard tick hosts. Its eggs can only develop in engorging or engorged nymphs (75, 76). If the tick immune system-borne encapsulation of the *I. hookeri* eggs is not successful, the eggs hatch. Larvae start consuming the tick tissues and thus cause the death of the host (75). Furthermore, Bohacsova and colleagues found that *A. nasoniae*, an endosymbiont of encyrtid wasps identified in high numbers in our nymph samples, is only detectable in tick nymphs parasitized by the wasp (72). According to these findings, nymphs harboring *A. nasoniae* do not often reach the adult stage due to the parasitoid wasp, *I. hookeri*. Thus *A. nasoniae* deriving reads demolish from the adult tick population specifically due to the death of nymph hosts. While the developmental stage dependence of *I. hookeri*-associated *A. nasoniae* is beyond question; the median abundance of this bacterial genus also showed differences in nymph groups of different climatic regions. Nymphs collected from warmer and rainier areas harbored more reads deriving from *Arsenophonus* spp. than ones collected from warmer areas. Attempts have been made to use *I. hookeri* as a means of biological control of ticks for approximately 100 years (76) and the consideration of climatic conditions as underlying causes for unstable control technique success rates may improve current biological control methods. Moreover, *A. nasoniae* is described to be male-killing in several wasp species (77), but in *I. hookeri* adult wasp males were also infected by *A. nasoniae*, although the emergence ratio of males to females was 1:3.6 in the infected populations (72). Nevertheless, the presence of this bacterium may underlie decreased *I. hookeri* numbers at certain habitats or could even have contributed to insufficient abundance rates by biological tick control purposes, attempted mass releases of the parasitoid wasps in the past (78, 79).

The genus of *Wolbachia*, having a significant, strong abundance rate shift to nymphs is reported to share several characteristics with *Arsenophonus* that affect tick life and, potentially, tick population size. One of the identified species, *Wolbachia pipientis*, is strongly related to the presence of *I. hookeri* (80, 81). Thus, the reason for the high number of wolbachial reads in nymphs may be the same as in the case of the *Arsenophonus* genus. *Wolbachia* spp. are also known to kill male hosts in some host insect species selectively (40, 82), while in others it appears to be non-male-killing (83). To the best of our knowledge, no studies exist on the effect of *Wolbachia* spp. on *I. hookeri*. Furthermore, similarly to the case of *Arsenophonus*, warmer and rainier areas had a slight positive effect on the number of wolbachial reads in nymphs although this effect was weaker than in the case of *Arsenophonus*. Due to the nymphal loads of reads from *Arsenophonus* and *Wolbachia*, the prevalence of *I. hookeri* in the samples and at the sampling points in Hungary can be strongly hypothesized. Evidence for the presence of these parasitoid wasps has recently been discovered in Hungary (45).

By *Candidatus Midichloria*, the strong, statistically significant difference that was observed for females among adult female and nymph ticks is in agreement with the

results of other research groups and has an explanation in the scientific literature. Studies exploring the inter-sex microbiome differences of adult *I. ricinus* ticks (35, 37, 84, 85) demonstrated much higher abundance rates of *Candidatus Midichloria* in females than in males independent of the regions of tick collection. The unique reason for this is that *Candidatus Midichloria mitochondrii* (CMM) invades the mitochondria of the cells within the ovaries of the female ticks. Despite the multiplication of the bacteria that consumes many ovarian mitochondria, the tick oocytes are expected to develop normally. CMM is described as being vertically transferred to all eggs (84, 86). Even though the nymphal sex ratio of ticks may not be exactly 1:1 (87), according to the available evidence, the presence of CMM does not result in sex ratio distortion in ticks (84). Simultaneously, it has been observed that CMM is transferred to both male and female larvae, but later, during the nymph stage, its specialization occurs toward females (85). Thus, a possible reason for the difference in CMM abundances among females and nymphs may be that many nymphs are males. Furthermore, the multiplication of CMM appears to increase after engorgement (85). Accordingly, the relatively low numbers of CMM might also be associated with the lack of engorgement among the nymphs collected for our study. Since tick collections occurred between the end of March and the middle of May, which represents the beginning of the activity period of nymphs (88), information on the overall engorgement status based on CMM counts appears to be realistic.

With the environmentally derived bacterial groups (*Bacillus*, *Curtobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, and *Staphylococcus*), the explanation of statistically significant life stage and climatic condition-wise differences in the bacterial composition appeared to be present.

Bacilli inhabit a broad range of environments, ranging from soils to insect guts and some species can be pathogens (36). The finding that the number of reads deriving from bacilli was significantly higher in nymphs than in adult females may originate from the choice to assess adult female ticks and exclude adult males from the study. While nymphs appeared in the study as a mixed population of males and females, the adult males collected at the sampling points were not represented due to their relatively low number compared to adult females. A study on the microbiome of *Rhipicephalus sanguineus* ticks describes a strong shift of *Bacillus* spp. toward the male tick population. Since our study only contained males in the nymph population, the observed shift may represent the male-relatedness to *Bacillus* spp. The nature of the relatedness of male ticks and *Bacillus* spp. has not yet been characterized (64). Nonetheless, *Bacillus* spp. are described not to be present in every tick microbiota (66, 89) and the detection rates of the members of this genus appear to show significant regional differences (37). Within our study, reads deriving from *Bacillus* spp. showed significantly higher abundances in nymphs from areas with more precipitation. Furthermore, Fernández-Ruvalcaba and colleagues reported that *Bacillus thuringiensis* strains significantly elevated mortality and demolished oviposition and egg hatch among adult, pesticide-resistant *Rhipicephalus microplus* ticks (90). Moreover, certain strains of *Bacillus wiedmannii* are known, while *B. thuringiensis* strains are even commercialized as insecticides or nematicides for biological pest control (91, 92), although the entomopathogenic effect of environmental strains proved to be more present (93). Both above-mentioned *Bacillus* species were present in our samples, which can decrease *I. ricinus* numbers, although potentially in both age groups. The key to the biopesticide characteristics of these bacilli is the formation of functional crystalline *Cry* proteins that are specific toxins. Other entomopathogenic bacteria such as *Bacillus cereus*, also present within our samples, are opportunistic as they act in secondary, non-specific pathways facilitated by certain weakening motives, such as the presence of *Cry* proteins (92). Thus, the shift in *Bacillus* abundance toward nymphs rather appears to occur due to the sex determination of the adult ticks included in the study.

Although within our samples, significantly more *Rhodococcus* reads are derived from adults than from nymphs, this result may be based on the following factor. The dominating species, *Rhodococcus fascians*, is a common bacterial phytopathogen that

interacts with a broad array of plants, causing their malformation (94). Older ticks may have had more opportunities for encounters with these environmental bacteria than younger ones. Higher *Rhodococcus* read counts in adults align with René-Martellet and colleagues' findings (64).

Association with *Staphylococcus* spp. that were significantly more abundant in adult females than in nymphs may be explained with similar reasoning. Staphylococci are common findings related to ticks (56, 65) that often appear on the skin and mucous membranes of the hosts of the ticks as well (56). Ticks carrying staphylococci may have already engorged and thus encountered these bacteria. Considering that the number of engorged nymphs appeared to be relatively low within our samples according to the reasons explained by the genus *Candidatus Midichloria*, fewer opportunities to encounter the members of this genus by nymphs are possible. Moreover, the relative abundance of *Staphylococcus* spp. also appears to be dependent on the region of tick collection (66) and, as described earlier, staphylococcal hits may also derive from contamination (37).

The finding that the members of another environmental bacterial genus, *Sphingomonas* (13) were identified with significantly higher abundance rates in nymphs than in adults is controversial to the hypotheses of *Rhodococcus* spp., *Staphylococcus* spp., and to the related findings of other authors (70). Another finding was that among adult females, the abundance rate of *Sphingomonas* spp. was significantly higher in warmer sampling areas than in cooler localities, while by nymphs, the temperature-wise difference was not relevant. Interestingly, another research group found that *Sphingomonas* spp. were much more abundant in adult ticks kept at 4°C than by those at 20°C, 30°C, or 37°C. Moreover, according to their study, *Sphingomonas* was among the most abundant bacterial genera at 4°C by males (65). The reason for this discrepancy with our results on temperature-wise abundance may rest on the species-level *Sphingomonas* composition. Additionally, interactions with other bacterial groups may also underlie our findings. In any case, further studies would be required to elucidate the findings of *Sphingomonas* populations and confirm or invalidate our hypotheses.

Statistically significant climatic condition-wise alterations in adult female and nymphal stage ticks only appeared in environmental bacterial genera, namely *Curtobacterium* and *Pseudomonas*. While the former appeared significantly more abundantly in warmer environments both for adult females and nymphs, a significant preference for little precipitation was observable in nymphs. This finding may be relevant in light of climate change, mostly because of a dominant species, *Curtobacterium flaccumfaciens* which is a phytopathogenic bacteria with economic significance (95) and has also been isolated from a child with fatal septicemia (96).

Reads from the genus of *Pseudomonas* were detected with significantly greater abundances at areas with higher GDD in adult females, while temperatures appeared not to influence the relative abundance of *Pseudomonas* spp. in nymphs. The formation of biofilms might explain this finding as some *Pseudomonas* strains have better biofilm formation capacities at warmer temperatures (97, 98). Thus, adult ticks from warmer environments that have already survived at least one summer, according to our knowledge of the tick life cycle, may harbor more bacteria from the *Pseudomonas* genus that managed to form biofilms and so became more steadily present. According to a study, the egg wax composition of the cattle tick, *Rhipicephalus microplus* is able to inhibit the biofilm formation of *Pseudomonas aeruginosa* (99). Thus, certain mechanisms may influence the abundance of *Pseudomonas* species at earlier tick life stages. The exact composition of *Pseudomonas* strains should be examined to evaluate this hypothesis as the genus is very versatile. At the same time, *Pseudomonas* spp. have been related to both nymphs and adults in other publications (37), while certain studies report more stable *Pseudomonas* spp. appearance rates in males than in females (35, 66).

All the above-mentioned bacterial groups share another factor, namely the interaction among the bacterial community members, this may be responsible for the microbial pattern assessed. In order to elucidate the possible influence of certain bacterial co-occurrences, the correlation analysis of the bacterial genera was performed

in each development stage and climatic category. Positive correlations among the taxa of microbial communities may be interpreted as the reflection of shared habitat or environmental condition preferences, cooperative activities, such as cross-feeding (100), or the representation of functional guilds performing complementary or similar functions (101). In contrast, negative correlations may indicate competition for limiting resources, niche partitioning, inequivalent resistance to losses, or active negative interactions (100, 101). In contrast to other studies (13), the number of positive and negative correlations was balanced within our samples.

All samples considered, *Arsenophonus* and *Wolbachia* correlated positively, which is assumed to have occurred due to the presence of *I. hookeri*. Furthermore, *Rickettsia* was observed to be negatively correlated with *Curtobacterium* spp. in several development stages and climatic groups, which may indicate positive public health consequences and a possible step toward a future tick control tool. In contrast, *Candidatus Midichloria* spp. which were previously detected in positive correlation with *Rickettsia* spp. (13) showed no correlation in our samples. Juxtaposing another publication in the field (13), neither the *Pseudomonas* and *Rickettsia* nor the *Bacillus* and *Rickettsia* pair showed any correlation. Correlations among environmental bacteria of various climatic categories are likely based on their similar environmental preferences and the previously mentioned interaction types. Due to the seasonal variability of environmental bacteria, the number of tick-borne, potentially pathogenic bacteria may increase or decrease correspondingly with the taxa of environmental origins (13). Accordingly, the presence or absence of certain environmental taxa may reflect the temporal dynamics of certain pathogens. The public health significance of this finding is particularly significant in light of climate change and potentially varying climatic conditions around the globe. Nevertheless, the interpretation of co-occurrence patterns and the nature of these correlations require further studies as abundance shifts depend on multiple factors. Thus, bacterial and parasitological interconnections are not exclusively responsible for the variations. Moreover, our results were obtained from the sequencing of entire tick individuals and thus lacked finer, e.g., organ scale considerations of the correlating taxa.

Conclusion

Here we reported the identification of the *I. ricinus* bacteriome-associated findings in adult females and nymphs collected from a climatically representative set of sampling points in Hungary. These results allowed us to show that (i) the *I. ricinus* bacteriome is dependent on the temperature and precipitation history of the geolocation of sampling; (ii) the *I. ricinus* bacteriome is not stable in the developmental stages of the ticks; (iii) based on the bacteriome patterns identified, the identified developmental stage-wise alterations may be associated with the presence of certain tick parasitoids that exclude the option of reaching the adult age; (iv) TBD pathogens are widely distributed at the climatically representative sampling points.

In the future, developmental stage and climate-associated microbial differences and correlations identified in this ecosystem study could be confirmed with experimental approaches and complemented with further metagenome studies to achieve sufficient data volumes of tick microbial inter-relatedness and exploit them as promising resources for novel tick control strategies.

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N.S. takes responsibility for the integrity of the data and the accuracy of the data analysis. N.S. and R.F. conceived the concept of the study. E.K., G.M., and M.G. performed sample collection and procedures. A.G.T., M.P., and N.S. participated in the bioinformatic and statistical analysis. A.G.T., H.Y., L.M., and N.S. participated in the drafting of the

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Adrienn Gréta Tóth, Data curation, Formal analysis, Writing – original draft, Writing – review and editing | Róbert Farkas, Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review and editing | Márton Papp, Formal analysis, Writing – review and editing | Oz Kilim, Writing – review and editing | Haeun Yun, Writing – original draft | László Makrai, Supervision, Writing – review and editing | Gergely Maróti, Methodology, Resources, Writing – review and editing | Mónika Gyurkovszky, Investigation | Eszter Krikó, Investigation | Norbert Solymosi, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

The short-read data of samples are publicly available and accessible through [PRJNA828115](https://www.ncbi.nlm.nih.gov/sra/PRJNA828115) from the NCBI Sequence Read Archive (SRA).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Fig. S1 (Spectrum01243-23-s0001.pdf). Bacterial genus abundance correlations.

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OPEN First detection of *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae) in *Ixodes ricinus* ticks (Acari: Ixodidae) from multiple locations in Hungary

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The parasitoid wasp, *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae), is the natural enemy of a wide range of hard and soft tick species. While these encyrtid wasps are supposed to be distributed worldwide, only a few studies report on their actual distribution around the globe. Within a shotgun sequencing-based metagenome analysis, the occurrence of *I. hookeri* was screened at multiple *Ixodes ricinus* (Acari: Ixodidae) tick sampling points in Hungary to contribute to the assessment of the distribution patterns of the parasitoid wasps in Central Europe. To our knowledge, the first report of the species in Hungary and the description of the southernmost *I. hookeri* associated geolocation in Central Europe took place within our study. *I. hookeri* infested *I. ricinus* nymphs were detected at five sampling points in Hungary. The results show that the exact distribution range of *I. hookeri* is still barely studied. At the same time, unprecedented public health issues being brought about by climate change might require steps toward the exploitation of the tick biocontrol potential and as an ecological bioindicator role of the parasitoid wasp in the future.

The emergence of vector-borne diseases that can affect human and animal populations is strongly influenced by climate change, urbanization, and globalization¹. Among the most significant arthropod vectors (e.g. ticks, fleas, black flies, mosquitoes, or sand flies), ticks transmit a markedly broad spectrum of pathogenic microorganisms, including various protozoa, rickettsiae, spirochaetes, and viruses². Since Hungary is situated in the southern part of Central Europe, climate change may facilitate the expansion of certain tick species to this landlocked country from the neighboring Mediterranean region. Currently, 27 hard tick species (Ixodidae) have been described in Hungary³. As part of the VectorNet project, the European Centre of Disease Prevention and Control (ECDC) currently monitors seven tick species, including *Ixodes ricinus*, that commonly transfer diseases to humans and animals⁴. Naturally, further tick species may also serve as vectors for microorganisms of human or veterinary medical significance⁵. Out of the seven tick species monitored by ECDC, all except for *Ornithodoros* spp. are hard ticks present in Hungary³.

Several studies report the incidence trends of tick-borne encephalitis (TBE) and Lyme borreliosis (LB), the most prevalent tick-borne infection in Europe^{6,7}. In the case of LB, which has higher country-wise incidence rates than TBE, decade-long trends of incidence rates are not consistent along the countries around the world, increasing, and decreasing tendencies both appear. On the other hand, reports of the geographic distribution of LB show a clear expansion, especially towards higher altitudes and latitudes^{6,7}. Although global trends of the incidence rates of these TBDs are not consistent^{6,7}, changes in the distribution range of European *I. ricinus* populations are. Enhanced surveillance and diagnostic measures raise awareness of the changing geographical distribution, density, and activity of the *I. ricinus*, the primary vector of TBE and LB in Europe⁸. As a consequence of climate change, *I. ricinus* expanded its distribution to areas of higher altitude and latitude apart from its prior range, and its northerly shift within the European continent has also been documented^{9–11}.

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As climate change, accompanied by various sociodemographic alterations, brings unprecedented challenges related to vector-borne diseases^{12,13}, the need for the development of control methods against tick populations is a public highlight. Several methods have been introduced to address this issue. These control methods often rest on either conventional chemical acaricides or on further alternatives, such as biological control methods assisted by the natural enemies of ticks^{14–18}. A line of biological control methods against ticks could be the Encyrtidae family members which are small-sized, parasitoid, or hyperparasitoid wasps distributed all around the globe. Due to their efficacy and target specificity, numerous wasps from this family are used as biological pest control, while several additional encyrtid species are documented as promising candidates for this role^{19–21}. *Ixodiphagus* spp., including *I. hookeri*²², are encyrtid wasps attacking a wide range of tick species that have received relatively much attention as a specific and effective, natural alternative for biological hard tick control^{23,24}. Interestingly, *I. hookeri* appears to have alternating preferences for the tick species and developmental stage of its hosts at geographically distant locations^{25,26}. In European settings, *Ixodiphagus* wasps are described to parasitize the larvae and nymphs of hard ticks with a clear predilection for unfed nymphs²⁴. If oviposition occurs in larvae, transstadial transmission through the molting of the ticks to nymphs can also occur²⁷. Wasp eggs start their embryonic development in engorging or engorged nymphs. Wasp larvae feed on tick tissues and emerge as fully grown adults causing the death of the host before it can reach the adult stage^{23,24}.

Ixodiphagus wasps have been associated with several hard and soft tick genera belonging to the families of Ixodidae and Argasidae, including *Ornithodoros*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*²³. Studies conducted in Europe revealed that *I. ricinus* appears to be the preferred species of the European *I. hookeri*, while another common tick species, *Dermacentor reticulatus* is supposed not to be chosen as a host by the European representatives of the parasitoid wasps²⁴.

While *Ixodiphagus* spp. have been detected in many countries and in a diverse range of hard and soft tick species, parasitoid wasps have been less studied in Hungary despite their potential to reduce tick populations and tick-borne disease cases. In the present study, our aim was to confirm the presence *I. hookeri* in a diverse set of locations in Hungary using a modern, sensitive metagenomic approach^{28,29}. Due to their high density in Hungary, high public health significance as TBD transmitters, and potential to host *Ixodiphagus* wasps, *I. ricinus* ticks were decided to be assessed for the parasitoids. Based on our approach, genomic information of the European populations of *I. hookeri* may also be obtained, which can serve as a reference for further studies.

Materials and methods

Between March and August of 2019, in two *I. ricinus* metagenome surveys, questing ticks, were collected from 21 geolocations in Hungary by flagging and dragging. One of the surveys was performed as a country-wide climatically designed sampling (17 sites)³⁰. Further samples were collected in 4 sites popular for outings and dog walking. The closest settlements of sampling points were: Kissziget (a), Sárvár (b), Mosonmagyaróvár (c), Sáska (d), Darány (e), Somogybabad (f), Péntesgyőr (g), Pécsvárad (h), Vértesszomsz (i), Németskér (j), Törökbálint (k), Normafa (l), Nagy-Hideg-Hegy (m), 10th district of Budapest (n), Pusztavacs (o), Kékes (p), Lillafüred (q), Aggtelek (r), Háromhuta (s), Nyíregyháza (t), Nyíradony (u). The collected ticks were frozen at -18°C . In the laboratory, the ticks were classified taxonomically using standard morphological keys³¹, and 10 nymphs and 10 adult females of *I. ricinus* per sampling sites (Fig. 2) were selected randomly. Before DNA extraction, the ticks were washed twice with 99.8% alcohol.

The blackPREP Tick DNA/RNA Kit (Analytik Jena GmbH) was used for the DNA isolation. Isolated total metagenome DNA was used for library preparation. In vitro fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

On the raw sequencing data, quality-based filtering and trimming were performed by TrimGalore (v0.6.6, <https://github.com/FelixKrueger/TrimGalore>), setting 20 as a quality threshold, retaining reads longer than 50 bp only. Using the remained reads, a de novo assembly was performed by MEGAHIT (v1.2.9)³² using default settings. The resulting contigs were taxonomically classified using Kraken2 ($k = 35$)³³ with the NCBI non-redundant nucleotide database³⁴. Contigs were predicted as *I. hookeri* by taxon classification and were checked with BLAST³⁵ on the partial sequence of *I. hookeri* 28S ribosomal RNA gene (MH077537.1) as a reference. Multiple sequence alignment was done by MAFFT (v7.490)³⁶. All data management procedures, analyses, and plottings^{37,38} were performed in the R environment (v4.2.1)³⁹.

Results

Of the 21 adult female samples (10 individuals per sample) examined, we did not find any contigs with reasonable evidence for *I. hookeri* origin. In five of the 21 nymph samples (10 individual nymphs per sample), namely sample b, c, d, g, n, contigs deriving from *I. hookeri* were found. The sequence identity of the contigs deposited in GenBank to the *I. hookeri* 28S rRNA gene was 378/386 (97.9%), 556/559 (99.4%), 439/447 (98.2%), 445/453 (98.2%), and 300/308 (97.4%) for samples b (accession id: OQ316579), c (OQ316577), d (OQ316581), g (OQ316578), and n (OQ312115), respectively. The multiple sequence alignments of the contigs with the partial reference sequence of *I. hookeri* 28S rRNA gene (MH077537.1) are shown in Fig. 1. The figure shows that the sequence of the generated contigs varies from the reference sequence at 8 positions (A434T, A491C, A499G, A666G, A670G, T677G, G678T, C688G). In the annotation of the altered positions, the first letter refers to the base in the reference sequence, the following numbers specify the genomic position of the polymorphism, and the last letter indicates the base detected in our samples. By position A434T, transversions were identified in sample b, c, d, and g. All our samples included A491C and A499G mutations. A666G, A670G, T677G, G678T, and C688G polymorphisms occurred in sample b, d, g, and n. In the easternmost sample (n), we found one position

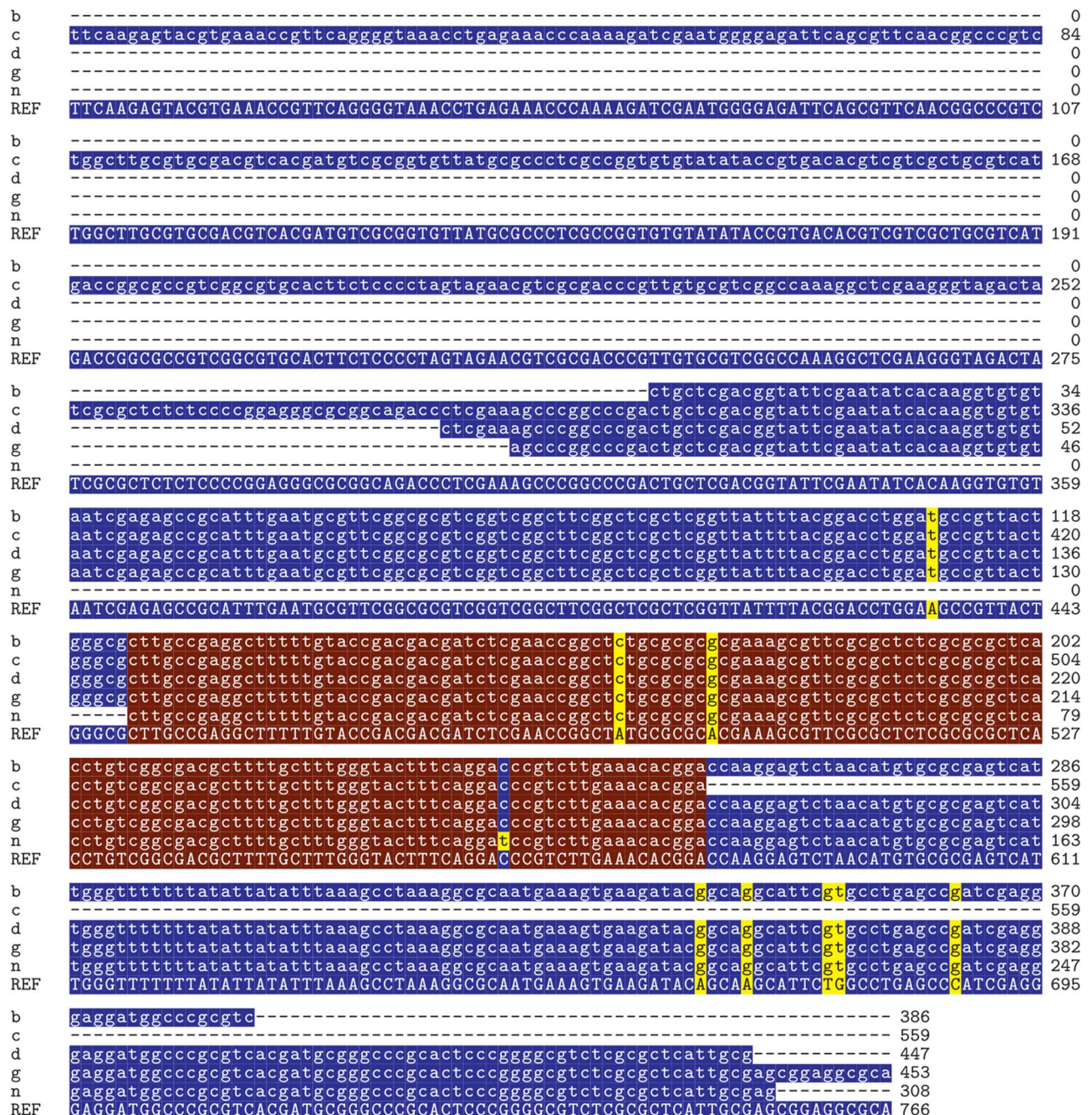


Figure 1. Multiple sequence alignments. The contigs were predicted to have *I. hookeri* origins based on the partial reference sequence of *I. hookeri* 28S ribosomal RNA gene (MH077537.1). Letters on a blue background indicate sequence identity from less than all the samples, and letters on a brown background indicate the parts where all 5 samples and the reference sequence are identical. Positions with yellow backgrounds indicate polymorphisms. The geopositions of the Hungarian samples are presented in Fig. 2.

(C565T) that differs from all other Hungarian samples and the reference sequence as well. The geoposition of the samples is presented in Fig. 2.

Discussion

The findings that no reads deriving from *I. hookeri* were detected in adult *I. ricinus* samples collected between the end of March and the middle of May, while nymphs were associated with this species, promote former theories of the life cycle of the parasitoid wasps^{23,24}. *I. hookeri* eggs may have been laid in the larvae or in nymphs before winter or, less probably, during spring, as the wasps were formerly associated with the possibility of surviving winter conditions^{40,41}.

To our knowledge, this is the first report on evidence of the presence of *Ixodiphagus* wasps, namely *I. hookeri* in Hungary. This finding expands the localities associated with *I. hookeri* within Europe. All except one sampling

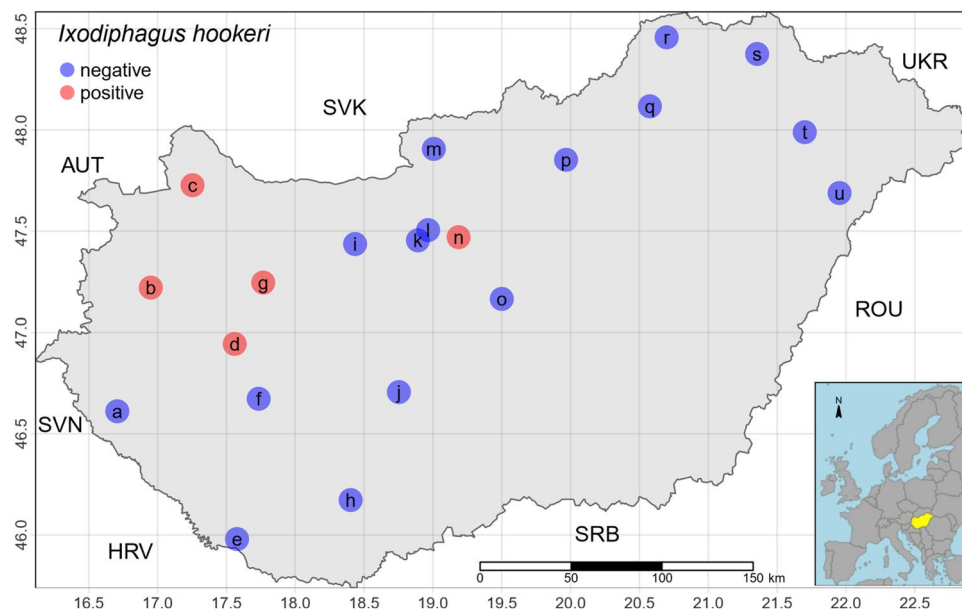


Figure 2. Geopositions of the metagenomically analyzed samples in Hungary. The red dots represent the sampling points we have found *I. hookeri* sequences, the blue ones where we have not. The inset map shows the study region, Hungary in Europe, colored yellow. Neighboring countries are presented by ISO 3 character codes (AUT: Austria, HRV: Croatia, ROU: Romania, SRB: Serbia, SVK: Slovakia, SVN: Slovenia, UKR: Ukraine).

points that were proven to host *I. hookeri* are located in western Hungary. The cluster of the four western Hungarian sampling points lays close to Austria's and Slovakia's borders. While the presence of *I. hookeri* has not been published in Austria; to our best knowledge, Slovakian reports of the occurrence of the wasps exist. *I. hookeri* has previously been identified at three locations within Slovakia; near Šoporňa, associated to *Haemaphysalis concinna*⁴², close to the capital of Slovakia, Bratislava, in *I. ricinus*^{43,44} and in the Slovak Karst, isolated from both *I. ricinus* and *H. concinna*⁴¹. According to our hypothesis, the western Hungarian cluster of sampling points b, c, d, and g (Sárvár, Mosonmagyaróvár, Sáska, and Pénteszgyőr, respectively) may be associated with the wasp populations described by Bratislava^{43,44} and by Šoporňa⁴² in Slovakia. Despite being situated close to areas where *I. hookeri* is present, no *I. hookeri* DNA was detected from ticks in sampling points m, p, q, r, and s (Nagy-Hideg-Hegy, Kékes, Lillafüred, Aggtelek, and Háromhuta, respectively). Shifting slightly to the east, sampling point n (Budapest) represented the closest occurrence of the wasps to Slovakia. Considering the physical proximity between sampling point r and the Slovak Karst, where the report of Buczek and colleagues was released, the possibility of receiving false negative results is raised. The basis of receiving false negative results will be described further on.

Other European countries where the presence of *I. hookeri* has been reported include the Czech Republic (detected in former Czechoslovakia)⁴⁵, Finland⁴⁶, France^{47,48}, the Georgia⁴⁹, Germany^{24,26,50}, Italy⁵¹, the Netherlands^{27,52}, the United Kingdom⁵³, Russia (detected in the Ussuri forest, in the Asian part of the former Soviet Union)⁵⁴ and Ukraine (detected in the former Soviet Union)⁵⁵. To our knowledge, sampling point d (Sáska) in Hungary represents the southernmost detection point of *I. hookeri* within Central Europe. The detection of *I. hookeri* in Hungary may serve as a novel hint regarding the potential distribution *I. hookeri* at the Balkan Peninsula, where the species appears to be little studied.

As mentioned above, wasp-negative sampling points can be wasp-invaded. Even though next-generation sequencing (NGS) based metagenomic approach appears to be just as or even more sensitive as polymerase chain reaction (PCR) based target detection techniques^{28,29,56}, certain limitations can be addressed. Within the pool of reads deriving from the shotgun-sequenced metagenome that contains genome fragments from every organism present in the sample, lower relative abundance rates of an individual species serve with relatively lower read counts from its genome. In other words, shotgun sequencing preserves the original relative read abundance rates of the various organisms of the samples and may represent fewer reads of certain species by non-targeted runs^{57,58}. Moreover, the *I. hookeri* reference sequence, that the metagenomic read sets were aligned to only represented a smaller fragment, namely the unique 28S ribosomal RNA gene of the full *I. hookeri* genome. Thus only *I. hookeri* reads deriving from this part of the genome could have been aligned, that further increases the chance of false-negative sampling points for the wasps. Besides the above-mentioned reasons, due to the low European tick parasitization rates of *I. hookeri*, the 10 nymphs collected at the sampling points may miss the wasps by chance alone.

Nevertheless, NGS-based approaches have a prospering future within the studies of parasitoids of public health significance, such as *I. hookeri*. According to Collatz and colleagues, large geographical distance and climatic differences (e.g. presence in Africa, Asia, Europe, and North America)^{24,25,59,60} may even underlie divergence and distinct taxonomic categorization of *I. hookeri* to different species, subspecies or at least strains²⁴. Concurrently, publications on *I. hookeri* indicate a certain extent of behavioral and host preferences at different

continents^{24,25,61}. To assess the basic variation in behavioral traits of *I. hookeri* or to identify specific characteristics of subgroups that can be better utilized by the biological control methods, the study of the *I. hookeri* genome or at least specific genome regions, such as 28S rRNA or 16S rRNA genes, may become inevitable, similarly to other weighty insect groups^{52,62,63}.

The improvement of our knowledge of *I. hookeri* with either traditional or genomic methods could facilitate the assessment of its potential as a means of biological control, while limitations and doubts about the wasps' biocontrol potential could be addressed with more research. Attempted mass releases of the parasitoid wasps in the U.S and in the former Soviet Union between 1920 and 1940^{40,64,65} were unsuccessful as far as causing noticeable reduction of tick populations. One reason for this may be that *I. hookeri* requires high tick host densities and superabundant tick populations to reach its ideal abundance^{61,66}. Inadequate numbers of parasitoids released compared to the geographical areas may have also undermined these trials²³. On the other hand, the parasitoid wasps have been transported to the sites of attempted mass releases from great distances, sometimes even from different continents (e.g. from France to the U.S.)^{40,64} without any considerations regarding their host preferences, climatic adaptations, or behavioral attributes, that have, since then turned out to be rather specific to their geographic locations of origin^{24,25,61}. In a global perspective, climate-associated occurrence rate alterations^{23,25} or differences in other characteristics of *I. hookeri*, such as the duration of its development, may also be underlain by host-related factors. Synchronization with the maturation of the tick host and, indirectly, with the main activity period of the vertebrate hosts of the ticks throughout the year may play a role in the life cycle of the wasps²⁴.

Furthermore, we do not know how great the tick populations would be without the endemic *I. hookeri* populations and how much the parasitoid wasps contribute to maintaining the equilibrium of the communities in which they are included⁶⁷. Nonetheless, the hypothesis regarding sufficiently high tick host densities and superabundant parasitoid host populations is in line with findings regarding the bioindication potential of certain insect species, including parasitoid wasps⁶⁸. If so, this potential may also be worth further observation.

Conclusively, assessment of existing populations and further examinations on entomologic and genomic traits along with ecological roles could help understand and exploit the *Ixodiphagus* wasps' potential as a biological tick control method or as a potential bioindicator species.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

N.S. takes responsibility for the data's integrity and the data analysis's accuracy. N.S. and A.G.T. conceived the concept of the study. E.K. and M.G. performed sample collection and procedures. N.S. participated in the bio-informatic and statistical analysis. A.G.T. and N.S. participated in the drafting of the manuscript. A.G.T., N.S. and R.F. carried out the manuscript's critical revision for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Antimicrobial resistance genes in raw milk for human consumption

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The increasing prevalence of antimicrobial resistance (AMR) is a significant threat to global health. More and more multi-drug-resistant bacterial strains cause life-threatening infections and the death of thousands of people each year. Beyond disease control animals are often given antibiotics for growth promotion or increased feed efficiency, which further increase the chance of the development of multi-resistant strains. After the consumption of unprocessed animal products, these strains may meet the human bacteriota. Among the foodborne and the human populations, antimicrobial resistance genes (ARGs) may be shared by horizontal gene transfer. This study aims to test the presence of antimicrobial resistance genes in milk metagenome, investigate their genetic position and their linkage to mobile genetic elements. We have analyzed raw milk samples from public markets sold for human consumption. The milk samples contained genetic material from various bacterial species and the in-depth analysis uncovered the presence of several antimicrobial resistance genes. The samples contained complete ARGs influencing the effectiveness of acridine dye, cephalosporin, cephamycin, fluoroquinolone, penam, peptide antibiotics and tetracycline. One of the ARGs, PC1 beta-lactamase may also be a mobile element that facilitates the transfer of resistance genes to other bacteria, e.g. to the ones living in the human gut.

The increasing prevalence of antimicrobial resistance (AMR) is a significant threat to global health. The wide-spread use of antibiotics, both in human healthcare and animal disease control^{1–3}, is increasingly shortening the time it takes for resistant strains to develop and more and more multi-drug-resistant bacterial strains cause life-threatening infections. There is increasing evidence that antimicrobial resistance genes (ARGs) responsible for the occurrence of phenotypically expressed antimicrobial resistance are widespread in various environmental samples^{4–7}. The pool of ARGs being present in a particular environmental sample is called the resistome⁸. In samples where the medical use of antibiotics can be excluded, normally, only a few ARGs are present^{9,10}. When antibiotics are extensively used for preventive and therapeutic purposes, bacterial strains respond to this selective pressure and will become increasingly resistant what finally leads to these agents' elevated prevalence. ARGs hosted by non-pathogenic bacteria can be transferred to pathogens with horizontal gene transfer (HGT) what elevates the latter group's resistance against antibiotics. The execution of HGT depends on several factors, albeit physical closeness of bacteria always increases the chances¹¹. The likelihood of HGT is even higher if ARGs are carried on mobile genetic elements (e.g. plasmids). In order to understand the chance of an ARG's horizontal gene transfer derived spread, studies aiming to assess the bacteria's resistome and the specific position of the identified ARGs¹² are very well-reasoned and necessary.

The microbiota of livestock products may come to direct contact with the human bacteriota, either during the processing steps or during the consumption of these products. The antibiotics used for farm animal disease control often share active substances with human medicines. Consequently, there is a risk that ARGs accumulated as a response to the high amount of antibiotics used in livestock farming may be transmitted to the human microbiota through animal products. Such spread of ARGs may reduce the efficacy of antibiotic therapies even more and may lead to the development of new multidrug-resistant strains. Fortunately, food processing typically

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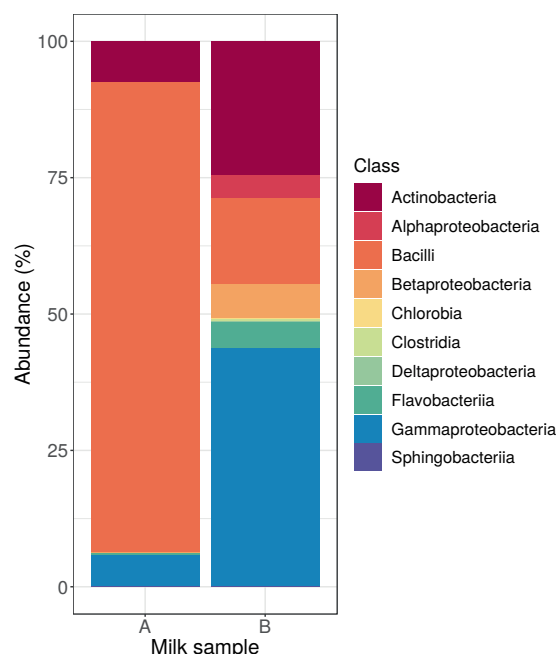


Figure 1. Core bacteriome composition. Relative abundance of bacteria classes by milk samples.

contains heat treatment steps that kill the majority of bacteria. Thus the role of active DNA-export mechanisms between the intestinal and the nutriment's bacteriome is lower¹³.

Raw milk is a product sold unprocessed; thus the presence or the grade of heat-treatment steps are upon the decision of consumers. In addition to this, the consumption of non-heat-treated raw milk justified by its favourable health effects is nowadays commonly set as a trend in the developed countries^{14,15}.

To our best knowledge, no previous study has investigated the possible presence of ARGs in raw milk, and we have found no data on the raw milk's resistome. This study aims to test the presence of ARGs in milk metagenome, investigate their genetic position and their linkage to mobile genetic elements^{16,17}.

Results

Metagenome. After DNA extraction and sequencing (see Methods), from sample A 17,773,004 while from sample B 8,425,326 paired-end reads were recovered. By the quality filtering, 0.20% and 0.80% of the reads were discarded from sample A and B, respectively. The reads were aligned to the host (*Bos taurus*) genome. As expected, most of the genetic material originated from the milking cow, from sample A 96.41% and from sample B 97.01% of the cleaned reads were filtered out due to host origin.

Of the reads, not aligning to the cow genome, we were able to classify 42.11% in sample A and 52.96% in sample B to known taxa. 185,982 reads of sample A and 11,437 reads of sample B were identified to belong to the kingdom of Bacteria. In sample A 93.54% of the reads were classified as Gram-positive bacteria, while in sample B this proportion was only 40.54%. The detailed composition of the core bacteriomes at class level is shown in Fig. 1.

ARGs and MGEs. Reads with overlapping pieces were assembled into longer DNA contigs by the metaSPAdes tool. The assembled contigs having a shorter length than 162 bp (sample A: 0.68%, sample B: 0.33% of all contigs) were excluded. The remaining contig's median length was 268 (IQR: 126.5) and 244 (IQR: 42) in sample A and B, respectively. Then, the contigs having any open reading frame (ORF) matched with an ARG in the Comprehensive Antibiotic Resistance Database (CARD) were collected. The detected ARGs and particular properties are presented in Table 1.

The identified ARGs were classified with the Resistance Gene Identifier (RGI) tool according to the ratio of their coverage in the samples and to the identity between the contigs assembled from the sequenced reads and the CARD ARG reference sequences. In Table 1 we list each ARG with perfect or strict hits predicted by RGI. We were able to identify three perfect ARG matches in sample A, *mepR*, *mgrA* and *Staphylococcus aureus* *norA*. According to the taxonomical classification of the contigs harbouring these ORFs their most likely origin is bacteria from *Staphylococcus* genus. The MGE analysis showed that none of these ORFs is mobile.

The sequence coverages of the strict matches in sample A ranged between 2.12% and 100%, with a mean of 36.61%. The identity of ORFs and CARD ARG reference sequences ranged between 95.02% and 100%, with a mean of 99.59%. Contigs containing ARG were classified on genus level and *Acinetobacter* (2.86%), *Carnobacterium* (11.43%), *Chryseobacterium* (2.86%), *Corynebacterium* (2.86%), *Kocuria* (11.43%), *Lactococcus* (8.57%), *Leuconostoc* (2.86%), *Macroccoccus* (2.86%), *Moraxella* (5.71%), *Staphylococcus* (37.14%) and *Streptococcus* (11.43%) genera were identified.

In the bacterial genome, ARGs may be located on chromosomes or on plasmids, the latter ones being more likely to translocate between bacteria. With the PlasFlow tool, we identified contigs harbouring chloramphenicol acetyltransferase, PC1 beta-lactamase (*blaZ*) and *tet(38)* ARGs that may be encoded on plasmids. The results of

ARG	Coverage %	Identity %	Genus	Species	Localization	MGE
Perfect RGI match in sample A						
mepR	100.00	100.00	<i>Staphylococcus</i>	<i>aureus</i>	chromosome	
mgrA	100.00	100.00	<i>Staphylococcus</i>	<i>aureus</i>	unclassified	
<i>Staphylococcus aureus</i> norA	100.00	100.00	<i>Staphylococcus</i>	<i>aureus</i>	chromosome	
Strict RGI match in sample A						
AAC(6')-IIc	30.05	100.00	<i>Carnobacterium</i>	<i>maltaromaticum</i>		
<i>Acinetobacter baumannii</i> AbaQ	17.97	100.00	<i>Leuconostoc</i>	<i>mesenteroides</i>		
APH(2'')-Ig	29.74	100.00	<i>Chryseobacterium</i>			
APH(3'')-Ia	7.35	100.00	<i>Acinetobacter</i>	sp. TTH0-4		
APH(3')-Ia	8.12	100.00				
APH(7'')-Ia	13.25	100.00	<i>Lactococcus</i>	<i>raffinolactis</i>		
arlR	74.89	95.12	<i>Staphylococcus</i>	<i>aureus</i>		
arlR	30.14	98.48	<i>Staphylococcus</i>	<i>aureus</i>		
arlS	29.93	100.00	<i>Staphylococcus</i>	<i>aureus</i>		
arlS	70.95	99.69	<i>Staphylococcus</i>	<i>aureus</i>	chromosome	
baeS	4.71	100.00				
BUT-1	11.59	100.00	<i>Moraxella</i>	<i>osloensis</i>		
<i>Campylobacter coli</i>						
chloramphenicol acetyltransferase	52.17	100.00	<i>Lactococcus</i>	<i>raffinolactis</i>	plasmid	
CatU	11.98	100.00	<i>Streptococcus</i>	<i>thermophilus</i>		
cfr(B)	24.36	100.00	<i>Streptococcus</i>	<i>urinalis</i>		
DHA-1	99.75	99.75	<i>Staphylococcus</i>	<i>aureus</i>	chromosome	
ErmW	10.61	100.00				
ICR-Mo	28.32	98.10	<i>Moraxella</i>	<i>osloensis</i>		
<i>Klebsiella pneumoniae</i> KpnF	68.81	100.00	<i>Corynebacterium</i>	<i>provincense</i>		
MCR-3.2	12.75	100.00	<i>Kocuria</i>	sp. BT304		
mecD	11.80	100.00	<i>Macrococcus</i>	<i>caseolyticus</i>		
mepA	100.00	99.78	<i>Staphylococcus</i>	<i>aureus</i>	chromosome	
mphM	27.09	100.00	<i>Carnobacterium</i>	<i>maltaromaticum</i>		
mphO	11.36	100.00	<i>Kocuria</i>			
MuxC	2.12	100.00				
norB	9.23	100.00				
OCH-2	10.26	100.00		<i>Brevibacterium</i>		
				phage Cantare		
PC1 beta-lactamase (blaZ)	100.00	95.02	<i>Staphylococcus</i>		plasmid	phage integrase
PEDO-1	20.98	100.00	<i>Lactococcus</i>	<i>lactis</i>		
PEDO-3	51.90	100.00	<i>Carnobacterium</i>	<i>maltaromaticum</i>		
QnrB42	20.09	100.00				
srmB	14.18	100.00	<i>Carnobacterium</i>	<i>maltaromaticum</i>		
<i>Staphylococcus aureus</i> LmrS	12.71	100.00	<i>Staphylococcus</i>	<i>aureus</i>	chromosome	
<i>Staphylococcus aureus</i> LmrS	86.25	99.27	<i>Staphylococcus</i>	<i>aureus</i>	unclassified	
tet(38)	100.44	99.33	<i>Staphylococcus</i>	<i>aureus</i>	plasmid	
tetS	13.42	100.00				
tetS	11.23	97.22				
vanJ	18.48	100.00	<i>Streptococcus</i>	<i>thermophilus</i>	unclassified	
vanRG	19.57	100.00	<i>Streptococcus</i>	<i>thermophilus</i>		
vanTN	6.43	100.00	<i>Kocuria</i>			
ykkC	25.89	100.00	<i>Kocuria</i>			
Strict RGI match in sample B						
mefE	5.71	100.00	<i>Delftia</i>	<i>tsuruhatensis</i>		
OXA-269	13.55	100.00				
OXA-442	9.12	100.00				
PEDO-1	24.83	100.00	<i>Acinetobacter</i>	sp. TTH0-4		

Table 1. ARGs identified in milk samples. The coverage column shows the fraction of CARD ARG reference sequence covered by the most similar ORF sequence. Identity represents the proportion of the identical nucleotides between the detected ORF and CARD ARG reference sequence. Species column shows the most likely species related to the ARG harbouring contig classified by Kraken2. For some contigs, the species level classification was ambiguous, genus reported only. The localization of contigs with ARG and longer than 1000 bp predicted by PlasFlow. Mobile genetic element domains coexisting with ARG are listed in column MGE.

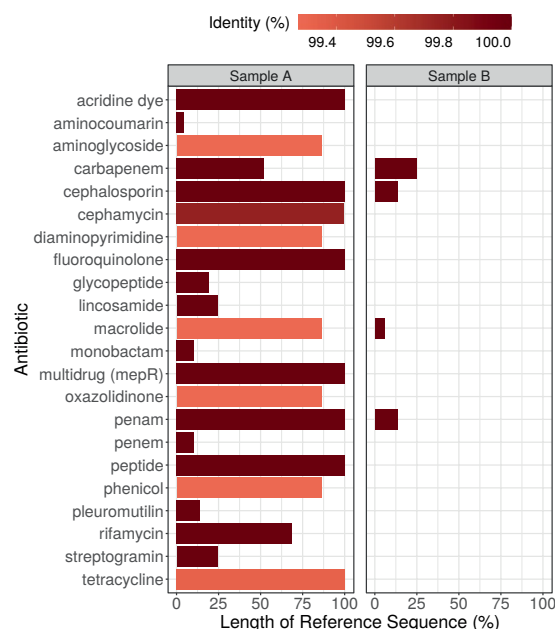


Figure 2. Maximal coverage and identity of detected ORFs by antibiotics. The ORF covered proportion of the reference ARG sequence (x axis) and the identity % of predicted protein (color).

MGE domain coexisting analysis showed that PC1 beta-lactamase (blaZ) ARG might be mobile since the contig had a phage integrase ORF within the distance of 10 ORFs.

There were no perfect matches in sample B that is not surprising since its overall bacterial nucleic acid content was less than 10% of that of sample A. The sequence coverages of the strict matches in this sample ranged between 5.71% and 24.83%, with the mean of 13.31%. The identity between ORFs and CARD ARG reference sequences was 100.00% in each detected ARG. Contigs containing ARGs were classified and genera *Acinetobacter* (50%) and *Delftia* (50%) were identified. None of the identified ARGs could be related to any mobile genetic elements.

The detected ARGs in both samples were matched to their corresponding antibiotics. Since one antibiotic compound may be related to more than one ORFs, we decided to select those to which we could link the ORFs with the broadest coverage and the highest identity to the reference ARG sequence. The maximal coverage and identity of detected ORFs are shown in Fig. 2. In sample A ARGs known to be decreasing the effectiveness of acridine dye, cephalosporin, fluoroquinolone, penam and peptide antibiotics were found in full length and with 100% identity. There were two other ARGs identified in sample A in full length and with identity above 99% that encoded resistance against further antibiotics (cephamycin and tetracycline).

Discussion

Antimicrobial resistance (AMR) is a natural feature of microorganisms that have originally occurred as a means of defence in the rivalry amongst the members of the microbiotas. The ubiquity of antimicrobial resistance genes (ARGs) is beyond question. Genes against antibiotics are present both in non-pathogenic and pathogenic bacteria. With the extended agricultural and clinical use of antibiotics, the number of ARGs are on the rise, and the growing number and spread of multi-resistant bacteria strains pose a global threat to global health. According to the CDC's Antibiotic Resistance Threats in the United States, 2019 report³, more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result. In addition, 223,900 cases of *Clostridioides difficile* occurred in 2017 and at least 12,800 people died. Dedicated prevention and infection control efforts in the U.S. and around the world are working to reduce the number of infections and deaths caused by antibiotic-resistant germs. However, the number of people facing antibiotic resistance is still too high. The AR Threats Report warns that not only people but also animals carry bacteria in their guts which may include antibiotic-resistant bacteria either with intrinsic or with acquired ARGs¹². Beyond disease control, animals may be given antibiotics for growth promotion or increased feed efficiency. Since bacteria are exposed to low doses of the drugs over a long period, this inappropriate antibiotic use can lead to the development of resistant bacteria³. The CDC report notes that when animals are slaughtered and processed for food, resistant germs in the animal gut can contaminate meat or other animal products, but do not mention the possible contamination of milk.

Detected ARGs in raw milk (Table 1) can be transferred from non-pathogens to pathogens via HGT. The over-expression of such genes, e.g. *norA* (regulated by *mgrA*) and *mepA* (regulated by *mepR*) coding multidrug efflux pumps confer resistance to fluoroquinolones (including norfloxacin or ciprofloxacin) and even disinfectants^{18–21}. Ciprofloxacin is a broad-spectrum antibiotic used to treat a variety of bacterial infections, including intra-abdominal, respiratory tract, skin, urinary tract, and bone and joint infections. Norfloxacin might be used for uncomplicated urinary tract infections (including cystitis) or the prevention of spontaneous bacterial peritonitis in cirrhotic patients, among others. *MepA* was also shown to result in resistance to tigecycline²², an antibiotic

that was developed to tackle complicated infections caused by multiresistant bacteria such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *E. coli*.

The two samples differ both in the composition of core bacteriome and the ARG abundance. In sample A the bacteriome was dominated by Gram-positive bacteria. Furthermore, most of the contigs harbouring ARG were classified taxonomically belonging to Gram-positive bacteria. In sample B, the Gram-negative bacteria governed the bacteriome. So the lower ARG abundance in sample B might come from the lower proportion of Gram-positives. Nevertheless, in sample B, not just the number of detected ARGs was lower, but the maximal coverage of the ARGs as well. One may find the reason for this phenomenon, the lower sequencing depth of sample B. The identity of these ORFs with the reference ARGs are very high so we may assume the assembled ORFs originated from ARGs. Accordingly, the possible reason of the lower coverage of ARGs may be caused by the insufficient read counts for assembly the complete ORFs. One possible argumentation of the ARGs' difference between sample A and B may be derived from the fact that health issues (e.g. mastitis) are relatively more common in large scale farms. Since the use of antibiotics is more permissive in veterinary practice - compared to human medicine - in the treatment of bacterial infections, it places a selective pressure on the bacteria of herds, what might increase the frequency and the diversity of ARGs.

Our results show that indeed ARGs can be present in raw milk. However, it should be the subject of further research to identify how resistant bacterial DNA gets into the milk, is it already there in the cow's udder or does it only mixed into the milk as contamination during or after milking.

At raw milk's environment of origin (dairy farms), the use of antimicrobial agents is widespread. Consequently, the microbiome of this product may show relatively high levels of resistance. Without heat-treatment, bacteria that are present in raw milk are not hindered from further multiplication what results in the amplification of their resistance genes either. Such a rise in the number of ARGs may increase the risk of horizontal gene transfer (HGT) events. This risk may even be higher in case of mobile ARGs (e.g. blaZ, which was detected on a plasmid and near to a phage integrase ORF).

Beyond human intervention, there are natural mechanisms that limit ARG-transfer¹¹. First of all, donor and recipient populations need to be present at the same physical space²³ and reach a specific critical density to ensure proper connectivity for a successful gene transfer event. Chances for a series of HGT events amongst two physically distant populations are relatively low except for the case when there is positive selection driven by any factors (e.g. selection by antibiotics). The second factor arises from the fact that genes encoding resistance against the same compounds may limit each other's spread. A population owning genes against a particular antibiotic is not under selective pressure to gain any other ARGs with the same effect. As a conclusion of earlier evolutionary steps, possession of resistance determinants of the same substrate profiles are possible. However, in a population where the distribution of these genes is stable, the chances of new recruitments are lower. Tertiary, acquisition of resistance genes sets metabolic costs deriving from the transfer and integration mechanisms needed. These costs vary by each ARG, and only affordable genes are spread¹¹.

Even though the bacterial compositions of milk are affected by the heat treatment^{24,25}, the question may arise whether the ARG content of raw and pasteurized milk are different? In water, DNA degradation starts by 90 °C²⁶. The HTST pasteurization (high temperature/short time) is performed at 72 °C for 15–40 seconds, while ultra-pasteurization (UHT) is at 135 °C for 1–2 seconds. Summarizing this information, one may conclude that the resistomes do not differ significantly in HTST and raw milk. On the other hand in UHT milk some DNA degradation might be suspected. Nevertheless, some aspects are broadening the picture, that are worth taking into consideration. First of all, in raw milk, the members of the bacteriota remain viable and may multiply depending on the storing temperature. The proliferation of bacterial cells increases the amount of the sample's extractable bacterial DNA content what appears in the results of the sequencing as raised bacterial read rates. Consequently, after the assembly of the reads, the likelihood of having contigs containing ARGs is higher. Pasteurization kills 99.99% of bacteria; thus, their multiplication has a low significance. Secondly, the bacteriome of milk consumers (humans and animals) may gain the ARGs of the milk-resistome by transformation and transduction only¹³, as pasteurization decreases the number of viable bacteria. In contrast, raw milk's higher viable bacterial cell count facilitates conjugation to the consumers' bacteriome while the above-mentioned horizontal gene transfer mechanisms¹³ are also kept. Of course, this phenomenon rather has an impact on the risk of HGT than on the resistome of raw or pasteurized milk.

Nevertheless, heat-treatment of raw milk seems to be an advantageous and a more than considerable step that besides inhibiting the amplification of genes having a potential risk, makes active gene transfer mechanisms lose their significance. On the other hand, even though it reduces the number of multiplication cycles, after the lysis of cells free DNA fragments appear in the sample that may still be uptaken by newly arriving bacteria.

However, the interpretation of resistome studies is yet to be deepened. The combination of next-generation sequencing, metagenomic and computational methods provides valuable data on the presence of ARGs. Moreover, it makes it possible to find genes in full coverage and length, and to identify their taxonomical classes of origin and their exact sequential surroundings. Synteny with mobile genetic elements is a fact to be taken into consideration when examining the risks meant by an ARG. Thus, the combination of methods mentioned above serves as a core component of today's necessarily expanded antimicrobial resistance research.

As a means of evolutionary pressure, the use of antibiotics selects bacterial strains that have antimicrobial resistance genes. Moreover, in the production animal sector, the application of such compounds increases not only the number of antibiotic-resistant bacterial strains but also the frequency of their appearance. After the consumption of animal products, these strains may meet the human microbiota, and the circumstances may be appropriate for the horizontal gene transfer derived spread of antimicrobial resistance genes amongst these populations. This phenomenon unfolds a possible source of acquisition of human pathogens' antimicrobial resistance other than the direct presence of antibiotic residuals in animal products.

Our findings suggest the antimicrobial resistance gene content of unprocessed animal products may play a role in the development of antimicrobial resistance of human pathogens. Nevertheless, the generalization of these findings requires more comprehensive studies to transcend our results that are based on a limited sample size.

Methods

Milk samples. Two times one litre of raw milk was purchased at a public market in the city of Budapest (sample A) and in the city of Szeged (sample B). Sample A and B originated from a large (with more than 250 dairy cattle) and a small scale (below 50 dairy cattle) dairy farm, respectively.

DNA extraction and metagenomics library preparation. Before the laboratory procedures, the milk samples were stored frozen. 120 mL of raw milk was centrifuged at 10,000 g for 10 min. Total DNA was extracted from the pellet using the ZR Fecal DNA Kit from Zymo Research. Paired-end fragment reads (2×150 nucleotides) were generated using the TG NextSeq 500/550 Mid Output Kits v2 sequencing kit with an Illumina NextSeq sequencer. Primary data analysis (base-calling) was carried out with bcl2fastq software (v.2.17.1.14, Illumina).

Bioinformatic analysis. Quality based filtering and trimming was performed by Adapterremoval²⁷, using 15 as a quality threshold. Only reads longer than 50 bp were retained. *Bos taurus* genome (ARS-UCD1.2) sequences as host contaminants were filtered out by Bowtie2²⁸ with *very-sensitive-local* setting minimizing the false positive match level²⁹. The remaining reads were taxonomically classified using Kraken2 ($k = 35$)³⁰ with the NCBI non-redundant nucleotide database³¹. The taxon classification data was managed in R³² using functions of package phyloseq³³ and microbiome³⁴. For further analysis, the reads assigned to Bacteria was used only³⁵. Core bacteria was defined as the relative abundance of agglomerated counts at class level above 0.1% at least one of the samples. By metaSPAdes³⁶ the preprocessed reads were assembled to contigs, with the automatically estimated maximal $k = 55$. From these contigs having a shorter length than the shortest resistance gene of the Comprehensive Antibiotic Resistance Database (CARD) were discarded^{37,38}. The ARG content of filtered contigs was analyzed with Resistance Gene Identifier (RGI) v5.1.0 and CARD v.3.0.6^{37,38}. Contigs harbouring ARG identified by RGI with perfect or strict cut-off were preserved and classified by Kraken2 on the same way as was described above. The plasmid origin probability of the contigs was estimated by PlasFlow v.1.1³⁹. To identify possible further mobile genetic element (MGE) homologs the predicted protein sequences of contigs were scanned by HMMER⁴⁰ against data of PFAM v32⁴¹ and TnpPred⁴². Following Saenz *et al.*³⁵ from the hits with lower than $E 10^{-5}$ the best was assigned to each predicted protein within the distance of 10 ORFs. The MGE domains coexisting with ARGs were categorized as phage integrase, resolvase, transposase or transposon.

Data availability

All data are publicly available and can be accessed through the PRJNA591315 from the NCBI Sequence Read Archive (SRA).

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Author contributions

N.S. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. L.M., I.C. and N.S. conceived of the study concept. G.M., L.M. and N.S. performed the sample collection and procedures. A.G.T., D.T., E.K. and N.S. participated in the bioinformatic and statistical analysis. A.G.T., A.V.P., L.M. and N.S. participated in the drafting of the manuscript. A.G.T., D.T., G.S., I.C. and N.S. carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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A glimpse of antimicrobial resistance gene diversity in kefir and yoghurt

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Antimicrobial resistance (AMR) is a global threat gaining more and more practical significance every year. The main determinants of AMR are the antimicrobial resistance genes (ARGs). Since bacteria can share genetic components via horizontal gene transfer, even non-pathogenic bacteria may provide ARG to any pathogens which they become physically close to (e.g. in the human gut). In addition, fermented food naturally contains bacteria in high amounts. In this study, we examined the diversity of ARG content in various kefir and yoghurt samples (products, grains, bacterial strains) using a unified metagenomic approach. We found numerous ARGs of commonly used fermenting bacteria. Even with the strictest filter restrictions, we identified ARGs undermining the efficacy of aminocoumarins, aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, elfamycins, fluoroquinolones, fosfomycins, glycyclines, lincosamides, macrolides, monobactams, nitrofurans, nitroimidazoles, penams, penems, peptides, phenicols, rifamycins, tetracyclines and triclosan. In the case of gene *ImrD*, we detected genetic environment providing mobility of this ARG. Our findings support the theory that during the fermentation process, the ARG content of foods can grow due to bacterial multiplication. The results presented suggest that the starting culture strains of fermented foods should be monitored and selected in order to decrease the intake of ARGs via foods.

Antimicrobial resistance (AMR) is a global threat gaining more and more practical significance every year. Although antimicrobial resistance genes (ARGs) have been present ever since the appearance of the first living microorganisms¹, the threat arising from AMR has stemmed from the daily use of antibiotics in human and animal healthcare. Even though antibiotic compounds are not directly responsible for the genetic changes behind the appearance of antimicrobial resistance, they place selective pressure towards the amplification of individual bacterial ARGs. Identifying the sources of intake of bacteria carrying ARGs is nowadays a biomedical priority. Bacteria appear in the newborn body right from birth², and later on, their invasion continues from the environment, from other humans and animals, or raw³ or processed⁴ food⁵. Bacteria reaching our gut through alimentary intake may share functional ARGs either with saprophytes or with pathogens in their physical proximity due to horizontal gene transfer (HGT). In the latter case, resistant or multi-resistant pathogenic strains may evolve, and therapeutic options for the treatment of bacterial infections may narrow. By the production and consumption of livestock products, the opportunity to establish transfer connections between bacteria is provided, as bacterial populations with different sets of ARGs may meet. Therefore, popular probiotic products (such as yoghurt and kefir), have the potential to allow encounters between their bacterial strains and those in the consumer. Yoghurt and kefir are probiotic foods with minor differences in their processing steps. Yoghurt is fermented with bacteria,

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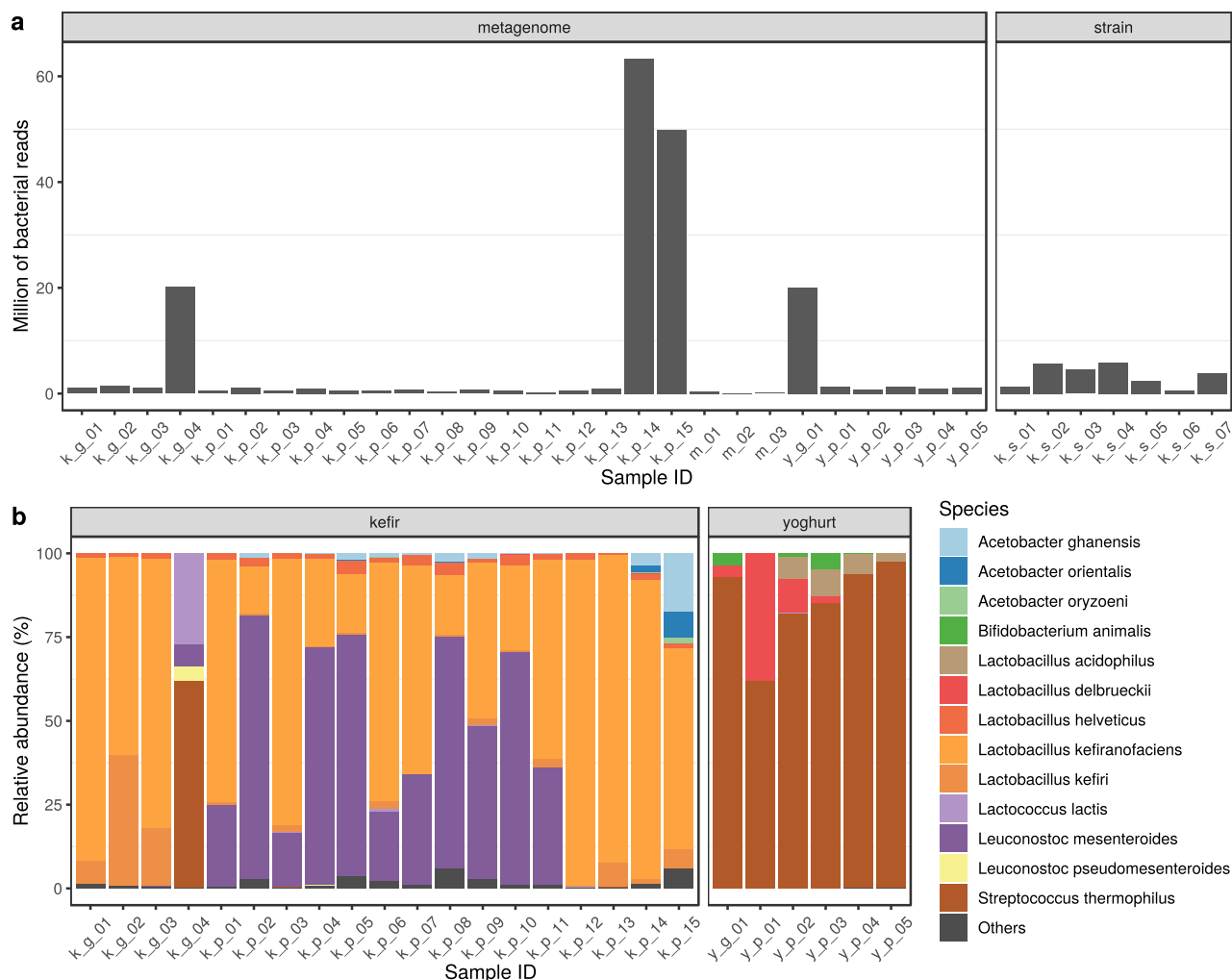


Figure 1. Bacterial content of the samples. **(a)** The number of reads classified bacterial by Kraken2 on the NCBI NT database. Metagenome includes the samples deriving from grains, milk or products. **(b)** Relative abundances of the most common bacterial species in the grain and product samples.

whereas the production of kefir requires fungi in addition. They have both been present in the human diet for a long time and still stand their ground in today's demanding, health-conscious society. Nevertheless, besides the health benefits, consumption of probiotic food may have an adverse effect. Along with the multiplication of bacteria during the fermentation process, the bacterial resistome also grows. If the intake of probiotic products occurs alongside the right triggers, a higher possibility of HGT is provided in the human gut. Our study examined the diversity of the ARG content of kefir and yoghurt products, their grains and bacterial strains using a unified metagenomic approach.

Results

The results were organised as follows. After describing the bacteriome of the samples, their resistome and mobilome are shown. This is followed by the summary of abundance changes during fermentation of some ARGs and the related bacteria carrying those ARGs. Finally, we present the non-nudged ARG findings.

Bacteriome. When classified by taxon, the number of reads aligning to bacterial genomes differed in the various samples (Fig. 1a). Two samples (k_g_04 and y_g_01 from bioproject PRJNA644779) contained ~20 million reads of bacterial origin. From bioproject PRJNA388572, sample k_p_15 had ~50 million bacterial reads, while k_p_14 contained more than 63 million. Excluding these four extremities, the average bacterial read count of the metagenomic samples was 6.7×10^5 (ranging between 7.3×10^4 and 1.4×10^6). The median sequencing depth of the strain k_s_01, k_s_02, k_s_03, k_s_04, k_s_05, k_s_06, k_s_07 were 46, 119, 115, 111, 6, 54, 108, respectively.

Figure 1b. demonstrates the relative abundances of the dominant bacterial species identified in the samples. 99% of all bacteria identified were related to these species. In kefir grains the dominant species were a *Lactobacillus kefirifaciens* ($57.7 \pm 40.5\%$), *Lactobacillus kefir* ($15.7 \pm 17\%$), *Streptococcus thermophilus* ($15.4 \pm 30.8\%$), *Lactococcus lactis* ($6.8 \pm 13.5\%$), *Leuconostoc mesenteroides* ($1.7 \pm 3.4\%$), *Leuconostoc*

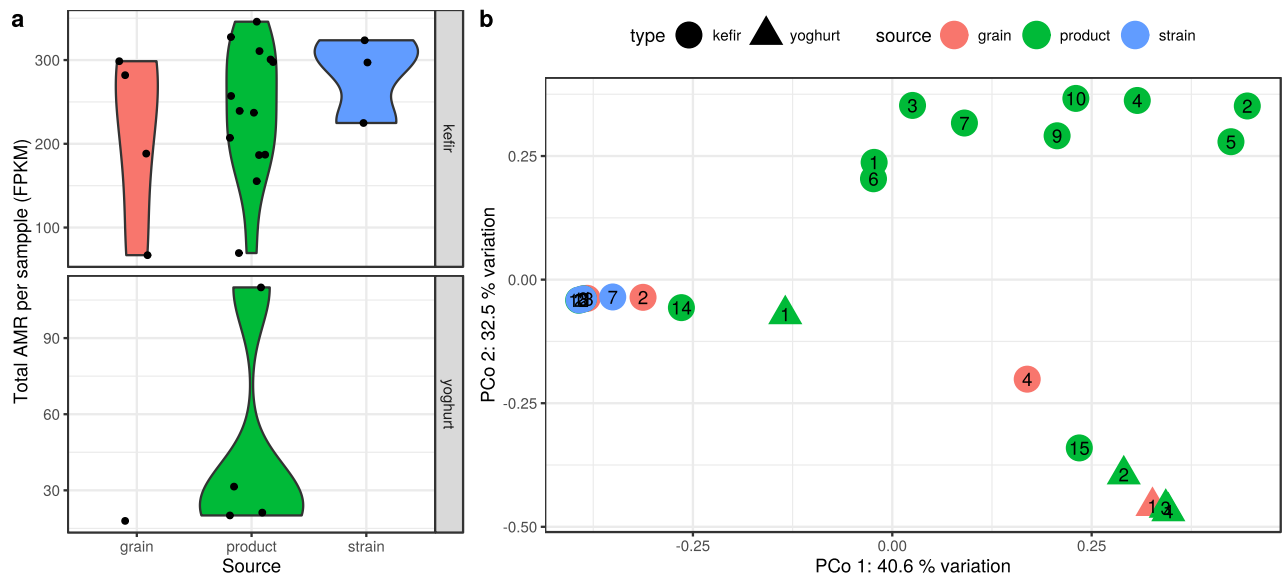


Figure 2. Antimicrobial resistance (AMR) abundance of the samples. **(a)** Violin plot representing the distribution of the total AMR fragments per kilobase per million fragments (FPKM) per sample, grouped by type and source. The horizontally jittered dots represent the FPKM of the samples. **(b)** The AMR abundance diversity (β -diversity) of the samples. It is plotted on the first two axes of principal coordinate analysis (PCoA) performed on Bray-Curtis distance which was calculated using the relative abundances of contigs harbouring ARGs. The symbols show the type, the colours the source, while the numbers correspond to the sequence number in the Sample ID. Some samples (k_s_01, k_s_04, k_s_05, k_s_06, k_p_08) are not shown as their ORFs did not meet filtering criteria.

pseudomesenteroides ($1\% \pm 2\%$) and *Lactobacillus helveticus* ($1\% \pm 0.7\%$) in descending order of abundance. The most significant species in the products overlapped with those in the kefir grains, although they had differences in their relative abundance (*L. kefirifaciens* ($55.4\% \pm 29\%$), *L. mesenteroides* ($35.7\% \pm 30\%$), *Acetobacter ghanensis* ($2.1\% \pm 4.4\%$), *L. helveticus* ($2.1\% \pm 1\%$), *L. kefir* ($1.8\% \pm 2\%$), *Acetobacter orientalis* ($0.6\% \pm 2\%$), *Acetobacter oryzoeni* ($0.2\% \pm 0.5\%$)). The one yoghurt grain examined was dominated by *Streptococcus thermophilus* (92.8%), *Bifidobacterium animalis* (3.6%) and *Lactobacillus delbrueckii* (3.5%) while the core bacteriome of the yoghurt product consisted of *S. thermophilus* ($83.9\% \pm 13.8\%$), *L. delbrueckii* ($10.1\% \pm 16.2\%$), *Lactobacillus acidophilus* ($4.6\% \pm 3.3\%$) and *B. animalis* ($1.2\% \pm 2.1\%$).

Resistome. According to our findings based on perfect and strict matches, AMR gene abundances show a great diversity in various types and sources of samples (Fig. 2a). Samples (k_s_01, k_s_04, k_s_05, k_s_06, k_p_08) that did not meet the ORF filtering criteria were not plotted on Figs. 2 and 3. The highest ARG abundance was observed in the kefir strain samples (average: 282 fragments per kilobase per million fragments (FPKM), sd: 51.1) followed by the kefir product (240 ± 78.6) and the kefir grains (209 ± 106). The yoghurt samples had lower abundances, in the only one grain, FPKM was 17.9, while in the products we found 45.7 ± 32.2 .

A Bray-Curtis distance-based principal coordinate analysis (PCoA) was performed to gain insight into the dissimilarity of the sample ARG abundances (Fig. 2b). With a permutational multivariate analysis of variance on the same distance matrix, we found that the type of the sample explains the 22.17% ($p < 0.001$) of dissimilarity among the sample resistomes. For the source grouping, the same measure was 18.92% ($p < 0.001$). Based on Fig. 2b one might conclude that the strongest effect on the dissimilarity is the bioproject of origin, as the analysis showed that it explains 35.56% ($p < 0.001$) of the dissimilarity variances.

In our kefir samples, we identified 22 ARGs in the product, 2 in the grain and 1 in the strain. In yoghurt, there was 1 ARG in the product and 2 in the grain (Fig. 3a). The relative abundances of antibiotic classes affected are shown in Fig. 3b for each sample. The detected ARGs and their most probable bacteria of origin are summarized on Fig. 4. The kefir ARGs identified in the product may help bacteria in the defence against aminocoumarins, aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, elfamycins, fluoroquinolones, fosfomycins, glycolcyclines, lincosamides, macrolides, monobactams, nitrofurans, nitroimidazoles, oxazolidinones, penams, penems, peptides, phenicols, pleuromutilins, rifamycins, streptogramins, tetracyclines and triclosan. Contigs containing these ARGs belonged to the genomes of *Enterobacter hormaechei* (genes: *acrB*; *acrD*; *ACT-36*; *bacA*; *baeR*; *CRP*; *emrB*; *emrR*; *Escherichia coli marR* mutant conferring antibiotic resistance; *E. coli soxS* with mutation conferring antibiotic resistance; *E. coli UhpT* with mutation conferring resistance to fosfomycin; *FosA2*; *marA*; *mdtB*; *mdtC*; *msbA*; *oqxA*; *oqxB*; *ramA*), *L. helveticus* (gene *poxA*), *L. kefirifaciens* (gene *poxA*) and *L. mesenteroides* (gene *APH(3')-IIb*). ARGs originating from the kefir grain may play a role in the appearance of resistance against lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins and tetracyclines and were found in the genomes of *L. kefirifaciens* (gene *poxA*) and *L. lactis* (gene *lmrD*). Gene *poxA* deriving from kefir strains (*L. kefirifaciens* and *L. plantarum*) confers resistance against

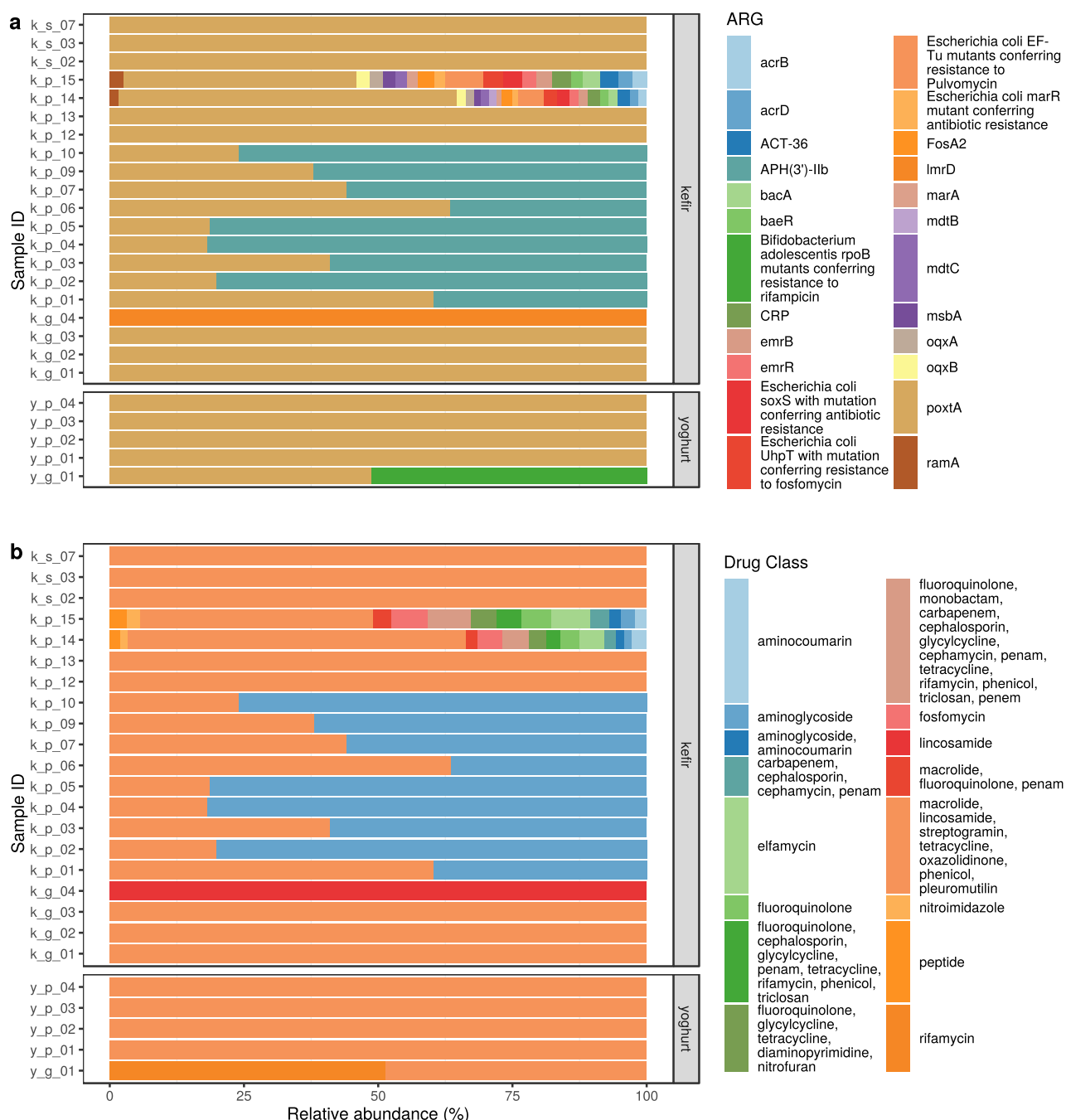


Figure 3. Antimicrobial resistance (AMR) abundance in kefir and yoghurt samples. **(a)** Relative abundance of ARGs. ORFs having at least 60% length and 90% base sequence identity with the reference ARG sequence are shown. Some samples (k_s_01, k_s_04, k_s_05, k_s_06, k_p_08) are not shown as their ORFs did not meet filtering criteria. **(b)** Relative abundance of drug classes related to the ARGs identified in the samples.

lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins and tetracyclines. Genes found in yoghurt grains encoded resistance against lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, rifamycins, streptogramins and tetracyclines, while the ARGs of the product itself may weaken the efficacy of lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins and tetracyclines. Contigs involving ARGs of the yoghurt product could have been connected to *L. acidophilus* (gene *pox tA*) and *L. delbrueckii* (gene *pox tA*). However, the ARGs of the grains aligned to the reference sequence of *B. animalis* (gene *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin) and *L. delbrueckii* (gene *pox tA*).

Based on the ARG abundances the proportion of resistance mechanisms was calculated for each sample. In the kefir product samples the dominant mechanism of identified ARGs was the antibiotic target protection (50.73%), followed by antibiotic inactivation (45.45%), antibiotic efflux (2.03%), antibiotic target alteration (1.07%), antibiotic efflux; reduced permeability to antibiotic (0.32%), antibiotic target alteration; antibiotic efflux; reduced

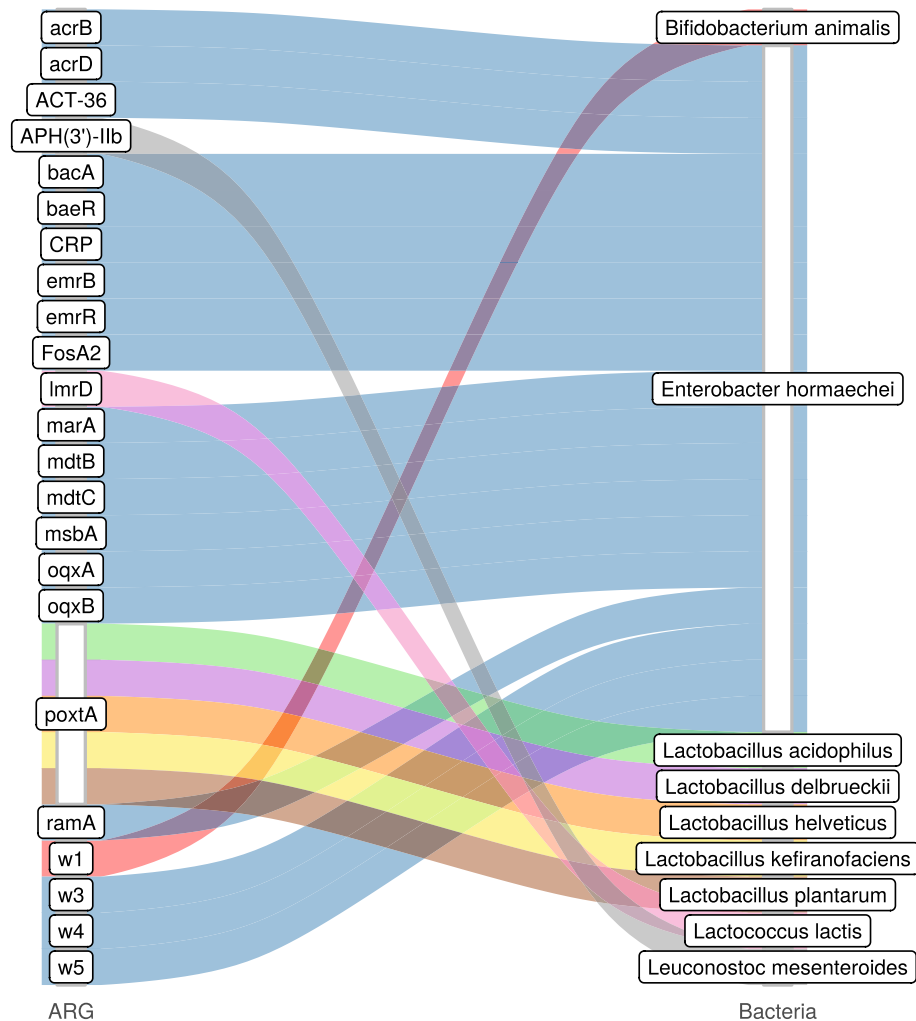


Figure 4. Identified ARGs and their most probable bacteria of origin. The gene names that are too long have been abbreviated (w1: *Bifidobacterium adolescentis* *rpoB* mutants conferring resistance to rifampicin; w3: *Escherichia coli* *marR* mutant conferring antibiotic resistance; w4: *E. coli* *soxS* with mutation conferring antibiotic resistance; w5: *E. coli* *UhpT* with mutation conferring resistance to fosfomycin).

permeability to antibiotic (0.27%), antibiotic target alteration and antibiotic efflux (0.13%). In the kefir grain samples, the main mechanisms were antibiotic target protection (91.98%) and antibiotic efflux (8.02%). In the kefir strains and yoghurt products, the antibiotic target protection was the only mechanism detected. In the one yoghurt grain sample, antibiotic target alteration; antibiotic target replacement (51.28%) and antibiotic target protection (48.72%) are the possible resistance mechanisms.

Mobile elements. The results of mobile genetic element (MGE) domain coexisting analysis showed that the ARG *lmrD* in sample k_g_04 might be mobile since the contig containing the gene had a transposase ORF within the distance of 10 ORFs. According to the analysis executed with PlasFlow⁶ there were not any identifiable contigs with plasmid origins harbouring ARGs.

ARG abundance changes during kefir fermentation. According to the metagenomic analysis published by Walsh et al.⁷, ARG abundances change during the fermentation process (Fig. 5a). In the case of all three grains (Fr1, Ick, UK3) *APH(3')-Iib* is present in the kefir fermented for 24 hours, while it is absent in all other time phases except for sample UK3 after 8 hours. *PoxA* was detectable in all samples except the 8 hour Fr1 kefir sample. The abundance fold change of 24 hours with respect to grain samples was 0.10, 0.59 and 0.26 for the starter culture Fr1, Ick and Uk3, respectively. Between the hour 8 and 24 samples, *poxA* abundance showed a 0.34-fold change in the Ick kefir, while in the case of the UK3 kefir sequence this value reached 0.62.

Contigs harbouring ARGs were classified taxonomically (Fig. 5c). All contigs containing the gene *APH(3')-Iib* were assigned to *L. mesenteroides*. Contigs with *poxA* were assigned to the reference genome of *L. helveticus* and *L. kefirifaciens*.

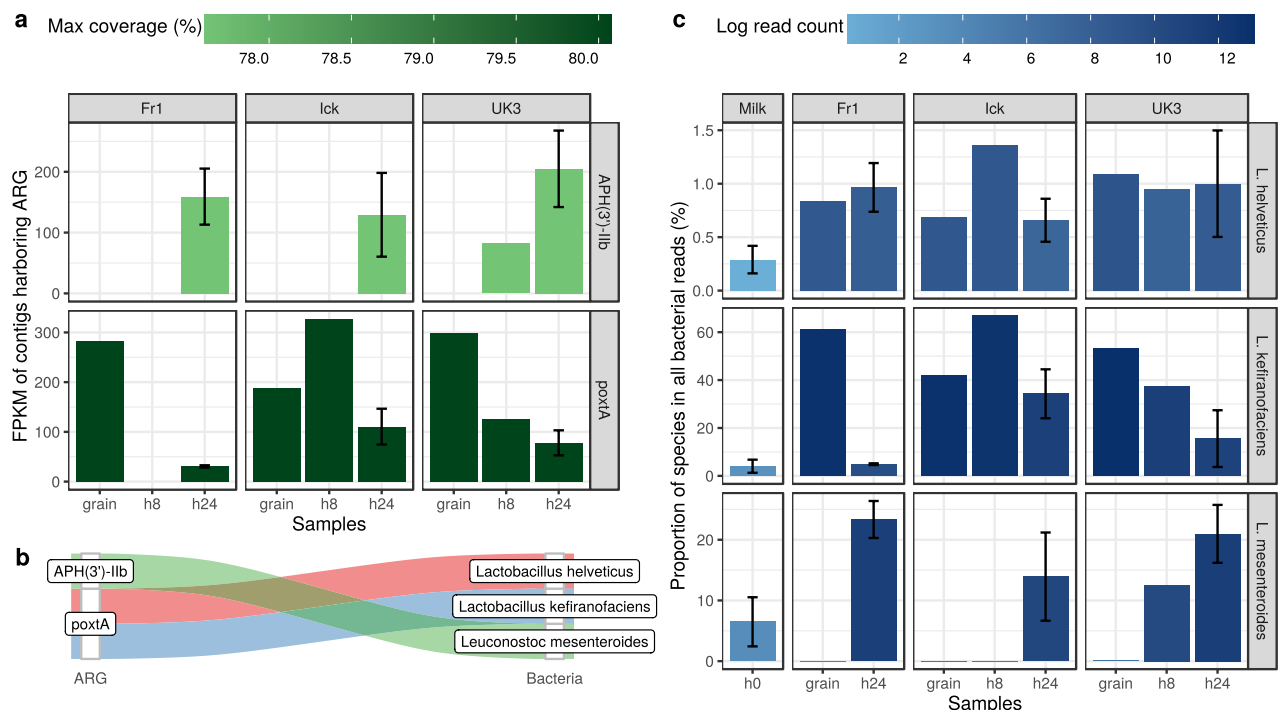


Figure 5. Changes during kefir fermentation. **(a)** Antimicrobial resistance gene (ARG) abundance expressed as fragments per kilobase per million fragments (FPKM) based on the alignment of bacterial reads to the ARG harbouring contigs. **(b)** ARGs and their most likely origins. **(c)** Relative abundances of bacteria with a probable ARG content.

All bacterial reads were then aligned to the reference genomes of bacteria mentioned above, and the hits are expressed proportionally (Fig. 5b). In contrast to *L. kefiranofaciens* that showed a decreasing tendency, an increase in time is observable by the relative abundances of *L. mesenteroides*. The proportion of reads assigned to *L. helveticus* shows no tendential increase or decrease in time. The increase of abundance of *APH(3)-IIB* shows a positive association with the relative abundance of *L. mesenteroides*. Similarly, *poxA* abundance is decreasing with the relative abundance of *L. kefiranofaciens*.

Excluding nudged hits. In order to set the alignment restrictions of ORFs to reference ARGs even stricter, we selected for a subgroup of reference ARGs that fit the ORFs from the starting base position on. Thus, nudging on the reference sequence by the alignment was avoided. With such a shrinkage, we reduced the number of detectable ARGs to four samples (Fig. 6). Sample k_g_04 from bioproject PRJNA644779 contained an ARG against lincosamides while the gene found in sample y_g_01 is responsible for resistance against rifamycin. Contigs harbouring these ARGs had the best alignment to *L. lactis* and *B. animalis*, respectively. Bioproject PRJNA388572 had two samples with similar matches, except for gene *mdtB* which appeared in full length in sample k_p_14 and was absent in k_p_15, this gene is responsible for aminocoumarin resistance. As some other ARGs of the sample also have the potential to confer resistance against aminocoumarin, the AMR profiles of the samples appeared to be the same, including aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, elfamycin, fluoroquinolone, fosfomycin, glycylicycline, macrolide, monobactam, nitrofurantoin, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline and triclosan resistance. Comparing this list to the nudged results oxazolidinone, pleuromutilin and streptogramin resistance genes were absent. Contigs containing ARGs had the best alignment to the genome of *E. hormaechei* in both cases⁸.

All four samples of both bioprojects included at least 20 million bacterial reads in the assembly of the contigs. The other samples consisted of significantly fewer reads. Consequently, as Sims et al.⁹ found, it is not possible to distinguish whether the absence of protein-coding genes or the disruption of open reading frames (ORFs) represent a deficiency of the assembly or real evolutionary gene loss. Examining the relationship between the number of bacterial reads and the length of identified ARGs (including nudges) with a linear model we found that after each extra 100,000 reads the coverage of reference gene raises by 7% by the ARG coding ORFs ($p < 0.0001$). In samples k_g_04, k_p_14, k_p_15 és a y_g_01, we randomly chose the average read number of the other samples (677,340) to reanalyze how much these results differ from the original ones executed on the full database. Contigs assembled contained one gene that was identified previously (excluding nudges), namely *lmrD* from sample k_g_04. ORFs predicted to be ARGs had a median coverage of 16.10% on the reference ARG sequences. In contrast, ORFs aligning to ARGs composed of the full read content of the four samples had a median coverage of 99.21%.

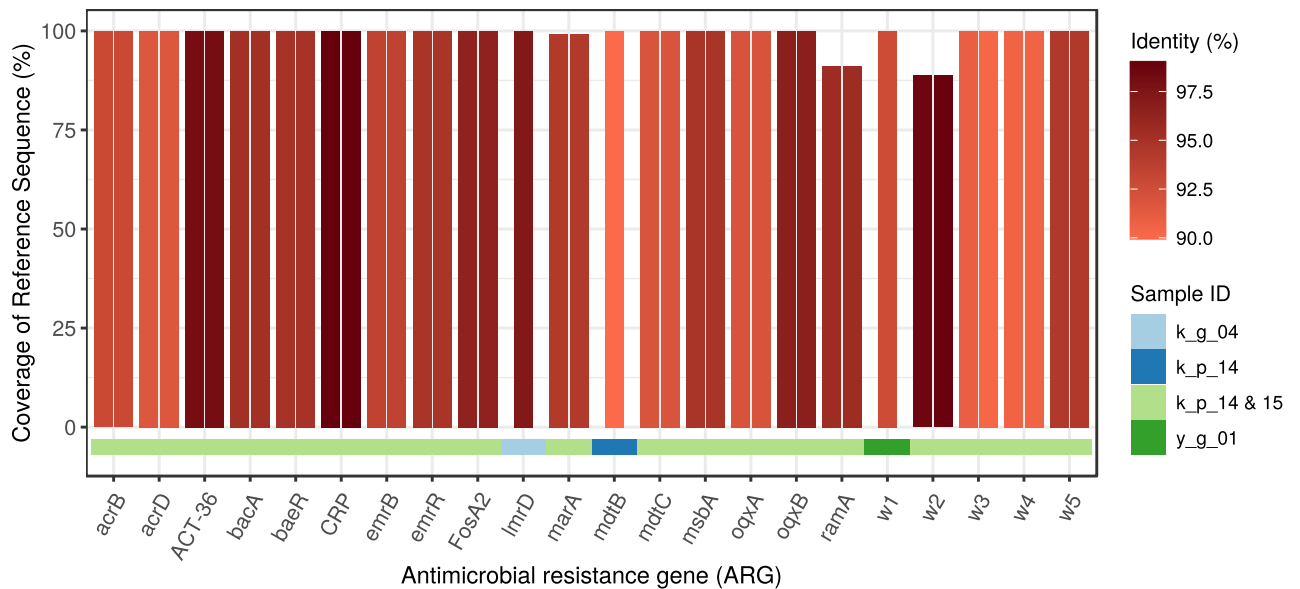


Figure 6. Identified ARGs excluding nudged findings. The coverage and identity of detected open reading frames (ORFs) by antimicrobial resistance genes (ARGs). The ORF covered proportion of the reference ARG sequence and the identity % of predicted protein (colour). The gene names which are too long have been abbreviated (w1: *Bifidobacterium adolescentis* *rpoB* mutants conferring resistance to rifampicin; w2: *Escherichia coli* EF-Tu mutants conferring resistance to Pulvomycin; w3: *E. coli* *marR* mutant conferring antibiotic resistance; w4: *E. coli* *soxS* with mutation conferring antibiotic resistance; w5: *E. coli* *UhpT* with mutation conferring resistance to fosfomycin).

Discussion

Studying ARGs that may enter the body with food, including fermented dairy products, can lead to critical health considerations. Characteristics of bacterial diversity and ARG abundance are well observable both in kefir and in yoghurt. ARG abundance is much higher in kefir than in yoghurt. One possible reason for this phenomenon could be the presence of fungi in kefir seed cultures. Since fungi may produce antibacterial toxins, bacteria having ARGs may gain a competitive advantage when coexisting with fungi.

Each bacterium (*Bifidobacterium animalis*¹⁰, *Enterobacter hormaechei*^{8,11}, *Lactobacillus acidophilus*^{11,12}, *Lactobacillus delbrueckii*^{11,12}, *Lactobacillus helveticus*^{11,12}, *Lactobacillus kefirifaciens*^{11–13}, *Lactobacillus plantarum*^{11,12,14}, *Lactococcus lactis*^{11,12,15,16}, *Leuconostoc mesenteroides*^{11,12,15,16}) obtained from the taxon classification of contigs containing ARGs is widely used in the production of fermented dairy products. Li et al.¹⁷ analyzed the ARG content of isolated bacteria from fermented drinks and yoghurts. According to their results, *APH(3'')-III*, *APH(6'')-APH(2'')*, *sul1*, *tet(M)* were detectable in *Lactobacillus bulgaricus* strains, while *APH(3'')-II*, *sul1*, *sul2*, *strA*, *strB*, *tet(M)* derived from *Streptococcus thermophilus*. In our study *APH(3'')-IIB* gene belonging to the *APH* gene family, supposedly originated from *L. mesenteroides*. Similarly, Carr et al.¹⁸ found a strong co-occurrence between *APH(3'')-Ia* and *L. mesenteroides* in Chinese saliva samples. Further similarity with the results of Carr et al.¹⁸ is that *lmrD* originated from *L. lactis*. Guo et al.¹⁹ detected ARGs in *Lactobacillus* strains of fermented milk products. They detected gene *erm(B)*, *gyrA*, *rpoB*, *vanE*, *vanX* in *Lactobacillus casei*, gene *APH(3'')-III*, *dfrD*, *erm(B)*, *gyrA*, *tet(W)*, *vanX* in *L. helveticus*, gene *erm(B)* and *vanX* in *L. plantarum*. We found the *poxA* gene associated with *L. helveticus* and *L. plantarum*. The *emrB* gene was identified in a contig from the genome of *E. hormaechei*.

During the fermentation of milk, the bacteria in seed cultures (and in milk) multiply and dominate the beverage. If any of these bacteria harbour ARGs, the amount of these genes will be increased in the final products. Based on data generated by Walsh et al.⁷ *L. helveticus* and *L. kefirifaciens* are the most probable origin of the contigs harbouring gene *poxA*. Sequences containing gene *APH(3'')-IIB* could have been stemmed from *L. mesenteroides*. According to Walsh et al.⁷, during fermentation the relative abundance of *L. kefirifaciens* and *L. mesenteroides* increased. Not surprisingly, in our reanalysis of the same data we found the same trends (Fig. 5b). While Marsh et al.²⁰ presented same changes of these species in kefir, Wurihan et al.²¹ showed opponent alterations in koumiss fermentation. ARG abundances (Fig. 5a) showed a positive association with the relative abundances of their most probable bacteria of origin (Fig. 5b). An increase in the relative abundance of *L. mesenteroides* was followed by the *APH(3'')-IIB* abundance. In contrast, *poxA* abundance dropped simultaneously with the decrease of the relative abundance of *L. kefirifaciens*.

The two most abundant ARGs were *poxA* and *APH(3'')-IIB*, which were both presents in yoghurt and kefir samples. *PoxA* (phenicol-oxazolidine-tetracycline resistance gene), an abundant ARG in Gram-positive bacteria, confers resistance to a wide range of critical antibiotics. The ABC-F class ATP binding ribosomal protection protein encoded by this gene is mainly present in Enterococci and Staphylococci. It was also identified in a methicillin-resistant *Staphylococcus aureus* (MRSA) strain that showed increased MIC to linezolid, a member of the oxazolidine class of antibiotics²². The study highlighted that Staphylococci, Enterococci and interestingly,

Pediococcus acidilactici harbouring the gene are all of animal origin and can be spread horizontally with the help of mobile genetic elements. In line with other paper²³ the study suggests that phenicols and other antiribosomal agents used in veterinary medicine might have played a role in the selection of *poxA*. This was also confirmed by Elghaieb et al.²⁴, who identified the gene in cow milk and animal wastewater. As oxazolidinones are prohibited in food animals, and phenicols are not permitted in dairy cattle in Europe, the source of these genes in Hungarian samples remains to be elucidated. *Pseudomonas aeruginosa* harbours an array of aminoglycoside-modifying genes, altering the drug by acetylation, adenylation or phosphorylation (APH). The presence of *APH(3')-IIB* in kefir samples is deliberately worrying as aminoglycoside 3'-phosphotransferases can mediate high-level resistance against several aminoglycosides. These genes might be plasmid-borne or chromosomally encoded; *APH(3')-IIB* is the latter, but a transposon-mediated mechanism has been suggested to be responsible for spreading the resistance genes^{25,26}. As the gene was almost exclusively described in *P. aeruginosa* previously, and the likely origin was *L. mesenteroides* in our study, the routes of resistance gene transfer related to this gene need to be further investigated. Although penicillins and cephalosporins are the most frequently used antibiotics for dairy cows, interestingly, the abundance of ARGs facilitating resistance against β -lactams is rather lacking. This phenomenon, together with the ARGs related to unused antibiotics in veterinary dairy medicine, raises the suspicion that the source of the abundant ARGs might not be a direct consequence of antibiotic use at dairy farms.

Bacteria entering the digestive tract with food, are provided with the opportunity of contacting other non-pathogenic and pathogenic bacteria. At the same time, one of the main prerequisites of HGT processes is the physical proximity of the participating bacteria. By virtue of the fulfilment of this requirement, various genes, including ARGs, can be exchanged by bacteria during horizontal gene transfer processes. If an ARG harbours on a mobile DNA-sequence, the probability of its HGT is higher. We found only one gene, namely *lmrD* in sample k_g_04, that is supposedly mobile. This deduction is based on the genomic environment of *lmrD*.

Antibiotic resistance caused by multidrug-resistant bacteria is a significant global public health threat²⁷. Infections with drug-resistant bacteria may result in major morbidity and mortality and increase the cost of health care when compared to infections by non-resistant strains of the same species. Even with the strictest filtering restrictions, we identified ARGs undermining the efficacy of aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, elfamycins, fluoroquinolones, fosfomycins, glycolcyclines, lincosamides, macrolides, monobactams, nitrofurans, nitroimidazoles, phenicols, rifamycins and triclosans. These findings raise several clinical considerations. For instance, carbapenems are broad-spectrum antibiotics used for the treatment of necrotizing pancreatitis²⁸ and severe intraabdominal infections. Tigecycline, a recently developed third-generation tetracycline antibiotic belonging to the glycolcycline class, is one of the few therapeutic options for carbapenem-resistant bacteria, like *Klebsiella pneumoniae*^{29,30} and carbapenem-resistant Enterobacteriaceae (CRE)³¹. Another group of ARGs identified in our study code resistance against fluoroquinolones. Emerging fluoroquinolone resistance in *Campylobacter* strains which are the leading cause of bacterial gastroenteritis in the world is a significant public health concern similarly to the rising incidence of fluoroquinolone-resistant cases of typhoid fever and invasive non-typhoidal *Salmonella* (iNTS) infections. We have also identified genes coding cephalosporin resistance in our samples. Cephalosporins belong to the most frequently used antibiotics globally. Intravenous third generation cephalosporins (e.g. ceftriaxone) are more potent against Gram-negative bacteria. They are frequently used in cholecystitis, spontaneous bacterial peritonitis or as a preventive measure in acute gastrointestinal haemorrhage³². ORFs harbouring ARGs that code resistance against macrolides may also raise serious public health concerns. Macrolide antibiotics absorb excellently from the gastrointestinal tract and have few side effects. Clarithromycin is still considered as a member of the first-line treatment protocol for *Helicobacter pylori* eradication in areas with a low resistance to clarithromycin³³. Azithromycin can contribute to the resolution of acute infections by immunomodulatory effects³⁴. It is frequently used for the treatment of acute watery or febrile diarrhoea and dysentery syndrome³⁵. Tetracycline resistance genes that we found predestine a potential loss in the efficacy of various tetracycline compounds. Once commonly used, nowadays rarely administered tetracycline has been recently rediscovered, as a component of *H. pylori* eradication regimen, partly due to increasing rate of resistance to other antibiotics (including the above-mentioned clarithromycin)³³.

As ARGs reaching the human body may originate from fermented dairy products, further examinations would be worthwhile to clarify the details and understand the practical medical significance. For this, it would be appropriate to analyze the samples of starter cultures and final products and register the results at set time points during the fermentation period. According to our findings, sequencing depth plays a significant role in the coverage of ORFs identified as ARGs, thus involving at least 20 million clusters is recommended by similar studies. The samples we examined and the studies we found in the literature^{3-5,17,19} confirm the hypothesis that foods of animal origin may contain significant amounts of diverse ARGs. The reason for the appearance of ARGs is complex, and the routes of appearance and spread are difficult to track. As sequencing techniques become cheaper, regular genetic monitoring of products of animal origin, including starter cultures, should be considered in addition to the strict control of antibiotics used in animal husbandry.

Methods

Data. The details of analyzed samples are listed in Table 1. One kefir and one yoghurt starter culture were shotgun sequenced (PRJNA644779) by the authors. The further short read datasets were obtained from NCBI SRA repository. A query was performed in SRA to find kefir or yoghurt related shotgun sequenced samples. As a result of this search further 33 datasets originating from 8 BioProjects were selected for the study. Except for the samples of BioProjects PRJEB15432 all others came from paired-end runs. The downloaded short reads originated from BioSamples of kefir grains (n = 4), kefir products (n = 15), kefir strains (n = 7), a yoghurt grain (n = 1) and yoghurt products (n = 5). In the unified names of the samples the first character corresponds to the type of the sample (k and y, kefir and yoghurt, respectively), the second character comes from the first letter

Sample ID	BioProject	Run	Type	Source	Sample
k_g_01	PRJEB15432	ERR1653138	kefir	Grain	Fr1 grain
k_g_02	PRJEB15432	ERR1653139	kefir	Grain	Ick grain
k_g_03	PRJEB15432	ERR1653140	kefir	Grain	UK3 grain
k_g_04	PRJNA644779	SRR12171332	kefir	Grain	kefir seed culture
k_p_01	PRJEB15432	ERR1653129	kefir	Product	UK3, 8 h
k_p_02	PRJEB15432	ERR1653130	kefir	Product	Fr1, 24 h (replicate 2)
k_p_03	PRJEB15432	ERR1653131	kefir	Product	Ick, 24 h (replicate 2)
k_p_04	PRJEB15432	ERR1653132	kefir	Product	UK3, 24 h (replicate 2)
k_p_05	PRJEB15432	ERR1653135	kefir	Product	Fr1, 24 h (replicate 3)
k_p_06	PRJEB15432	ERR1653136	kefir	Product	Ick, 24 h (replicate 3)
k_p_07	PRJEB15432	ERR1653137	kefir	Product	UK3, 24 h (replicate 3)
k_p_08	PRJEB15432	ERR1653141	kefir	Product	Fr1, 24 h (replicate 1)
k_p_09	PRJEB15432	ERR1653142	kefir	Product	Ick, 24 h (replicate 1)
k_p_10	PRJEB15432	ERR1653143	kefir	Product	UK3, 24 h (replicate 1)
k_p_11	PRJEB15432	ERR1653145	kefir	Product	Fr1, 8 h
k_p_12	PRJEB15432	ERR1653146	kefir	Product	Ick, 8 h
k_p_13	PRJNA288044	SRR2082409	kefir	Product	KEFIR.shotgun
k_p_14	PRJNA388572	SRR7287342	kefir	Product	Metagenome from probiotic beverage K03
k_p_15	PRJNA388572	SRR8282406	kefir	Product	Metagenome from probiotic beverage K02
k_s_01	PRJDB4955	DRR064132	kefir	Strain	<i>Lactobacillus parakefiri</i> JCM 8573
k_s_02	PRJNA222257	SRR1151211	kefir	Strain	<i>Lactobacillus kefirifaciens</i> subsp. <i>kefirifaciens</i> DSM 5016
k_s_03	PRJNA222257	SRR1151212	kefir	Strain	<i>Lactobacillus kefirifaciens</i> subsp. <i>kefirgranum</i> DSM 10550
k_s_04	PRJNA222257	SRR1151213	kefir	Strain	<i>Lactobacillus kefiri</i> DSM 20587
k_s_05	PRJNA222257	SRR1151226	kefir	Strain	<i>Lactobacillus parakefiri</i> DSM 10551
k_s_06	PRJNA635855	SRR11965732	kefir	Strain	<i>Acetobacter syzygii</i> str. K03D05
k_s_07	PRJNA635872	SRR11966381	kefir	Strain	<i>Lactobacillus plantarum</i> K03D08
m_01	PRJEB15432	ERR1653133	milk	Milk	0 h (replicate 1)
m_02	PRJEB15432	ERR1653134	milk	Milk	0 h (replicate 2)
m_03	PRJEB15432	ERR1653144	milk	Milk	0 h (replicate 3)
y_g_01	PRJNA644779	SRR12171305	yoghurt	Grain	yoghurt seed culture
y_p_01	PRJEB30083	ERR2982980	yoghurt	Product	Yoghurt-A
y_p_02	PRJEB30083	ERR2982981	yoghurt	Product	Yoghurt-B
y_p_03	PRJEB30083	ERR2982982	yoghurt	Product	Yoghurt-C
y_p_04	PRJEB30083	ERR2982983	yoghurt	Product	Yoghurt-D
y_p_05	PRJEB30083	ERR2982984	yoghurt	Product	Yoghurt-E

Table 1. The list of analyzed samples obtained from NCBI SRA. In the unified names of the samples the first character corresponds to the type of the sample (k and y, kefir and yoghurt, respectively), the second character comes from the first letter of the source (g, p and s for grain, product and strain, respectively), while the last tag is a sequence number. The last column shows the available attribute data of the biosamples.

of the source (g, p and s for grain, product and strain, respectively), while the last tag is a sequence number. Of the collected projects, a peer-reviewed publication is available for the PRJNA222257³⁶, PRJEB15432⁷ and PRJEB30083³⁷. For all other samples, the only accessible metadata were the attributes in SRA. In PRJEB15432 Walsh et al.⁷ followed the microbial changes during the fermentation process of kefir. They used full-fat pasteurized milk inoculated by three different grains (Fr1, Ick, and UK3 from France, Ireland and United Kingdom, respectively). The pasteurized milk (with three replications) and grains (without replication) were sampled at hour 0. In the fermentation from kefir at hour 8 (without replication) and hour 24 (with three replications), further specimens were taken.

DNA extraction and metagenomics library preparation for PRJNA644779. Total metagenome DNA of kefir (k_g_04) and yoghurt (y_g_01) samples were extracted using the UltraClean Microbial DNA Isolation kit from MoBio Laboratories. The quality of the isolated total metagenomic DNA was checked using an Agilent TapeStation 2200 instrument. The DNA samples were used for in vitro fragment library preparation. In vitro fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Read numbers were the following: 22,044,496 and 20,895,112 for kefir and yoghurt, respectively. Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

Bioinformatic and statistical analysis. Quality based filtering and trimming was performed by Trimmomatic³⁸, using 15 as a quality threshold. Only reads longer than 50 bp were retained. The remaining reads were taxonomically classified using Kraken2 ($k = 35$)³⁹ with the NCBI non-redundant nucleotide database⁴⁰ with two different confidence setting. The first run was performed with the default settings to select all possible bacterial reads. The following taxon classification was performed with the `-confidence 0.5` parameter to get more precise species level hits. The taxon classification data was managed in R⁴¹ using functions of the package phyloseq⁴² and microbiome⁴³. For further analysis, only reads assigned to Bacteria Kingdom was used⁴⁴. The preprocessed bacterial reads were assembled to contigs by MEGAHIT (v1.2.9)⁴⁵ using default settings. From the contigs, all possible open reading frames (ORFs) were gathered by Prodigal⁴⁶. The protein translated ORFs were aligned to the ARGs of database CARD v.3.0.9^{47,48} by Resistance Gene Identifier (RGI, v5.1.0) with Diamond⁴⁹. The ORFs classified as perfect or strict were further filtered with 90% identity and 60% coverage. The findings were presented including and excluding the nudged hits. For the analysis of ARG abundance changes during kefir fermentation, only ARGs with maximal coverage greater than 75% in samples taken at different time points were included. Contigs harbouring ARGs were classified by Kraken2 using the NCBI RefSeq⁵⁰ complete bacterial genomes database. In keeping with Hendriksen et al.¹ the ARG abundance was expressed as fragments per kilobase per million fragments (FPKM)⁵¹ of contigs containing ARGs. For the i th contig $FPKM_i = q_i / (l_i \times Q) \times 10^6$, where q_i is the number of reads that mapped to the contig, l_i is the length of contig and Q is the total number of mapped reads. To calculate q values, all bacterial reads were aligned to the contigs by Bowtie2⁵² with the parameter of `-very-sensitive-local`. To identify possible further mobile genetic element (MGE) homologs the predicted protein sequences of contigs were scanned by HMMER⁵³ against data of PFAM v32⁵⁴ and TnpPred⁵⁵. Similar to Saenz et al.⁴⁴ from the hits with lower than $E 10^{-5}$ the best ones were assigned to each predicted protein within the distance of 10 ORFs. The MGE domains coexisting with ARGs were categorized as phage integrase, resolvase, transposase or transposon. The plasmid origin probability of the contigs was estimated by PlasFlow v.1.1⁶. According to the ARG abundance of the samples, a dissimilarity matrix was calculated using the Bray-Curtis index⁵⁶ with package vegan⁵⁷. With the same library and the same matrix, a permutational multivariate analysis of variance was applied to quantify the associations between the dissimilarity and independent variables (type, source, BioProject). For the visualization of the sample distances based on this matrix, a principal coordinate analysis (PCoA) was performed with package ape⁵⁸. The relationship between the detected ORF length and the sequencing depth was explored using a linear model. All analyses and plotting were done in R-environment⁴¹.

Data availability

The short read data of sample data are publicly available and can be accessed through the PRJDB4955, PRJEB15432, PRJEB30083, PRJNA22257, PRJNA288044, PRJNA388572, PRJNA635855, PRJNA635872, PRJNA644779 from the NCBI Sequence Read Archive (SRA).

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Author contributions

N.S. takes responsibility for the integrity of the data and the accuracy of the data analysis. A.G.T., I.C., L.M. and N.S. conceived the concept of the study. G.M. and N.S. performed sample collection and procedures. A.G.T., N.S. and S.A.N. participated in the bioinformatic and statistical analysis. A.D., A.G.T., A.J., A.V.P., G.M., G.S., K.B. and N.S. participated in the drafting of the manuscript. A.D., A.G.T., A.J., A.V.P., G.M., G.S., I.C., K.B., M.F.J.

and N.S. carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Article

Mobile Antimicrobial Resistance Genes in Probiotics

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Abstract: Even though people worldwide tend to consume probiotic products for their beneficial health effects on a daily basis, recently, concerns were outlined regarding the uptake and potential intestinal colonisation of the bacteria that they carry. These bacteria are capable of executing horizontal gene transfer (HGT) which facilitates the movement of various genes, including antimicrobial resistance genes (ARGs), among the donor and recipient bacterial populations. Within our study, 47 shotgun sequencing datasets deriving from various probiotic samples (isolated strains and metagenomes) were bioinformatically analysed. We detected more than 70 ARGs, out of which *rpoB* mutants conferring resistance to rifampicin, *tet(W/N/W)* and potentially extended-spectrum beta-lactamase (ESBL) coding *TEM-116* were the most common. Numerous ARGs were associated with integrated mobile genetic elements, plasmids or phages promoting the HGT. Our findings raise clinical and public health concerns as the consumption of probiotic products may lead to the transfer of ARGs to human gut bacteria.

Keywords: antimicrobial resistance; bacteriome; resistome; mobilome; probiotics



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1. Introduction

Probiotics and probiotic products have gained a worldwide reputation and popularity in our everyday lives irrespective of cultural background, geographic location or social standards. Beneficial health effects assigned to probiotics have been reported in several studies [1]. What these studies have in common is that they state that microbes carried in probiotics must remain present in the intestinal tract for a shorter or longer period of time to exert the expected beneficial effects. Nevertheless, the success of colonisation depends on several factors, thus the certainty of its realisation varies from individual to individual [2]. Recently, however, the possibility of some unfavourable or sometimes even adverse effects of probiotic consumption have also been raised [3]. Several publications indicate that bacterial strains included in probiotic compounds, powders and capsules may contain antimicrobial resistance genes (ARGs) [4–7]. Recognising that ARGs may enter into the human body by food (e.g., probiotic products), studies on the genetic characteristics of microorganisms (including bacteria) used in the food chain have been recommended by European Food Safety Authority (EFSA) in recent years [8,9].

Genes, including ARGs of the probiotic bacteria, can be transmitted to bacteria within the intestinal tract of the consumers by horizontal gene transfer (HGT). If such ARGs are received by pathogenic bacteria, the efficacy of antibiotic therapy prescribed as medical

intervention for the diseases they cause may lessen. HGT can take place by transformation, conjugation or transduction. All these processes have one important property in common, namely, a DNA fragment getting introduced to a recipient cell. Apart from transformation, by which any gene can be taken up by the bacterium from its environment, the routes of HGT require special active delivery processes. By conjugation, cell-to-cell contact provides the opportunity for a copy of a plasmid to translocate to a recipient bacterium [10]. In contrast, transduction negates the necessity for cell-to-cell contact, as in this case bacteriophages act as a conduit for shuttling genes among bacteria [11]. The genetic environment of the genes, possibly ARGs, involved in the transfer has a significant influence on the efficacy of the latter two HGT processes, i.e., on the genes' mobility. The transferability of ARGs is facilitated by the presence of mobility genes in their tight genetic environment. If the genes harbour on plasmids or prophages, the chance of their transfer increases. By probiotics with supposedly mobile ARGs, the likelihood of gene transmission to other bacteria in the intestinal tract increases. In our work, we aimed to gain insight into the mobility of ARGs in probiotics for human consumption using freely available samples sequenced by other research groups or by ourselves. Currently, the few accessible data on probiotic ARG mobility originate from studies with diverse methodologies [6,12–16]. Therefore, we intended to analyse the next-generation sequencing datasets of different probiotics and probiotic isolated bacterial strains with a unified bioinformatics approach.

2. Materials and Methods

In this study, we followed the FAO/WHO definition of probiotics, i.e., live microorganisms, which confer a health benefit on the host when administered in adequate amounts [17].

2.1. Data

For the study, we selected freely available samples from the sequencing of probiotic products for human consumption or from bacterial strains isolated from such products. The details of analysed samples are listed in Table 1. One probiotic capsule was shotgun sequenced (PRJNA644361) by the authors. All further short read datasets were obtained from NCBI SRA repository.

Table 1. The list of analysed samples obtained from NCBI SRA. In the unified names of the samples the first character corresponds to the type of the sample (s and m, isolate and metagenome, respectively), the second tag is a sequence number. Except the signed (*) all samples were paired end sequenced. The last column shows the available information about the biosamples.

Sample ID	BioProject	Run	Description
<i>Isolates</i>			
s01	PRJEB14693	ERR1554589	<i>Lactiplantibacillus plantarum</i>
s02	PRJEB14693	ERR1554590	<i>Lactiplantibacillus plantarum</i>
s03	PRJEB14693	ERR1554591	<i>Lactiplantibacillus plantarum</i>
s04	PRJEB38007	ERR4421718	<i>Pseudomonas</i> sp. RGM2144
s05	PRJNA312743	SRR3205957	<i>Limosilactobacillus fermentum</i>
s06	PRJNA347617	SRR4417252	<i>Limosilactobacillus fermentum</i>
s07	PRJNA635872	SRR11966381	<i>Lactiplantibacillus plantarum</i>
s08	PRJNA639653	SRR12037315	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>
s09	PRJNA639653	SRR12037316	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>
s10	PRJNA639653	SRR12037890	<i>Streptococcus thermophilus</i>
s11	PRJNA649814	SRR12375795	<i>Enterococcus faecalis</i>
s12	PRJNA649814	SRR12375796	<i>Enterococcus faecalis</i>
s13	PRJNA649814	SRR12375797	<i>Enterococcus faecalis</i>
s14	PRJNA650131	SRR12376423	<i>Escherichia coli</i>
s15	PRJNA650131	SRR12376425	<i>Escherichia coli</i>
s16	PRJNA650131	SRR12376427	<i>Escherichia coli</i>
s17	PRJNA650131	SRR12376429	<i>Escherichia coli</i>

Table 1. Cont

Sample ID	BioProject	Run	Description
s18	PRJNA650131	SRR12376431	<i>Escherichia coli</i>
s19	PRJNA650131	SRR12376433	<i>Escherichia coli</i>
s20	PRJNA639653	SRR12412204	<i>Lactocaseibacillus rhamnosus</i>
<i>Microbiota</i>			
m01	PRJNA474998	SRR8132838	probiotic powder (FC13678)
m02	PRJNA475000	SRR8138827	probiotic powder (FC13669)
m03	PRJNA474989	SRR8140233	probiotic powder (FC13655)
m04	PRJNA474995	SRR8140386	probiotic powder (FC13628)
m05 *	PRJNA508569	SRR8289759	probiotic product (2)
m06	PRJNA508569	SRR8289760	probiotic product (1)
m07 *	PRJNA508569	SRR8289761	probiotic product (4)
m08 *	PRJNA508569	SRR8289762	probiotic product (3)
m09 *	PRJNA508569	SRR8289763	probiotic product (6)
m10 *	PRJNA508569	SRR8289764	probiotic product (5)
m11	PRJNA542229	SRR9040978	dietary supplement (PB4)
m12	PRJNA542229	SRR9040979	dietary supplement (PB10)
m13	PRJNA542229	SRR9040980	dietary supplement (PB11)
m14	PRJNA542229	SRR9040981	dietary supplement (PB2)
m15	PRJNA542229	SRR9040982	dietary supplement (PB14)
m16	PRJNA542229	SRR9040983	dietary supplement (PB13)
m17	PRJNA542229	SRR9040984	dietary supplement (PB16)
m18	PRJNA542229	SRR9040986	dietary supplement (PB18)
m19	PRJNA542229	SRR9040987	dietary supplement (PB17)
m20	PRJNA542229	SRR9040988	dietary supplement (PB8)
m21	PRJNA542229	SRR9040989	dietary supplement (PB19)
m22	PRJNA542229	SRR9040990	dietary supplement (PB12)
m23	PRJNA542229	SRR9040991	dietary supplement (PB9)
m24	PRJNA542229	SRR9040992	dietary supplement (PB6)
m25	PRJNA542229	SRR9040993	dietary supplement (PB5)
m26	PRJNA542229	SRR9040994	dietary supplement (PB7)
m27	PRJNA644361	SRR12153424	probiotic capsule

2.2. DNA Extraction and Metagenomics Library Preparation for PRJNA644361

Total metagenome DNA of the probiotic capsule sample was extracted using the UltraClean Microbial DNA Isolation kit from MoBio Laboratories. The quality of the isolated total metagenome DNA was checked using an Agilent TapeStation 2200 instrument. The DNA sample was used for in vitro fragment library preparation. In vitro fragment library way prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

2.3. Bioinformatic Analysis

Quality based filtering and trimming of the raw short reads was performed by Trim-Galore (v0.6.6, <https://github.com/FelixKrueger/TrimGalore>, accessed on 22 March 2021), setting 20 as a quality threshold. Only reads longer than 50 bp were retained and taxonomically classified using Kraken2 (v2.1.1) [18] and a database created (24 March 2021) from the NCBI RefSeq complete archaeal, bacterial and viral genomes. For this taxon assignment, the -confidence 0.5 parameter was used to obtain more precise species level hits. The taxon classification data was managed in R [19] using functions of the packages phyloseq [20] and microbiome [21]. The preprocessed reads were assembled to contigs by MEGAHIT (v1.2.9) [22] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. From the contigs having more than 500 bp, all possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3) [23].

The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.1.1) [24,25] by Resistance Gene Identifier (RGI, v5.1.1) with Diamond [26]. The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. All nudged hits were excluded. The integrative mobile genetic element (iMGE) content of the ARG harbouring contigs was analysed by MobileElementFinder (v1.0.3) [27]. Following the distance concept of Johansson et al. [27] for each bacterial species, those with a distance threshold defined within iMGEs and ARGs were considered associated. In the MobileElementFinder database (v1.0.2) for *Escherichia coli*, the longest composite transposon (cTn) was the Tn1681. In the case of this species, its length (24,488 bp) was taken as the cut-off value. For *Lactococcus lactis*, this threshold was the length of the Tn5721 transposon, 11,256 bp. For *Enterococci*, the database contained cTn, the Tn6246 (5147 bp) transposon, in *E. faecium* only. The same threshold was used for *E. faecalis* contigs. As the database neither contains species-level, nor genus-level cTn data for *Bacillus*, *Bifidobacterium* and *Streptomyces* species, a general cut-off value was chosen for the contigs of these species. This value was declared as the median of the longest cTns per species in the database (10,098 bp). The average nucleotide identity (ANI) was calculated for the region of iMGE and associated ARGs by FastANI (v1.32) [28]. The plasmid origin probability of the contigs was estimated by PlasFlow (v1.1) [29]. The phage content of the assembled contigs was predicted by VirSorter2 (v2.2.1) [30]. The findings were filtered for dsDNA phages and ssDNAs. All data management procedures, analyses and plottings were performed in R environment (v4.0.4) [19].

3. Results

The analysis of the sequencing data from 20 isolates and 27 metagenomic (multi-microorganism) samples (Table 1) is summarised in three sections. Following the presentation of the bacteriome and the identified AGRs (resistome), predictions regarding the mobility potential of ARGs were also summarized based on genetic characteristics that may play a significant role in HGT. If integrated mobile genetic elements (iMGE) are identified in the sequence context of an ARG, its greater mobility can be assumed. The case is the same if the contig harbouring an ARG are derived as plasmid or phage originated. In the mobilome section, we summarise these results.

3.1. Bacteriome

By taxon classification, the number of reads aligning to bacterial genomes differed in the various samples. The median bacterial read count of the metagenomic samples was 8.2×10^6 (IQR: 4.4×10^6). The median sequencing depth of the isolated strains was 220 (IQR: 94.8). The taxonomic origin of the short reads generated from isolates is shown in Table 1. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the metagenomic samples is shown in Figure 1. These dominant genera (with mean prevalence) in descending order were *Lactobacillus* (40%), *Enterococcus* (35%), *Bifidobacterium* (34%), *Limosilactobacillus* (34%), *Lactococcus* (32%), *Lacticaseibacillus* (31%), *Bacillus* (26%), *Weizmannia* (22%), *Ligilactobacillus* (19%), *Streptococcus* (18%), *Lactiplantibacillus* (12%) and *Sphingobacterium* (2%).

3.2. Resistome

The median length of the filtered contigs harbouring ARGs constructed by de novo assembly was 102,711 bp (IQR: 105,696). The number of ARGs found on the contigs ranged from 1 to 12. Besides 182 perfect ARG matches, a further 225 hits were classified strict (RGI) and met the criteria of having 90% coverage and 90% sequential identity.

ARGs were detected in all metagenomic samples and in few isolates (Figure 2). The majority of isolates (s01, s02, s03, s04, s05, s06, s07, s08, s09, s10, s20) contained no ARG. The highest number of ARGs was found in samples s14–s19, obtained from sequencing six *Escherichia coli* strains isolated from the same probiotic product. It is important to highlight that we also found the *H-NS* gene in these samples which is not indicated in the figure, as its

effect is anti-AMR. The most common ARGs were the *rpoB* mutants conferring resistance to rifampicin, *TEM-116* and *tet(W/N/W)* genes, detected in 18, 15 and 13 samples, respectively.

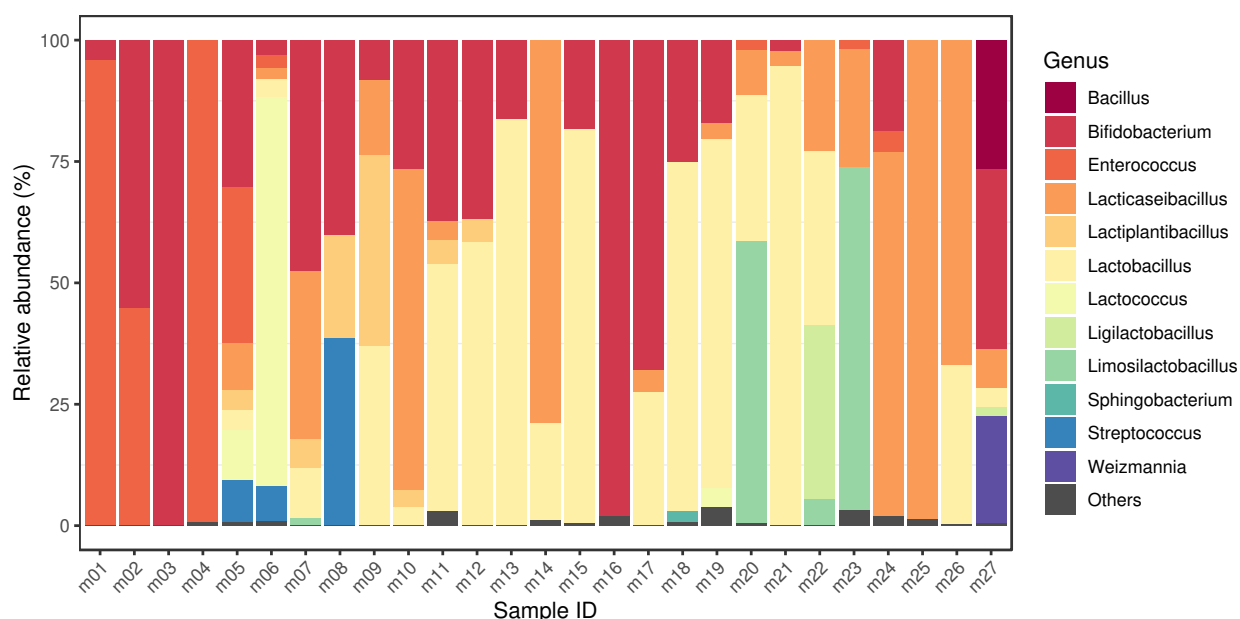


Figure 1. Bacteriome of samples. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the metagenomic samples. The dominant genera (with mean prevalence) in descending order were *Lactobacillus* (40%), *Enterococcus* (35%), *Bifidobacterium* (34%), *Limosilactobacillus* (34%), *Lactococcus* (32%), *Lacticaseibacillus* (31%), *Bacillus* (26%), *Weizmannia* (22%), *Ligilactobacillus* (19%), *Streptococcus* (18%), *Lactiplantibacillus* (12%) and *Sphingobacterium* (2%). Sample accession numbers for the Sample IDs are listed in Table 1.

The proportion of resistance mechanisms was calculated based on the ARG diversity. The dominant mechanism of identified ARGs was the antibiotic efflux (58.33%), antibiotic inactivation (11.11%), antibiotic target alteration (11.11%), antibiotic target protection (9.72%), antibiotic target alteration and antibiotic efflux (4.17%), antibiotic efflux and reduced permeability to antibiotic (1.39%), antibiotic target alteration and antibiotic efflux and reduced permeability to antibiotic (1.39%), antibiotic target alteration and antibiotic target replacement (1.39%) and antibiotic target replacement (1.39%).

There was no detectable ARG in the studied samples originating from *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Pseudomonas* sp. RGM2144 or *Streptococcus thermophilus* species.

The identified ARGs associated with bacteria by species are as follows. *Bacillus subtilis*: *aadK*, *B. subtilis mprF*, *B. subtilis pgsA* with mutation conferring resistance to daptomycin, *bmr*, *lmrB*, *mphK*, *vmlR*, *ykkC*, *ykkD*. *Bifidobacterium animalis*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *tet(W/N/W)*. *B. bifidum*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *B. bifidum ileS* conferring resistance to mupirocin, *tet(W/N/W)*. *B. breve*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *tetW*. *B. longum*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *tet(W/N/W)*. *Enterococcus faecalis*: *dfrE*, *efrA*, *efrB*, *emeA*, *lsaA*, *tetM*. *E. faecium*: *AAC(6′)-II*, *eatAv*, *msrC*. *Escherichia coli*: *acrB*, *acrD*, *acrE*, *acrF*, *acrS*, *bacA*, *baeR*, *baeS*, *cpxA*, *CRP*, *emrA*, *emrB*, *emrK*, *emrR*, *emrY*, *eptA*, *E. coli acrA*, *E. coli acrR* with mutation conferring multidrug antibiotic resistance, *E. coli ampC* beta-lactamase, *E. coli ampC1* beta-lactamase, *E. coli ampH* beta-lactamase, *E. coli emrE*, *E. coli GlpT* with mutation conferring resistance to fosfomycin, *E. coli marR* mutant conferring antibiotic resistance, *E. coli mdfA*, *E. coli soxR* with mutation conferring antibiotic resistance, *E. coli soxS* with mutation conferring antibiotic resistance, *evgA*, *evgS*, *gadW*, *gadX*, *kdpE*, *marA*, *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtF*, *mdtG*, *mdtH*, *mdtM*, *mdtN*, *mdtO*,

mdtP, *msbA*, *PmrF*, TEM-116, *TolC*, *ugd*, *YojI*. *Lactococcus lactis*: *lmrD*. *Streptomyces albulus*: AAC(3)-IV.

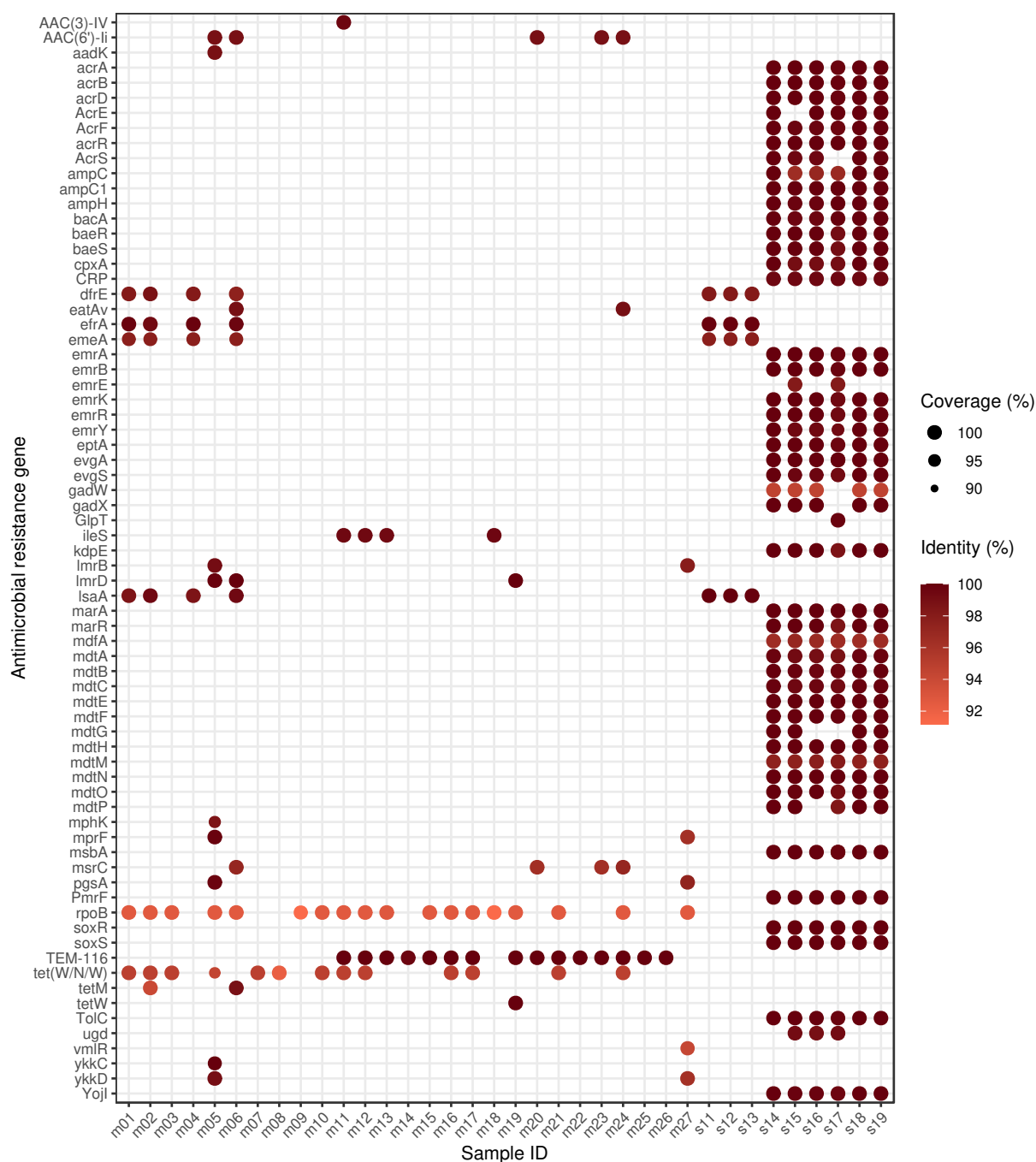


Figure 2. Identified antimicrobial resistance genes (ARGs) by samples. For each sample–ARG combination, only the best finding is plotted. The size and the colour of the dots correspond to the coverage and the sequence identity of hits on reference genes, respectively. In samples s01–s10 and s20, there was no identifiable ARG. The gene names that are too long have been abbreviated (*acrA*: *Escherichia coli* *acrA*; *acrR*: *E. coli* *acrR* with mutation conferring multidrug antibiotic resistance; *ampC*: *E. coli* *ampC* beta-lactamase; *ampC1*: *E. coli* *ampC1* beta-lactamase; *ampH*: *E. coli* *ampH* beta-lactamase; *emrE*: *E. coli* *emrE*; *GlpT*: *E. coli* *GlpT* with mutation conferring resistance to fosfomycin; *ileS*: *Bifidobacterium bifidum* *ileS* conferring resistance to mupirocin; *marR*: *E. coli* *marR* mutant conferring antibiotic resistance; *mdfA*: *E. coli* *mdfA*; *mprF*: *Bacillus subtilis* *mprF*; *pgsA*: *B. subtilis* *pgsA* with mutation conferring resistance to daptomycin; *rpoB*: *Bifidobacterium adolescentis* *rpoB* mutants conferring resistance to rifampicin; *soxR*: *E. coli* *soxR* with mutation conferring antibiotic resistance; *soxS*: *E. coli* *soxS* with mutation conferring antibiotic resistance).

The ARGs belonging to the genome of *Bacillus subtilis* may play a role in the appearance of resistance against aminoglycosides, lincosamides, macrolides, oxazolidinones, peptides, phenicols, pleuromutilins, streptogramins, tetracyclines; *Bifidobacterium animalis*: rifamycins, tetracyclines; *Bifidobacterium bifidum*: mupirocins, rifamycins, tetracyclines; *Bifidobacterium breve*: rifamycins, tetracyclines; *Bifidobacterium longum*: rifamycins, tetracyclines; *Enterococcus faecalis*: acridine dye, diaminopyrimidines, fluoroquinolones, lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, rifamycins, streptogramins, tetracyclines; *Enterococcus faecium*: aminoglycosides, lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins, tetracyclines; *Escherichia coli*: acridine dye, aminocoumarins, aminoglycosides, benzalkonium chlorides, carbapenems, cephalosporins, cephamycins, fluoroquinolones, fosfomycins, glycylicyclines, lincosamides, macrolides, monobactams, nitroimidazoles, nucleosides, penams, penems, peptides, phenicols, rhodamines, rifamycins, tetracyclines, triclosans; *Lactococcus lactis*: lincosamides; *Streptomyces albulus*: aminoglycosides.

3.3. Mobilome

The frequencies of iMGEs, phages and plasmids associated with ARGs by bacteria of origin are summarised in Figure 3.

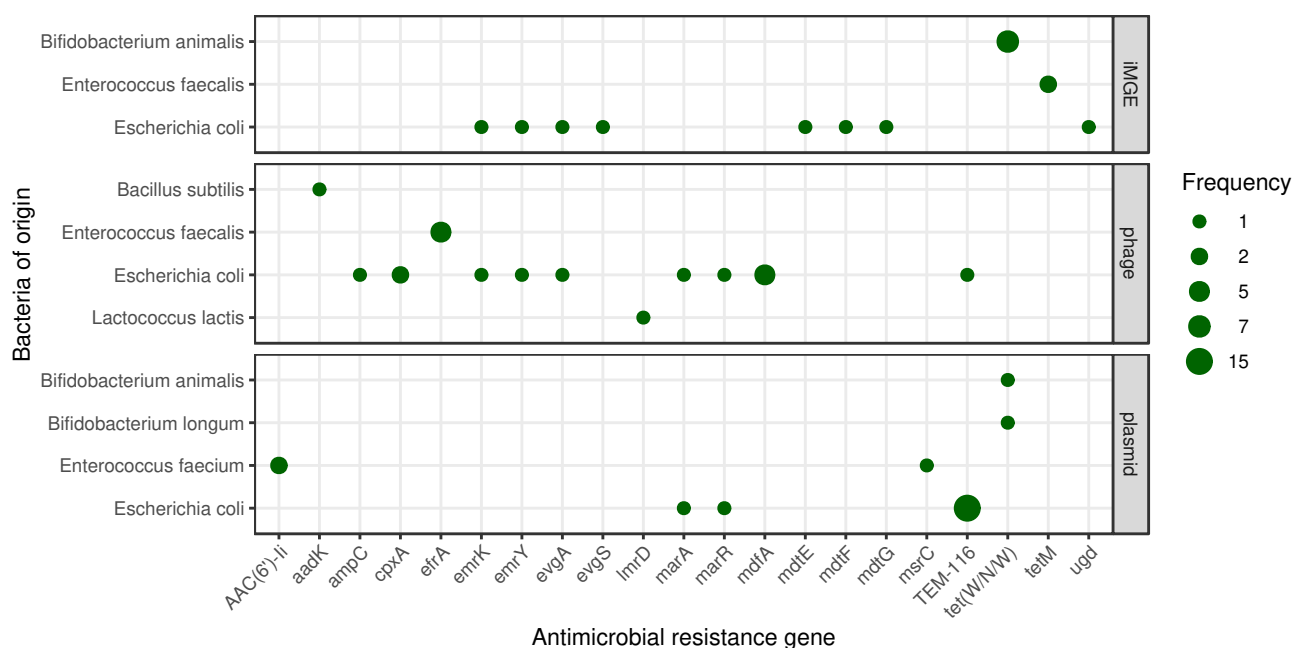


Figure 3. Mobile antimicrobial resistance gene frequency by bacteria of origin. The size of the dots indicates the occurrence frequency of the given gene flanked by iMGE, positioned in plasmid or phage.

3.4. Coexistence of ARGs and iMGEs

Based on the distance method proposed by Johansson et al. (2021) [27] iMGE associated ARGs were detected in three species (*Bifidobacterium animalis*, *Enterococcus faecalis* and *Escherichia coli*). In seven metagenomic samples (m01, m02, m03, m07, m16, m17, m24) we found tet(W/N/W) associated with ISBian1 insertion sequence on contigs classified as *B. animalis* originated. In two further samples (m02, m06) on *E. faecalis* originated contigs, tetM is linked to the transposon Tn6009. The ARG mdtG in the *E. coli* sample s14 and the ARG ugd in s15 are associated with IS3 and IS100, respectively. On two different contigs in the sample s17, multiple ARGs were detected with iMGE. One of them has the ISKpn24 associated with mdtE and mdtF. The other one has the IS102 linked to emrY, emrK, evgA and evgS genes. According to the average nucleotide identity (ANI) analysis most of the

contig region of iMGE and associated ARGs had a high level of conservation (ANI > 97%). Nevertheless, both contigs classified as *E. faecalis* originated showed ANIs below 80%.

3.5. Plasmids

In samples m08 and m21, we identified one-one plasmid associated contig with *tet(W/N/W)* classified as *Bifidobacterium longum* and *Bifidobacterium animalis* originated, respectively. In the samples m20 and m23 on *Enterococcus faecium*, classified contigs of plasmids *AAC(6′)-II* were detected. Further, *E. faecium* classified contigs of the sample m23 contained the gene *msrC*. In the samples m11, m12, m13, m14, m15, m16, m17, m19, m20, m21, m22, m23, m24, m25 and m26, *Escherichia coli* originated contigs from plasmids harboured the gene *TEM-116*. In the *E. coli* isolate sample s15, one contig of plasmid had the *marA* and *marR* genes.

3.6. Phages

By phage prediction, only dsDNAphages were detected. One contig, classified as *Bacillus subtilis* from the m05 metagenomic sample, contained prophage harbouring gene *aadK*. One prophage in predicted *Enterococcus faecalis* originated contig was found in sample m04 having gene *efrA*. The same content was detected in sample m01 on contigs classified as *E. faecalis*. All three *E. faecalis* isolates (s11, s12, s13) contained contigs harbouring the gene *efrA* within a prophage. In sample m17, one *E. coli* classified contig had the gene *TEM-116*, while a *Lactococcus lactis* classified one carried the gene *lmrD* within a prophage. All the *E. coli* isolates contained contigs with prophages harbouring ARG. In the sample s17 and s19 the *mdfA* gene is presented within a prophage. The sample s15 contains contigs harbouring prophage with the gene *marA*, *marR*. The sample s16 harbours contigs with prophage having genes *emrK*, *emrY*, *evgA*. The gene *ampC* was found in sample s15, while the gene *cpxA* in samples s14 and s18 within prophages.

4. Discussion

The results presented demonstrate that the bacteria of probiotics may not only carry significant amounts of ARGs, but in numerous cases, those genes may also be mobile, thereby contributing to their spread to other bacteria and having possible consequences on the antibiotic treatment efficacy.

Bacterial genera identified in the metagenomic samples also appear in many probiotic related articles of the current international literature. Various species of *Bacilli*, *Bifidobacteria*, *Enterococci*, *Lacticaseibacilli*, *Lactiplantibacilli*, *Lactobacilli*, *Lactococci*, *Ligilactobacilli*, *Limosilactobacilli* and *Streptococci* are the core members of commercial probiotic bacterial communities [31–40]. Two identified bacterial genera (*Sphingobacterium*, *Weizmannia*) in the various samples are less frequent probiotic components. The possibility of exploiting *Sphingobacteria* in probiotic foods was previously mentioned based on the characterization of flour and batter samples of sorghum and pearl millet [41]. Members of the genus were detected by the high-throughput sequence analyses of fermented beverages [42]. Probiotic *Weizmannia* species (e.g., former *Bacillus coagulans*) have recently been reclassified [43], and have an unquestionable probiotic significance [44]. It is important to note that there may be notable differences in the gene pool between strains of particular species, so the results presented do not mean that all strains of a given species contain the genes identified here.

While at least one ARG was found in each metagenomic sample, less than half of the isolates contained any of them. No ARG was detected in *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Pseudomonas* sp. RGM2144 or *Streptococcus thermophilus*. Contigs originating from *Bacillus subtilis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Lactococcus lactis* and *Streptomyces albulus* each contained at least one ARG.

The available literature was screened to evaluate our findings and gain reliable knowledge of the ARGs that could have been attached to bacteria at the species level. All ARGs

found in *Bacillus subtilis* (*aadK*, *B. subtilis mprF*, *B. subtilis pgsA* with mutation conferring resistance to daptomycin, *bmr*, *lmrB*, *mphK*, *vmlR*, *ykkC*, *ykkD*) have previously been identified in *B. subtilis* and many of them were even reported to be specific for this species or the *Bacillus* genus [45–51]. In the *Bifidobacterium* genus, ARGs were associated with four species (*B. animalis*, *B. bifidum*, *B. breve* and *B. longum*). None of the *B. animalis*, *B. bifidum*, *B. breve* and *B. longum* related *B. adolescentis* *rpoB* mutants conferring resistance to rifampicin and *tet(W/N/W)* are specific for the identified species but both genes have previously been described in them [6,52–55]. *B. bifidum ileS* conferring resistance to mupirocin reported in *B. bifidum* supposedly cannot be exclusively linked to this species of the genus, but it had been identified in it before [56]. Out of the *Enterococcus faecalis* deriving genes, *dfrE* was first identified in *E. faecalis* [57], but according to a recent study it is not exclusive to this species any more [58]. The genes *efrA* and *efrB* have been described in *E. faecalis* and *E. faecium* [59,60]. Gene *emeA* has only been identified in *E. faecalis* so far [59]. Apart from *E. faecalis*, *lsaA* has been attached to *Streptococcus agalactiae*, while *tetM* appears in a broad spectrum of bacterial species [61–65]. All three ARGs (*AAC(6′)-Ii*, *eatAv*, *msrC*) associated with *E. faecium* have been previously published as appearing in this species, and the first two are even specific for it [66–69]. All ARGs originating from *Escherichia coli* (*acrB*, *acrD*, *acrE*, *acrF*, *acrS*, *bacA*, *baeR*, *baeS*, *cpxA*, *CRP*, *emrA*, *emrB*, *emrK*, *emrR*, *emrY*, *eptA*, *E. coli acrA*, *E. coli acrR* with mutation conferring multidrug antibiotic resistance, *E. coli ampC* beta-lactamase, *E. coli ampC1* beta-lactamase, *E. coli ampH* beta-lactamase, *E. coli emrE*, *E. coli GlpT* with mutation conferring resistance to fosfomycin, *E. coli marR* mutant conferring antibiotic resistance, *E. coli mdfA*, *E. coli soxR* with mutation conferring antibiotic resistance, *E. coli soxS* with mutation conferring antibiotic resistance, *evgA*, *evgS*, *gadW*, *gadX*, *kdpE*, *marA*, *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtF*, *mdtG*, *mdtH*, *mdtM*, *mdtN*, *mdtO*, *mdtP*, *msbA*, *PmrF*, *TEM-116*, *TolC*, *ugd*, *YojI*) have previously been described in this species and many of them are even specific to it, according to the Comprehensive Antibiotic Resistance Database (CARD) [24,25]. Gene *lmrD*, the only ARG deriving from *Lactococcus lactis* has been identified in this species along with some others [70,71]. Even though *AAC(3)-IV* has been identified in several studies [72,73], according to our knowledge this is the first time it has been detected in *Streptomyces albulus*.

Gene *TEM-116*, which is often referred to as a clinically significant extended-spectrum beta-lactamase (ESBLs), was the most frequently identified finding in our study. ESBLs are most commonly defined as the members of a ubiquitous enzyme family that is capable of conferring resistance to penicillins, first-, second- and third-generation cephalosporins and aztreonam, and of being impeded by beta-lactamase inhibitors such as clavulanic acid [74]. The 400 *TEM* variants that have been identified so far, can be disclosed in two clusters with one deriving from *TEM-1* (the first *TEM* protein to be described) and one linked to *TEM-116* as a progenitor [75]. In line with our findings, gene *TEM-116* is reported to be present worldwide harbouring in the conjugative plasmids of a wide range of Gram-negative bacteria. Despite its wide geographical dissemination, establishment on multiple plasmids and centrality in the *TEM* family network indicating it is a naturally occurring enzyme with microbiologically proven ESBL characteristics [76,77], some concerns have arisen about its designation, after the gene was found in non-ESBL producing *Klebsiella pneumoniae* strains [78]. Moreover, commercial Taq polymerases used in PCRs may be contaminated with *bla_{TEM-116}* DNA which could lead to the erroneous identification of the gene in samples that do not actually contain it [79,80]. In our study, each sample in which this gene was detected originated from the same bioproject (PRJNA542229). As the samples come from different dietary supplements, one may interpret that this finding is an artefact or contamination as a consequence of some sample preparation steps. Nevertheless, as more detailed information on sample preparation is not available, this issue cannot be resolved.

As seen above, and as described in other publications [81], there is still a great deal of variation in details which need to be clarified by the interpretation of ARGs. Nevertheless, the suspicion that the identified ARGs may undermine the efficacy of several antibiotic

classes, including acridine dye, aminocoumarins, aminoglycosides, benzalkonium chloride, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, fluoroquinolones, fosfomycins, glycylicyclines, lincosamides, macrolides, monobactams, mupirocins, nitroimidazoles, nucleosides, oxazolidinones, penams, penems, peptides, phenicols, pleuromutilins, rhodamines, rifamycins, streptogramins, tetracyclines and triclosans raises some clinical considerations. According to the latest CDC report on antimicrobial use in the U.S., amoxicillin (penam), azithromycin (aminoglycoside), amoxicillin and clavulanic acid (penam, increased activity), cephalexin (cephalosporin) and doxycycline (tetracycline) are the most commonly administered compounds [82]. Moreover, based on the latest WHO report on global antimicrobial use, amoxicillin (penam), ciprofloxacin (fluoroquinolone), sulphamethoxazole and trimethoprim are the most commonly prescribed oral drugs and ceftriaxone (cephalosporin), gentamicin (aminoglycoside) and benzylpenicillin (penam) are the most commonly used parenteral compounds in 4 surveyed countries of the African region. In six countries of the region of the Americas, amoxicillin (penam), cefalexin (cephalosporin) and doxycycline (tetracycline) are the antibiotics with the highest oral consumption rates and ceftriaxone (cephalosporin), oxacillin (penam) and gentamicin (aminoglycoside) are the ones with the highest parenteral use. In the European region, reports were made of 46 countries. Among orally administered antibiotics, amoxicillin (penam), amoxicillin and beta-lactamase inhibitors (penam, increased activity) and doxycycline (tetracycline) are the top 3 compounds, while ceftriaxone (cephalosporin), gentamicin (aminoglycoside), and cefazolin (cephalosporin) are the most common parenteral ones. Amoxicillin (penam), azithromycin (macrolide) and amoxicillin and beta-lactamase inhibitors (penam, increased activity) are the most commonly consumed oral antibiotics and ceftriaxone (cephalosporin), benzathine benzylpenicillin (penam) and procaine benzylpenicillin (penam) are the top 3 parenterally administered agents in the Eastern Mediterranean region. In the six surveyed countries of the Western Pacific region amoxicillin (penam), doxycycline (tetracycline) and amoxicillin and beta-lactamase inhibitors (penam, increased activity) are the most commonly prescribed oral antibiotics, while cefazolin (cephalosporin), ceftriaxone (cephalosporin) and cefuroxime (cephalosporin) are the most frequently used parenteral compounds [83]. Many of the most highly prioritized antibiotics could be affected by the presence of the detected ARGs. Meanwhile, out of the 15 antibiotic groups mentioned in the latest WHO report on critically important antimicrobials (CIA) for human medicine, nine (aminoglycosides, carbapenems and other penems, cephalosporins, glycylicyclines, macrolides, monobactams, oxazolidinones, penicillins of various categories, quinolones) could possibly be affected by the ARGs identified in the various samples [83].

It is important to underline that all the six *E. coli* isolates contained the gene *H-NS*, which plays a crucial role in the global gene regulation of various bacteria, including this species. The expression of a wide variety of genes is repressed by *H-NS*, and its deletion increases AMR and decreases drug accumulation. Even though this gene is stored in CARD [24,25], its functional effect is adverse to that produced by ARGs [84].

If ARGs are transmitted from probiotic bacteria to pathogenic bacteria within the consumer's body, they may reduce the effectiveness of antibiotic therapy on the diseases participating pathogenic bacteria cause. The execution of gene transfer processes is more likely among bacteria that are in close physical proximity to each other and if the ARGs are associated to a mobile genetic environment. According to our results a considerable number of ARGs, such as those which are iMGES-linked or have resided in plasmids or prophages.

The co-occurrence of *tet(W/N/W)* and ISBian1 is in line with the findings of Rozman et al. [6], according to which all genomes of *B. animalis* (*subspecies lactis* or *animalis*) ($n = 42$) available in 2019 contained this gene. Moreover, by the investigation of the mobility characteristics of *tetW*, out of the transposases belonging to the family of the insertion sequences, ISBian1 seemed to be *subspecies* dependent in *B. animalis subsp. lactis* and flanking *tetW* in the majority of the strains [6]. Our results of *tetM* linking to the transposon Tn6009 in *E. faecalis* is consistent with finding of Zangue et al. in South-African fecal samples [85].

In two samples, contigs harbouring *tet(W/N/W)* originating from *Bifidobacterium longum* and *Bifidobacterium animalis* were predicted to belong to plasmids. Several studies reported a wide prevalence of the *tetW* gene in *Bifidobacteria* [6,12,86,87]. While the co-occurrence of *tetW* and its flanking transposase is a common genetic feature of *B. animalis*, previous reports lack the identification of plasmids in *B. animalis*, even though the gene was associated with plasmids in other bacterial species [88]. Despite *AAC(6′)-II* deriving from *E. faecium* being located in the chromosome in previous studies and it being defined as a chromosome-borne ARG on CARD [24,25,89], our research indicates it may take place in a plasmid. An *E. faecium*-associated contig contained gene *msrC*. According to the available literature, *msrC* is a chromosomal-encoded gene that is mentioned as an intrinsic property of *E. faecium* strains [24,25,90]. While the expected bacterial species of origin was confirmed, our finding raises the likelihood of the gene being connected to a plasmid as well. In 15 samples, *E. coli*-originated contigs harboured the gene *TEM-116*. Plasmid origin is a common feature of ESBL genes such as *TEM-116* according to several publications and is often referred to as a feature to facilitate their quick spread [91–93]. In the *E. coli* isolate sample s15, one contig had the *marA* and *marR* genes. These widespread multiple antibiotic resistance genes had been identified on plasmids before [94]. The gene *efrA* harbouring in contigs with a prediction of phage origins were identified in all publicly available *E. faecalis* genome sequences by Panthee and colleagues too, along with a large set of phages in the genomes [95].

As our results derive from in silico data analysis, it is only possible to describe the features that prove and facilitate presence and mobility of the genes. Whether or not the identified genes operate in the bacterial strains of a given probiotic cannot be determined. In order to clarify this, additional functional, e.g., gene expression studies, should be performed.

An important aspect to take into consideration by the interpretation of the ARG occurrence in probiotics is that constituent strains can often naturally be, or rendered multiresistant, so that they can be co-administered with oral antibiotics and reduce gastrointestinal side effects [96,97]. In our study we could not distinguish whether the examined samples contained the ARGs for this purpose. Moreover, as ARGs were found in the vast majority of the samples tested, not a negligible proportion of them, it is possible that the presence of ARGs in bacteria may also play a role in their probiotic effect. ARGs play a role in defence against antibiotics and may provide general fitness against specific toxic effects for bacteria [98,99]. One may make an analogy with earlier practice. In livestock farming, antibiotics have been widely used as feed supplements for yield enhancement on a purely empirical basis. By this practice, antibiotics have put pressure on the gut bacteria and selected for resistant strains. As a result, animal feed efficiency and production indicators have improved. When probiotics are consumed, the expectation is that the “good” microorganisms, bacteria will colonise the gut. In numerous animal husbandry areas (e.g., broiler chicken production), the producers try to achieve this by continuous probiotic feeding. If these probiotics also contain bacterial strains harbouring ARGs, they achieve very similar results as before with the selective effect of antibiotic utilisation. If it is true that certain ARGs are essential for the efficacy of probiotic bacteria, then the selection of strains should be carried out with consideration of the human health consequences. That is, bacterial strains that contain ARGs having no significant influence on human antimicrobial therapy efficiency should be used. However, based on our results, it can also be suggested that bacteria that do not contain ARGs at all can be used as probiotic components. To have a more detailed insight into this topic, several further studies would be needed. For instance, they could also focus on reducing the mobility of genes whose presence may be necessary for the probiotic nature of particular bacteria. Based on the results, we consider it essential to monitor the ARG content of probiotic preparations and their mobility characteristics in the fight against antimicrobial resistance.

Author Contributions: N.S. takes responsibility for the integrity of the data and the accuracy of the data analysis. A.G.T., I.C., N.S. and S.S. conceived the concept of the study. G.M., N.S. and S.S. performed sample collection and procedures. Á.B., A.G.T. and N.S. participated in the bioinformatic analysis. Á.B., A.G.T., G.M., I.C., M.F.J. and N.S. participated in the drafting of the manuscript. Á.B., A.G.T., G.M., I.C., M.F.J., N.S. and S.S. carried out the critical revision of the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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A survey on antimicrobial resistance genes of frequently used probiotic bacteria, 1901 to 2022

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Background: Antimicrobial resistance (AMR) is caused by AMR determinants, mainly genes (ARGs) in the bacterial genome. Bacteriophages, integrative mobile genetic elements (iMGEs) or plasmids can allow ARGs to be exchanged among bacteria by horizontal gene transfer (HGT). Bacteria, including bacteria with ARGs, can be found in food. Thus, it is conceivable that in the gastrointestinal tract, bacteria from the gut flora could take up ARGs from food. **Aim:** The study objective was to gain insight into the ARG set carried by commonly used probiotic bacteria that may enter the human body with non-fermented foods, fermented foods, or probiotic dietary supplements (FFPs) and to assess ARG mobility. **Methods:** Next generation sequencing whole genome data from 579 isolates of 12 commonly employed probiotic bacterial species were collected from a public repository. Using bioinformatical tools, ARGs were analysed and linkage with mobile genetic elements assessed. **Results:** Resistance genes were found in eight bacterial species. The ratios of ARG positive/negative samples per species were: *Bifidobacterium animalis* (65/0), *Lactiplantibacillus plantarum* (18/194), *Lactobacillus delbrueckii* (1/40), *Lactobacillus helveticus* (2/64), *Lactococcus lactis* (74/5), *Leucoconstoc mesenteroides* (4/8), *Levilactobacillus brevis* (1/46), *Streptococcus thermophilus* (4/19). In 66% (112/169) of the ARG-positive samples, at least one ARG could be linked to plasmids or iMGEs. No bacteriophage-linked ARGs were found. **Conclusion:** The finding of potentially mobile ARGs in probiotic strains for human consumption raises awareness of a possibility of ARG HGT in the gastrointestinal tract. In addition to existing recommendations, screening FFP bacterial strains for ARG content and mobility characteristics might be considered.

Introduction

Antimicrobial resistance (AMR) is one of the foremost threats hindering the treatment of infectious diseases worldwide, both in human and animal medicine. Due to the clear relatedness of excess antimicrobial use (AMU) and elevated AMR rates, measures are required on a permanent basis. Despite the interventions to reduce AMU, high levels of antibiotic consumption in both animals and humans are still being reported in several countries [1]. It is currently estimated that 700,000 people are dying per annum from AMR-related issues worldwide, with projections forecasting this number to rise to 10 million by 2050 [2]. Identifying potential sources of AMR is thus of utmost importance. AMR can be acquired by bacteria via gene mutations or horizontal gene transfer (HGT) [3]. HGT occurs primarily by transformation, conjugation, or transduction and involves small fragments of DNA being transferred between bacteria [4]. The transfer of AMR genes (ARGs) is enhanced through bacteriophages, integrative mobile genetic elements (iMGEs) and plasmids [5,6].

In our previous work based on metagenomics, and in agreement with the results of other research groups [7-11], we found that non-fermented [12] and fermented [13] foods or probiotic dietary supplements [14] contain a considerable number of ARGs, some of which are mobile. When bacteria with mobile ARGs are consumed through these foods, the ARGs can enter the digestive tract, where it is conceivable that they may be transferred to non-pathogenic bacteria and facultative pathogenic bacteria. This process might even be facilitated if the ingested bacteria are prone/able to colonise the intestinal tract. Since probiotic bacteria are expected to colonise the gut, their AMR determinants can contribute to the gut resistome [15-17].

The aim of our study was to gain insights into the set of ARGs and their mobility potential in prominent probiotic bacterial strains (from the Bifidobacteriales and

KEY PUBLIC HEALTH MESSAGE

What did you want to address in this study?

Antimicrobial resistance (AMR) challenges treating infections. In bacteria, AMR relies on antibiotic resistance genes (ARGs), some of which can be mobile. In certain conditions, bacteria with mobile ARGs may transfer their ARGs to other bacteria. If bacteria with mobile ARGs are in food, they may upon ingestion pass on these ARGs to the bacteria that are present in the digestive tract of humans. We wished to shed light on ARGs in probiotic bacterial species, particularly their mobility characteristics.

What have we learnt from this study?

Among 12 probiotic species of interest, we analysed in detail 10 species commonly used in non-fermented/fermented foods or probiotic dietary supplements (FFPs). Using bioinformatics, we screened their genetic data for ARGs and then assessed if the ARGs were mobile. Overall, several types of ARGs were found. Their occurrence varied between species, with no ARGs detected in two species. Among samples of bacteria with ARGs, a considerable proportion had ARGs that were likely mobile.

What are the implications of your findings for public health?

Eating foods that contain bacteria with mobile ARGs may allow such bacteria to come near other bacteria found in the human body. This proximity could facilitate mobile ARGs' transfer from the food bacteria to other bacteria in the gut, even pathogenic ones. While acquiring mobile ARGs does not always confer AMR, extending current recommendations to detect potential functional traits of concern in bacteria used for food might be considered, with screening for mobile ARGs in probiotic bacteria.

Lactobacillales order) isolated from food (fermented and non-fermented) and probiotics using a unified bio-informatic pipeline.

Methods

Our work is based on next generation sequencing (NGS) data from isolates of 12 commonly used probiotic bacterial species that have been isolated in other studies. The bacterial species were selected based on a non-systematic review. This involved a search of the PubMed database (<https://pubmed.ncbi.nlm.nih.gov/>) using the keywords 'kefir', 'yogurt', 'probiotic', and 'bacteria', for papers published in English after 2000. From the hits, we selected reports with relevant data (see below) on probiotic bacterial species [18-23].

Data used

Data that met the following criteria: having genomic library source, being whole genome sequenced and being Illumina platform based were downloaded from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository.

The selected species (and sample download dates in day/month/year) were: *Bifidobacterium animalis* (4/12/2022), *Lactocaseibacillus casei* (4/12/2022), *Lactocaseibacillus paracasei* (4/12/2022), *Lactiplantibacillus plantarum* (3/12/2022), *Lactobacillus delbrueckii* (25/12/2022), *Lactobacillus helveticus* (4/12/2022), *Lactobacillus kefirianofaciens* (25/12/2022), *Lactobacillus kefir* (25/12/2022), *Lactococcus lactis* (6/12/2022), *Leucoconostoc*

mesenteroides (25/12/2022) *Levilactobacillus brevis* (2/12/2022), *Streptococcus thermophilus* (3/12/2022). The source data collected on samples were grouped into three categories: non-fermented food or fermented food or probiotic (FFP) bacteria, intestinal bacteria, and others. The FFP group included samples with the following origins: fermented beverage (n=12), fermented food (n=69), fermented food of dairy nature (n=124), fermented food of non-dairy nature (n=141), milk with source non-specified (n=10), milk from farm animal (n=14), milk of human origin (n=34), milk/dairy product (n=26), probiotic dietary product (n=1), probiotic dietary supplement (n=146), starter culture (n=2). The detailed metadata for each sample are available at: <https://doi.org/10.6084/m9.figshare.21877134.v2>.

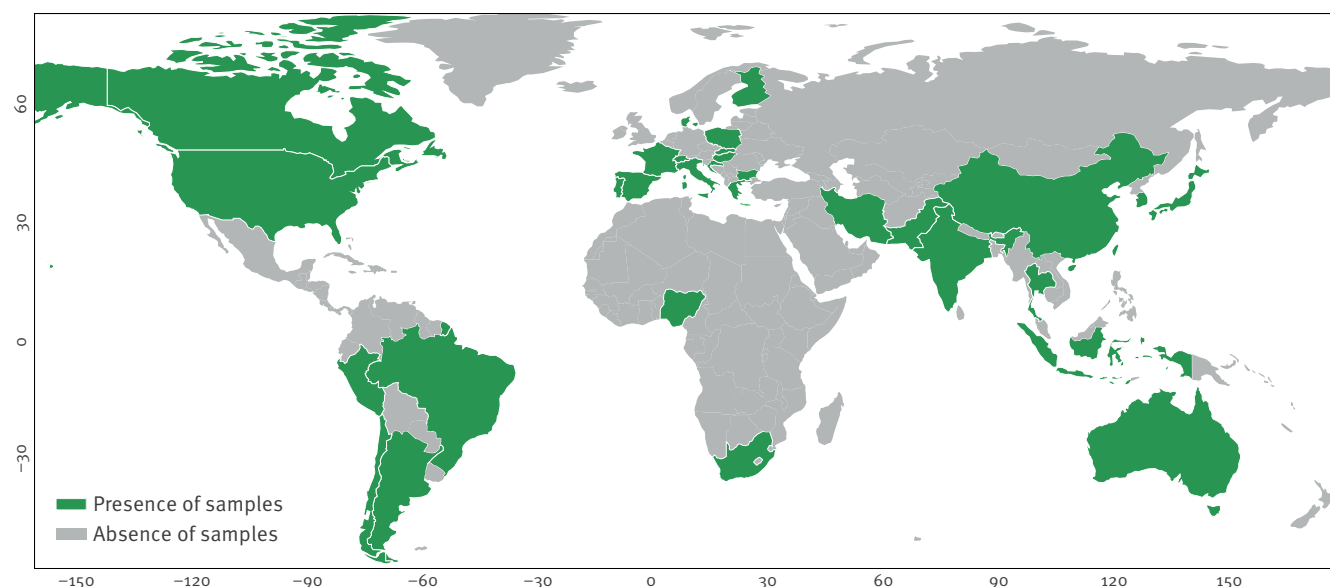
Bioinformatic and statistical analysis

Quality based filtering and trimming of the raw short reads was performed with TrimGalore (v.o.6.6, <https://github.com/FelixKrueger/TrimGalore>), setting 20 as a quality threshold. Only reads longer than 50 bp were retained.

Cleaned reads from each bacterial species were aligned to the representative reference genome for the corresponding bacterium species (*B. animalis*: NC_017216.2; *L. casei*: NZ_AP012544.1; *L. paracasei*: NC_022112.1; *L. plantarum*: NZ_CP028221.1; *L. delbrueckii*: NZ_CP018218.1; *L. helveticus*: ASM2283254v1; *L. kefirianofaciens*: NZ_CP061341.1; *L. kefir*: NZ_CP029971.1; *L. lactis*: NZ_CP059048.1; *L. mesenteroides*: NZ_CP028251.1; *L. brevis*: NZ_CP015398.1; *S. thermophilus*: NZ_LR822015.1) for each

FIGURE 1

Geographical origin of bacterial samples from food or fermented food or probiotics analysed the study, 1901–2022 (n = 561 samples)^a



^a For 18 samples, the country of origin was not available.

TABLE 1

Proportions of ARG-positive bacterial samples^a found in the study, stratified by country, 1901–2022 (n = 561 isolates)^b

Country	Number of samples with ARGs /total number of samples	Country	Number of samples with ARGs /total number of samples
Argentina	6/10	Iran	0/3
Australia	1/20	Italy	6/8
Brazil	0/1	Japan	0/3
Bulgaria	0/3	Korea	0/2
Canada	0/2	Nigeria	0/3
Chile	0/2	Pakistan	0/1
China	60/198	Peru	3/3
Croatia	2/10	Poland	2/2
Denmark	3/4	Portugal	0/2
Finland	1/1	Slovakia	0/14
France	1/2	South Africa	0/1
Greece	0/7	Spain	4/5
Hungary	0/1	Switzerland	4/6
India	4/10	Thailand	0/1
Indonesia	0/3	United States	67/232

ARG: antimicrobial resistance gene.

^a Samples were from food or fermented food or probiotics.

^b For 18 samples, the country of origin was not available, five of which were ARG-positive. In one sample from Taiwan, no ARG was detected.

TABLE 2

Description per species, of identified ARGs with the antibiotics they potentially confer resistance to, and resistance mechanisms, 1901–2022 (n = 579 isolates)

Bacterial species (number of samples with ARG (n); total number of samples (N))		ARG (number of samples bearing the ARG)		Antibiotics the ARGs may confer resistance to ^a	Mechanisms of resistance potentially involved (proportion of ARG-containing-samples with a given mechanism) ^a
<i>Bifidobacterium animalis</i>	(n = 65; N = 65)	<i>B. adolescentis</i> <i>rpoB</i> mutations conferring rifampicin resistance	(n = 65)	Phenicols, rifamycins, tetracyclines	Antibiotic inactivation (1/65); antibiotic target alteration and target replacement (65/65); antibiotic target protection (65/65)
		<i>Limosilactobacillus reuteri</i> <i>cat-TC</i>	(n = 1)		
		<i>tet(W)</i>	(n = 65)		
<i>Lactiplantibacillus plantarum</i>	(n = 18; N = 212)	<i>AAC(6')-li</i>	(n = 2)	Aminoglycosides, cephalosporins, lincosamides, macrolides, monobactams, penams, phenicols, streptogramins, tetracyclines	Antibiotic efflux (1/18); antibiotic inactivation (14/18); antibiotic target alteration (1/18); antibiotic target protection (10/18)
		<i>ANT(3'')-IIa</i>	(n = 6)		
		<i>ANT(6)-Ia</i>	(n = 1)		
		<i>catA8</i>	(n = 1)		
		<i>eatAv</i>	(n = 2)		
		<i>ErmB</i>	(n = 1)		
		<i>lnuA</i>	(n = 2)		
		<i>msrC</i>	(n = 2)		
		<i>TEM-1</i>	(n = 1)		
		<i>TEM-181</i>	(n = 1)		
		<i>tet(C)</i>	(n = 1)		
		<i>tet(M)</i>	(n = 5)		
		<i>tet(S)</i>	(n = 1)		
<i>Lactobacillus delbrueckii</i>	(n = 1; N = 41)	<i>TEM-116</i>	(n = 1)	Cephalosporins, monobactams, penams, penems	Antibiotic inactivation (1/1)
<i>Lactobacillus helveticus</i>	(n = 2; N = 66)	<i>lnuA</i>	(n = 2)	Lincosamides	Antibiotic inactivation (2/2)
<i>Lactococcus lactis</i>	(n = 74; N = 79)	<i>ErmB</i>	(n = 1)	Lincosamides, macrolides, streptogramins, tetracyclines	Antibiotic efflux (71/74); antibiotic target alteration (1/74); antibiotic target protection (5/74)
		<i>lmrD</i>	(n = 71)		
		<i>tet(M)</i>	(n = 1)		
		<i>tet(S)</i>	(n = 4)		
<i>Leucoconstoc mesenteroides</i>	(n = 4; N = 12)	<i>ANT(3'')-IIa</i>	(n = 3)	Aminoglycosides,	Antibiotic efflux (1/4); antibiotic inactivation (3/4)
		<i>lmrD</i>	(n = 1)	lincosamides	
<i>Levilactobacillus brevis</i>	(n = 1; N = 47)	<i>lnuA</i>	(n = 1)	Lincosamides	Antibiotic inactivation (1/1)
<i>Streptococcus thermophilus</i>	(n = 4; N = 23)	<i>ErmB</i>	(n = 2)	Lincosamides, macrolides, streptogramins, tetracyclines	Antibiotic target alteration (2/4); antibiotic target protection (2/4)
		<i>tet(S)</i>	(n = 2)		

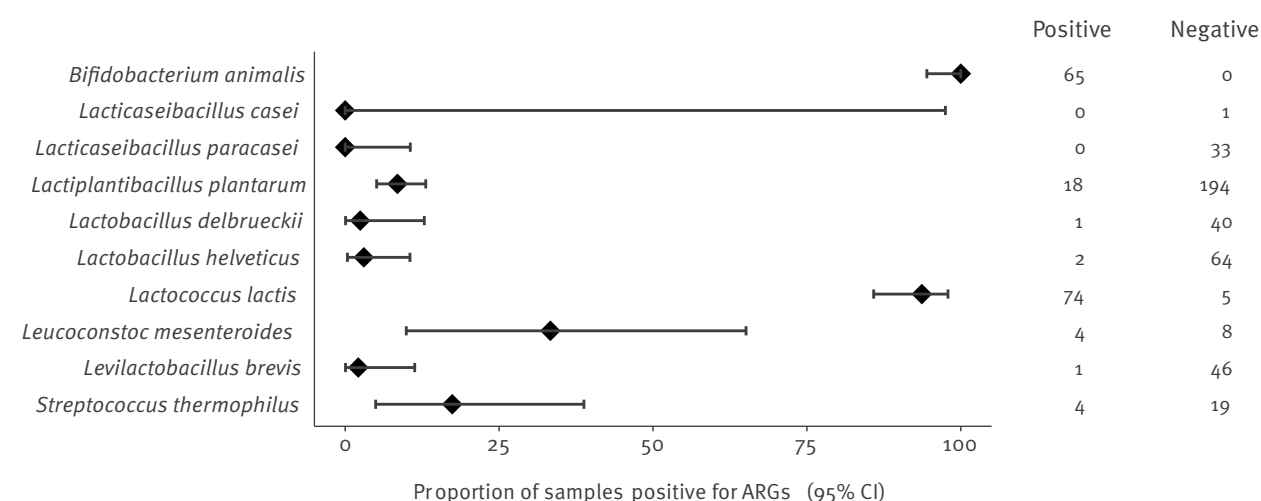
ARG: antimicrobial resistance gene.

^a Information on the antimicrobial resistances that the genes may contribute to, and the mechanisms of action originates from the Comprehensive Antibiotic Resistance Database (CARD) database [27,28].

For *Lactiplantibacillus plantarum*, one sample had two ARGs, two had three ARGs and one had four ARGs. For *Lactococcus lactis* three samples had two ARGs. For *Leucoconstoc mesenteroides*, four samples had one ARG. For *Streptococcus thermophilus*, four samples had one ARG.

FIGURE 2

Proportion of ARG positive samples, stratified by species, 1901–2022 (n = 579 isolates)



ARG: antimicrobial resistance gene; CI: confidence interval.

The numbers of ARG positive and negative samples for each species are listed beside the prevalence (diamond) and its 95%CI (horizontal line).

sample by Bowtie2 [24]. Reads from samples that covered at least 80% of the respective reference genome were de novo assembled with MEGAHIT (v1.2.9) [25] using default settings.

From the contigs all possible open reading frames (ORFs) were gathered with Prodigal (v2.6.3) [26]. The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v3.2.5) [27,28] by Resistance Gene Identifier (RGI, v6.0.0) with Diamond [29]. The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. Moreover, it was taken into account that when the bit-score of a hit is below the gene-specific predetermined cut-off value while the identity exceeds the 95% threshold, the RGI for ARG annotation classifies the hit as strict and uses the nudged notation. As a resistance gene prediction, which is a nudged, is more prone to be a false positive result, all nudged hits were excluded.

The iMGE content of contigs harbouring ARGs was analysed with MobileElementFinder (v1.0.3) and its database (MGEdB v1.0.2) [6]. Following the distance concept of Johansson et al. [6], an ARG was considered to be associated with an iMGE if it was within a given distance of this iMGE. In the MGEdB we found data only for *L. lactis*, the longest composite transposon (cTn) for that species was the *Tn5721*, its length (11,256 bp) was taken as the cut-off value. For the rest of the species, a general threshold value was declared as the median of the longest cTns per species in the database (10,098 bp). The plasmid origin probability of the contigs was estimated by PlasFlow (v1.1) [30]. The phage content of the assembled contigs was predicted by VirSorter2 (v2.2.1) [31]. The findings were filtered for

dsDNAPhages and ssDNAs. For MobileElementFinder, PlasFlow and VirSorter2 the default settings were used.

The 95% confidence intervals (CI) were estimated using the exact method for prevalence [32]. All data management procedures, analyses and plots were performed in R environment (v4.2.1) [33].

Results

Sample characteristics

For the 12 species, a total of 2,244 samples were downloaded. After fitting to the reference genomes, 1,452 of these samples were retained, as the obtained sequence covered at least 80% of the reference genome. Of these, 579 samples were from FFP isolates, 559 had intestinal origin and 314 originated from other sources. The data by sample is presented separately (<https://doi.org/10.6084/m9.figshare.21877134.v2>).

In non-FFP samples, *L. kefirifaciens* (n=5 samples) and *L. kefiri* (n=2 samples) species were present. In contrast, no *L. kefirifaciens* or *L. kefiri* species could be observed in the FFP samples. The 579 FFP samples comprised the following species: *B. animalis* (n=65 samples), *L. casei* (n=1 samples), *L. paracasei* (n=33 samples), *L. plantarum* (n=212 samples), *L. delbrueckii* (n=41 samples), *L. helveticus* (n=66 samples), *L. lactis* (n=79 samples), *L. mesenteroides* (n=12 samples), *L. brevis* (n=47 samples) and *S. thermophilus* (n=23 samples).

All FFP samples with available collection date had been obtained between 1901 and 2022. Eight of the 579 samples had no year recorded for the time of their collection. In contrast, none of the samples had the release

TABLE 3

ARGs linked to an integrative mobile genetic element or plasmid, according to ARG-positive bacteria species (n = 169 samples)

Species (number of samples with ARG)	ARG (number of samples with ARG on mobile element)
iMGE^a	
<i>Bifidobacterium animalis</i> (n = 65)	<i>tet(W)</i> (n = 62)
<i>Lactiplantibacillus plantarum</i> (n = 18)	<i>tet(M)</i> (n = 1), <i>tet(S)</i> (n = 1) ^b
Plasmid^c	
<i>Bifidobacterium animalis</i> (n = 65)	<i>Bifidobacterium adolescentis rpoB</i> mutants conferring resistance to rifampicin (n = 30), <i>Limosilactobacillus reuteri</i> cat-TC (n = 1), <i>tet(W)</i> (n = 40)
<i>Lactiplantibacillus plantarum</i> (n = 18)	<i>ANT(3'')-IIa</i> (n = 2), <i>ANT(6)-Ia</i> (n = 1), <i>catA8</i> (n = 1), <i>ErmB</i> (n = 1), <i>lnuA</i> (n = 2), <i>msrC</i> (n = 1), <i>TEM-1</i> (n = 1), <i>TEM-181</i> (n = 1), <i>tet(C)</i> (n = 1), <i>tet(M)</i> (n = 5), <i>tet(S)</i> (n = 1)
<i>Lactobacillus delbrueckii</i> (n = 1)	<i>TEM-116</i> (n = 1)
<i>Lactobacillus helveticus</i> (n = 2)	<i>lnuA</i> (n = 2)
<i>Lactococcus lactis</i> (n = 74)	<i>ErmB</i> (n = 1), <i>lmrD</i> (n = 20), <i>tet(M)</i> (n = 1), <i>tet(S)</i> (n = 4)
<i>Levilactobacillus brevis</i> (n = 1)	<i>lnuA</i> (n = 1)
<i>Streptococcus thermophilus</i> (n = 4)	<i>ErmB</i> (n = 2), <i>tet(S)</i> (n = 2)

ARG: antimicrobial resistance gene; iMGE: integrated mobile genetic element.

^a iMGE-associated ARGs can be on a plasmid or not.

^b The *tet(M)* and *tet(S)* ARGs were present in one sample each.

^c ARGs borne by a plasmid can be associated to an iMGE or not.

date missing. The release dates of the whole genome sequenced data were between 10 February 2014 and 15 November 2022. For 561 of the FFP samples, the country of origin was described. The countries from which samples originated are shown in Figure 1 and Table 1.

Resistome

Of 579 FFP samples, 169 were ARG-positive (29%; 95%CI: 26–33). The proportions of ARG-positive samples stratified by their country of origin are presented in Table 1 and by species in Figure 2. The names of the ARGs identified and their number per species are summarised in Table 2. Among the samples analysed, ARGs were neither identified in the single sample of *L. casei* nor in the 33 samples of *L. paracasei* species (Figure 2).

Table 2 also lists the antibiotics that the ARGs may contribute resistance to. All 65 samples of *B. animalis* had 15 (V516E, E525V, D532E, A533V, E543K, K552E, Q554E, A557V, V559D, G560A, E561A, E562G, V565E, S570E, S571M) of the 16 *B. adolescentis rpoB* mutations in the rifampicin pocket, which confer resistance to rifampicin, the only absent mutation being A443V [34]. The resistance mechanisms, that the identified ARGs have previously been shown to be involved in, included antibiotic efflux, antibiotic inactivation, antibiotic target alteration and/or antibiotic target replacement and antibiotic target protection (Table 2).

Mobilome

No ARGs that could be linked to bacteriophages were found in any sample. On the other hand, in 66% (112/169) of the ARG-containing samples, at least one gene could be linked to a plasmid or iMGE. The ARGs determined to be on a mobile element (whether plasmid or iMGE) are presented according to species in Table 3. For each bacterial species, and for each ARG, the proportion of samples with the mobile ARG in question, among all positive samples for this ARG are shown in Figure 3.

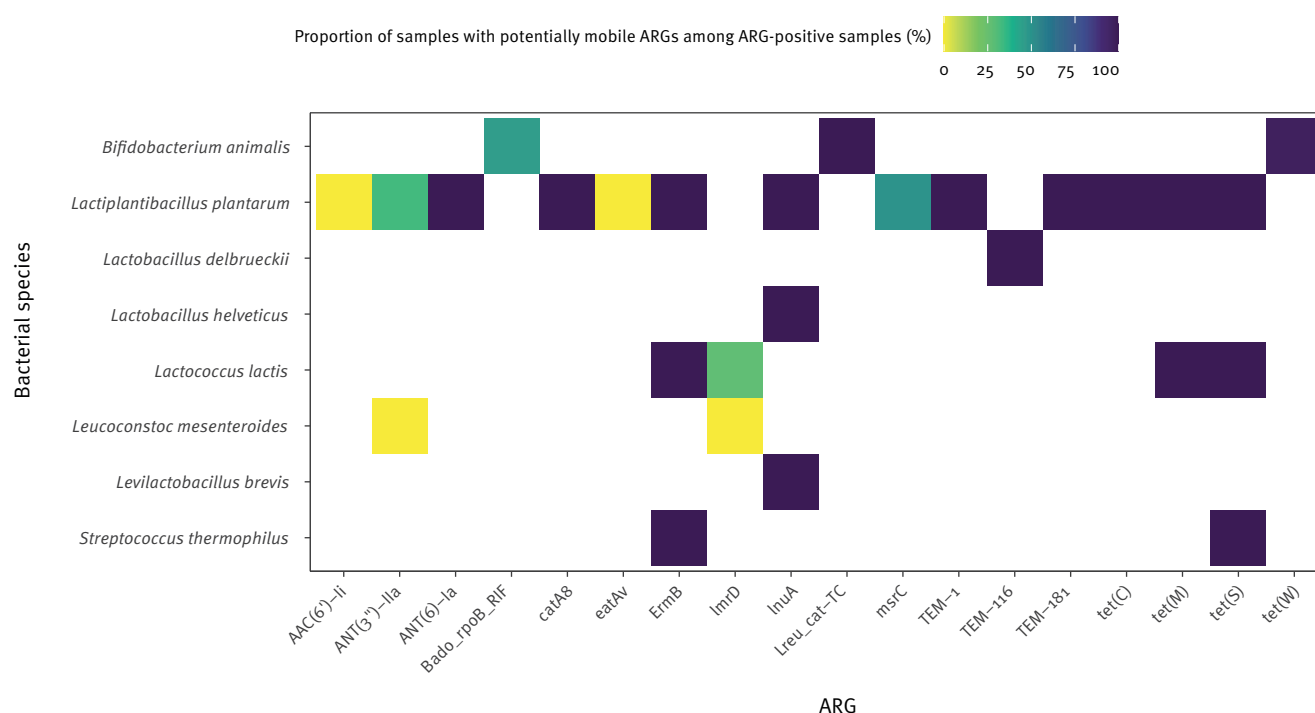
Discussion

Our study confirms that numerous ARGs are present in probiotic bacterial species constituting the bacteriome of edible products and that many of them are mobile. Thus, the application and intake of certain probiotic bacterial strains could have the potential to contribute to the appearance and spread of AMR.

The proportions of samples containing ARGs among those that we examined varied across bacterial species. Samples of *B. animalis* and *L. lactis* appeared to be relatively richer in ARGs. Nevertheless, in the case of *B. animalis* the high proportion of samples with ARG was underlain by the constant detection rate of two genes, namely *tet(W)* and *B. adolescentis rpoB* mutants conferring resistance to rifampicin. Tetracycline-resistance-encoding *tet(W)* is regularly associated with probiotic *B. animalis* strains [35–37]. Moreover, in certain subspecies, this ARG is

FIGURE 3

Proportion of samples found with potentially mobile ARGs among ARG-positive samples, 1901–2022 (n = 169 samples)



Bado_rpoB_RIF: *Bifidobacterium adolescentis* *rpoB* mutants conferring resistance to rifampicin gene; Lreu_cat-TC: *Limosilactobacillus reuteri* cat-TC gene.

The proportion is the number of samples containing potentially mobile (linked to iMGE or plasmid) ARGs divided by the total number of samples containing ARGs.

considered to be innate, phylogenetically distinct from that of other bacterial species and as having a negligible risk of transfer [35]. At the same time, *tet(W)* is commonly flanked by transposase genes [37]. Interestingly, among our 65 ARG-positive *B. animalis* samples, the majority of *tet(W)*-containing samples (n=62) had this gene associated with iMGEs that were cTns. The interpretation of the mobility potential of the cTn-associated genes is, however, unsure and would require further investigation. Consequently, the public health implications of these findings are not clear. On the other hand, there were 40 samples with *tet(W)* on plasmids in *B. animalis*. For *L. lactis*, lincosamide-resistance encoded *ImrD* showed the highest rate within the examined samples. This gene, in interaction with *ImrC*, is considered to have notable importance in phenotypically multidrug-resistant (MDR) *L. lactis* strains [38]. Just as in most (51/71) of our *ImrD*-positive samples, *ImrD* has been described as chromosomally encoded [39]. Nevertheless, 20 of our samples were associated with plasmid-borne *ImrD*. The plasmidome of *L. lactis* is highly dynamic and of high economic value [40]. Our findings may have public health relevance and may raise awareness of the potential need for more advanced surveillance measures.

In contrast, no *L. casei* or *L. paracasei* strains contained any ARGs, and in *L. delbrueckii*, *L. helveticus* and *L. brevis*, ARGs were relatively less frequent. As the presence

of AMR markers is an undesired trait for any microbe introduced in humans as a potential probiotic [41], these species might be favoured in alimentary products. On the other hand, despite the low ARG numbers and diversity, each ARG detected in the above-mentioned species appears to be plasmid-associated, and as such, potentially transferable.

Compared to species where ARGs seemed more prevalent, and species with little occurrence of ARGs in our analyses, the proportions of ARG-positive samples among *L. plantarum*, *L. mesenteroides* and *S. thermophilus* were intermediary in our study, but for these species, the ARGs appeared more diverse. Whereas genomes from *L. mesenteroides* contained no ARGs associated with mobile-genetic-element (plasmid or iMGE), the resistome of *S. thermophilus* was predicted to be highly mobile. The uptake of plasmids carrying ARGs, including *ErmB*, encoding the macrolide-lincosamide-streptogramin B (MLSB) phenotype is not without precedent for streptococci [42]. The samples with *L. plantarum* were more numerous, which may have led to the highest ARG diversity among the species we examined. All but two *L. plantarum* ARGs (AAC(6') II and *eatAv*) were located on mobile genetic elements. Among the 18 ARG-positive samples, bacteria in 17 had ARGs on plasmids and in two samples the ARGs were flanked by iMGEs. Consequently, potential

implications for public health associated with the *L. plantarum* strains appears non-negligible.

The ARGs identified overall in the current study may affect the activity of several classes of antibiotics, such as aminoglycosides, cephalosporins, lincosamides, macrolides, monobactams, penams, penems, phenicols, pleuromutilins, rifamycins, streptogramins and tetracyclines. They may be involved in resistance mechanisms against many antibiotics used in human and animal medicine. The World Health Organization (WHO) regularly publishes an updated list of human antimicrobials according to their importance, with in 2019, three categories: (i) Critically Important Antimicrobial (CIA) that are the last resort in the treatment of human disease, (ii) Highly Important Antimicrobial (HIA) and (iii) Important Antimicrobial (IA). The CIAs are further subdivided into high priority CIAs and highest priority CIAs. The latter group, abbreviated as HPCIA, includes cephalosporins (3rd, 4th and 5th generation), glycopeptides, ketolides and macrolides, polymyxins and quinolones [43]. Of the five HPCIA drug groups, we found ARGs that potentially could compromise the effectiveness of two (cephalosporins, ketolides and macrolides). We also found ARGs that have the potential to interfere with the activity of five high priority CIAs (aminoglycosides, monobactams, penams, penems, rifamycins), six HIAs (cephalosporins, lincosamides, penams, phenicols, streptogramins, tetracyclines) and one IA (pleuromutilins). The European Medicines Agency (EMA) also produced a list in 2019 aimed at restricting the veterinary use of antimicrobials that are important for human medicine [44]. The antimicrobials are listed under the categories: Avoid, Restrict, Caution and Prudence. We found ARGs that may confer resistance to three drug groups listed as Avoid (cephalosporins, monobactams, streptogramins), one listed as Restrict (cephalosporins), seven as Caution (rifamycins, aminoglycosides, phenicols, lincosamides, macrolides, pleuromutilins, cephalosporins) and two as Prudence (tetracyclines, penams). In addition, the World Organisation for Animal Health (OIE) has a list of critical antimicrobial agents used in veterinary medicine. The OIE uses three categories; Veterinary Critically Important Antimicrobial Agents (VCIA), Veterinary Highly Important Antimicrobial Agents (VHIA) and Veterinary Important Antimicrobial Agents (VIA) [45]. The ARGs we found can have an effect on six VCIA (tetracyclines, aminoglycosides, phenicols, macrolides, cephalosporins, penams), four VHIA (rifamycins, lincosamides, pleuromutilins, cephalosporins) and one VIA (streptogramins). Thus, many important human and animal medicine antibiotics could be affected by the ARGs we detected in probiotic bacterial strains from products for human consumption. Nevertheless, it is important to highlight that the presence of ARGs does not necessarily result in the phenotypical appearance of AMR. Further gene expression studies or phenotypical probes (e.g. the assessment of minimal inhibitory concentration values) would

be required to evaluate the expressed AMR traits of the examined probiotic bacteria.

Although we consider our results important in the absence of a similar survey study with such a large sample size and a uniform methodology, we must mention their shortcomings and limitations. Our study relies on retrospective data collection. The samples from which data were retrieved had been initially collected over a large period of time and were not obtained from different areas in a systematic way. Therefore, the results are not representative of any particular geographical location at a given time. Moreover, data obtained were based on the NCBI SRA system, which is quite permissive regarding the completeness of the uploaded metadata of samples. Hence, some detailed information can be missing in the metadata, hindering a more thorough presentation and interpretation of the results. We believe that one of the main problems with the extendibility of our results is that the exact types of isolation sources were not available for all samples. Furthermore, it would be very important to know under which conditions (e.g. medium, temperature) each strain was isolated and cultured. It is also unknown whether any antimicrobial agents were used in the cultures to control competing species. If any antimicrobials were used during the culturing process, subpopulations with ARGs could be propagated. The generalisability of our result would also increase if the age of the cultures from which the sequenced strains were isolated was known. Optimally, in a prospective study, these factors could be controlled. Thus, the noise in the variation between species and isolation sources could be reduced. Nonetheless, our work could raise awareness of the need for controlled prospective studies.

Conclusions

Our results suggest that some probiotic bacterial species may contain a higher proportion of ARGs, while others may represent a lower proportion. We also observe that a considerable proportion of the ARGs that we identified were mobile. In the European Union [46], there are recommendations with methodological suggestions for the whole genome sequencing analysis of microorganisms in the food chain. However, these recommendations do not provide detailed guidelines for the analysis of the mobilome. Since our results suggest that the prevalence of mobile ARGs might not be negligible, it might be worthy to consider the development of guidelines to monitor these mobile ARGs.

Ethical statement

Only anonymised data from a publicly available repository were used, which does not require ethical approval.

Funding statement

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Conflict of interest

None declared.

Authors' contributions

NS takes responsibility for the integrity of the data and the accuracy of the data analysis. AGT, MFJ, MP, NS, and SÁN conceived the concept of the study. AGT, MP, NS, and SÁN participated in the bioinformatic analysis. AGT, MFJ, and NS participated in the drafting of the manuscript. AGT and NS carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Antimicrobial resistance determinants in silage

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Animal products may play a role in developing and spreading antimicrobial resistance in several ways. On the one hand, residues of antibiotics not adequately used in animal farming can enter the human body via food. However, resistant bacteria may also be present in animal products, which can transfer the antimicrobial resistance genes (ARG) to the bacteria in the consumer's body by horizontal gene transfer. As previous studies have shown that fermented foods have a meaningful ARG content, it is indicated that such genes may also be present in silage used as mass feed in the cattle sector. In our study, we aspired to answer what ARGs occur in silage and what mobility characteristics they have? For this purpose, we have analyzed bioinformatically 52 freely available deep sequenced silage samples from shotgun metagenome next-generation sequencing. A total of 16 perfect matched ARGs occurred 54 times in the samples. More than half of these ARGs are mobile because they can be linked to integrative mobile genetic elements, prophages or plasmids. Our results point to a neglected but substantial ARG source in the food chain.

In intensive cattle farming, silage is an essential component of feed. An average dairy cow consumes 25–27 kg of this forage a day, reaching up to a silage consumption of 12,500 kg per lactation^{1,2}. Silage is most commonly produced from maize or legume plants by the process of anaerobic fermentation. Throughout the fermentation process, fermenting microorganisms, including bacteria, multiply. As a result of this biochemical transformation, the silage is enriched with beneficial nutrients. If bacteria that are involved in the process harbor antimicrobial resistance genes (ARGs), the amount of these genes in the silage will increase in parallel with the bacterial counts. Consequently, silage, as a mass feed may continuously supply the gastrointestinal tract of animals with bacteria carrying ARGs. Bacteria entering the digestive system may come into contact with the host microbiota that facilitates the exchange of bacterial genes (e.g. ARGs) by horizontal gene transfer (HGT). HGT may take place as a result of three different processes: conjugation, transduction and transformation. Except for transformation, by which a bacterium can take up any gene from its environment, the routes of HGT require particular active delivery processes. By conjugation, cell-to-cell contact provides the opportunity for a copy of a plasmid to translocate to a recipient bacterium³. Transduction negates the condition of cell-to-cell contact, as in this case, bacteriophages act as a conduit for shuttling genes among bacteria⁴. The genetic environment of the genes involved in the transfer significantly influences the efficacy of the latter two HGT processes, i.e., the genes' mobility. The reason why the mobility characteristics of ARGs involved in silage are worth taking into consideration is the following. If ARGs from silage are transmitted to pathogenic bacteria within an animal's body, efficacy of antibiotic (AB) treatment may be reduced on the consequent bacterial diseases. In addition, in case of the gut colonization of silage-borne bacteria that carry ARGs, the appearance and enrichment of bacterial ARGs may take place in the animals' environment after defecation. Decreased efficacy of AB treatments may result in economic loss, and the increased environmental ARG level may have additional veterinary and human health consequences. It is proven in former publications that the number of ARGs in fermented dairy products can increase due to the multiplication of fermenting bacteria⁵. Nevertheless, the description of this phenomenon cannot be found for silage in the literature. Our study aimed to examine the diversity, bacterial relatedness and mobility potential of ARGs deriving from silage. For this purpose, freely available next-generation sequencing (NGS) shotgun metagenome datasets were analyzed by a unified bioinformatics pipeline.

Results

Based on the taxon classification performed on a database containing complete reference genomes of plants, the most dominant plants in the silage belong to the *Medicago* genus and most likely to the alfalfa (*M. sativa*) species. Further results of the analysis of the 52 shotgun metagenomic sequenced samples (Table 2) are summarised in the following sections. After presenting the bacteriome and the identified AGRs (resistome), predictions

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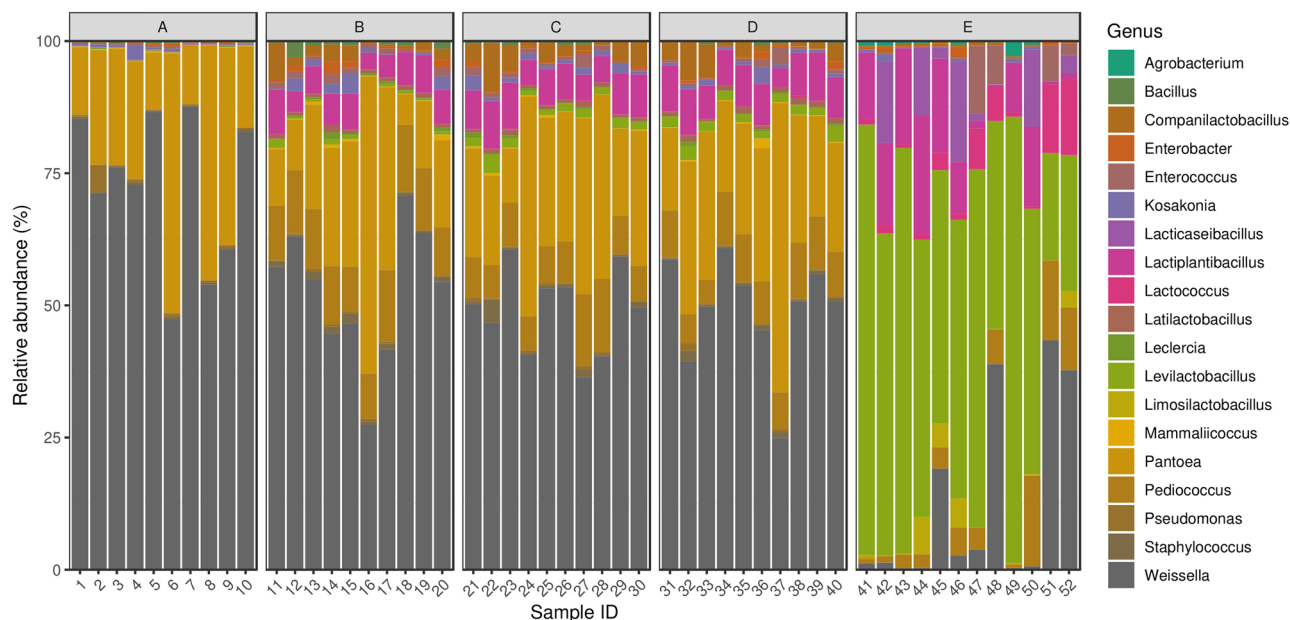


Figure 1. Silage core bacteriome. Relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples. The elements of the PRJNA495415 dataset were taken on days 0, 7, 14 and 28 were classified into groups A, B, C and D, respectively. All items from BioProject PRJNA764355 are assigned to group E.

regarding the mobility potential of ARGs were also resumed based on genetic characteristics that may play a significant role in HGT.

Bacteriome. By taxon classification, the number of reads aligning to bacterial genomes varied by samples (median: 20.6×10^6 , IQR: 2.9×10^6). The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples are shown in Fig. 1.

The dominant bacterial genera (with mean abundance) in descending order were *Weissella* (45.7%), *Pantoea* (18.5%), *Levilactobacillus* (13.5%), *Pediococcus* (6.7%), *Lactiplantibacillus* (6.3%), *Companilactobacillus* (1.7%), *Lactocaseibacillus* (1.3%), *Enterococcus* (1.2%), *Lactococcus* (1%), *Kosakonia* (0.8%), *Staphylococcus* (0.6%), *Enterobacter* (0.5%), *Latilactobacillus* (0.5%), *Bacillus* (0.4%), *Limosilactobacillus* (0.4%), *Pseudomonas* (0.4%), *Leclercia* (0.2%), *Mammaliicoccus* (0.2%), *Agrobacterium* (0.1%).

Resistome. The median length of the filtered contigs harboring ARGs constructed by de novo assembly was 4,204 bp (IQR: 2,832). The number of ARGs found on the contigs ranged from 1 to 2. The identified 16 ARG types appeared 54 times in the analyzed 52 samples. These ARGs were the following: *aadA2*, *ant(6)-Ia*, *ant(9)-Ia*, *aph(3')-IIa*, *aph(3')-IIIa*, *dfrG*, *erm(44)v*, *lmrD*, *lsaE*, *poxA*, *qnrD1*, *qnrS1*, *sul1*, *sul2*, *tet(K)*, *vatE* (Fig. 2). The resistance mechanism of identified ARGs was the antibiotic inactivation (48.1%), antibiotic target protection (20.3%), antibiotic efflux (13.0%), antibiotic target alteration (9.3%), antibiotic target replacement (9.3%) in descending order of frequency. Table 1 shows the bacterial species to which the ARG harboring contigs were assigned based by the taxon classification. In addition, the table also presents which drug classes are affected by the ARGs.

Mobilome. We found a total of 53 ARGs that are assumably mobile. Ten of these ARGs are linked to integrative mobile genetic elements (iMGE). A further two ARGs were detected in prophages and forty-one on plasmids. The frequencies of ARGs associated with iMGEs, phages and plasmids are summarized in Fig. 3 by bacterial species of origin.

Following the distance method proposed by Johansson et al.⁶, integrated mobile genetic element associated ARGs were detected in five samples (30, 45, 46, 48, 52) and five species (*B. subtilis*, *E. hormaechei*, *E. faecium*, *L. paracasei*, *L. plantarum*). *B. subtilis* associated *aph(3')-IIa* in sample 30, and *poxA* of *E. faecium* in sample 45, of *L. plantarum* in sample 52, and of *L. paracasei* in sample 46 were detected as iMGE linked gene. *Sul1* and *aadA2* were detected in *E. hormaechei* co-existed with integrated mobile elements in sample 48. Two prophage-linked ARGs were identified, the contig harboring *erm(44)v* classified to *S. pseudoxylus* by VirSorter2 was found to be of dsDNA phage origin while the contig of *lsaE* from *S. suis* was predicted as ssDNA derived. These phage associated ARGs were detected in sample 37 and sample 45 respectively. Contigs with ARGs were predicted to belong to plasmids in 19 samples (Nr. 11, 12, 17, 30, 32, 36, 37, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52).

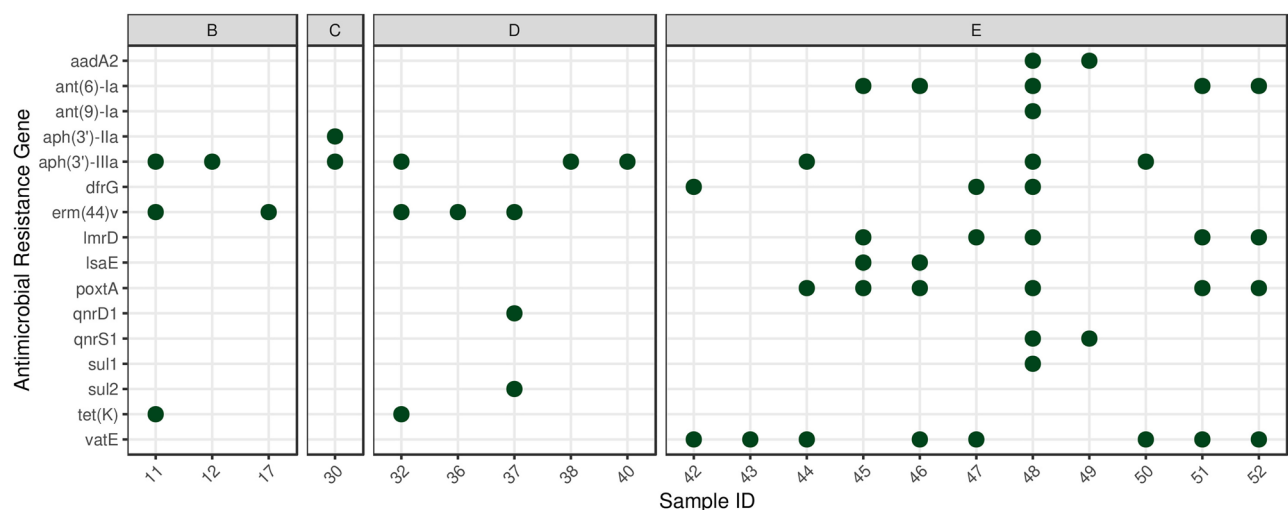


Figure 2. Identified antimicrobial resistance genes (ARGs) by samples. Perfect ARG matches were plotted by samples. The data of the PRJNA495415 taken on days 0, 7, 14 and 28 were classified in groups A, B, C and D, respectively. All samples from BioProject PRJNA764355 were assigned to group E.

Bacteria	ARG(s)	Drug class
<i>Acinetobacter baumannii</i>	aadA2	Aminoglycoside
<i>Amyllobacillus amylophilus</i>	ant(6)-Ia	Aminoglycoside
<i>Bacillus subtilis</i>	aph(3')-IIa	Aminoglycoside
<i>Cronobacter</i> sp. JZ38	qnrS1	Fluoroquinolone
<i>Enterobacter hormaechei</i>	aadA2, sul1	Aminoglycoside, sulfonamide
<i>Enterococcus faecalis</i>	aph(3')-IIIa	Aminoglycoside
<i>Enterococcus faecium</i>	poxtA	Lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>Escherichia coli</i>	sul2	Sulfonamide
<i>Gracilibacillus</i> sp. SCU50	dfrG	Diaminopyrimidine
<i>Lactocaseibacillus manihotivorans</i>	ant(6)-Ia	Aminoglycoside
<i>Lactocaseibacillus paracasei</i>	poxtA	Lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>Lactiplantibacillus plantarum</i>	aph(3')-IIIa, poxtA, vatE	Aminoglycoside, lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>Lactococcus lactis</i>	lmrD	Lincosamide
<i>Levilactobacillus brevis</i>	poxtA	Lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>Ligilactobacillus acidiphilicis</i>	ant(9)-Ia	Aminoglycoside
<i>Providencia rettgeri</i>	qnrD1	Fluoroquinolone
<i>Staphylococcus aureus</i>	ant(6)-Ia, tet(K)	Aminoglycoside, tetracycline
<i>Staphylococcus carnosus</i>	erm(44)v	Lincosamide, macrolide, streptogramin
<i>Staphylococcus pseudoxylus</i>	erm(44)v	Lincosamide, macrolide, streptogramin
<i>Staphylococcus saprophyticus</i>	erm(44)v	Lincosamide, macrolide, streptogramin
<i>Streptococcus suis</i>	lsaE	Lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>Tetragenococcus halophilus</i>	aph(3')-IIIa	Aminoglycoside
<i>Weissella paramesenteroides</i>	ant(6)-Ia	Aminoglycoside

Table 1. Identified ARGs and the drug classes affected by them per bacterial species of origin.

Discussion

Throughout our study, numerous perfect ARG matches were identified in the metagenome of *Medicago* silage samples. All but group A of the analyzed subsets had at least one sample containing one or more ARG. Among the PRJNA495415 Bioproject samples, the highest number of ARGs were found in group D. The interpretation of this finding is limited due to the lack of detailed information on the samples. Interestingly, all but one of the PRJNA764355 bioproject samples contained ARGs. Due to the lack of metadata, it is hard to find any reason

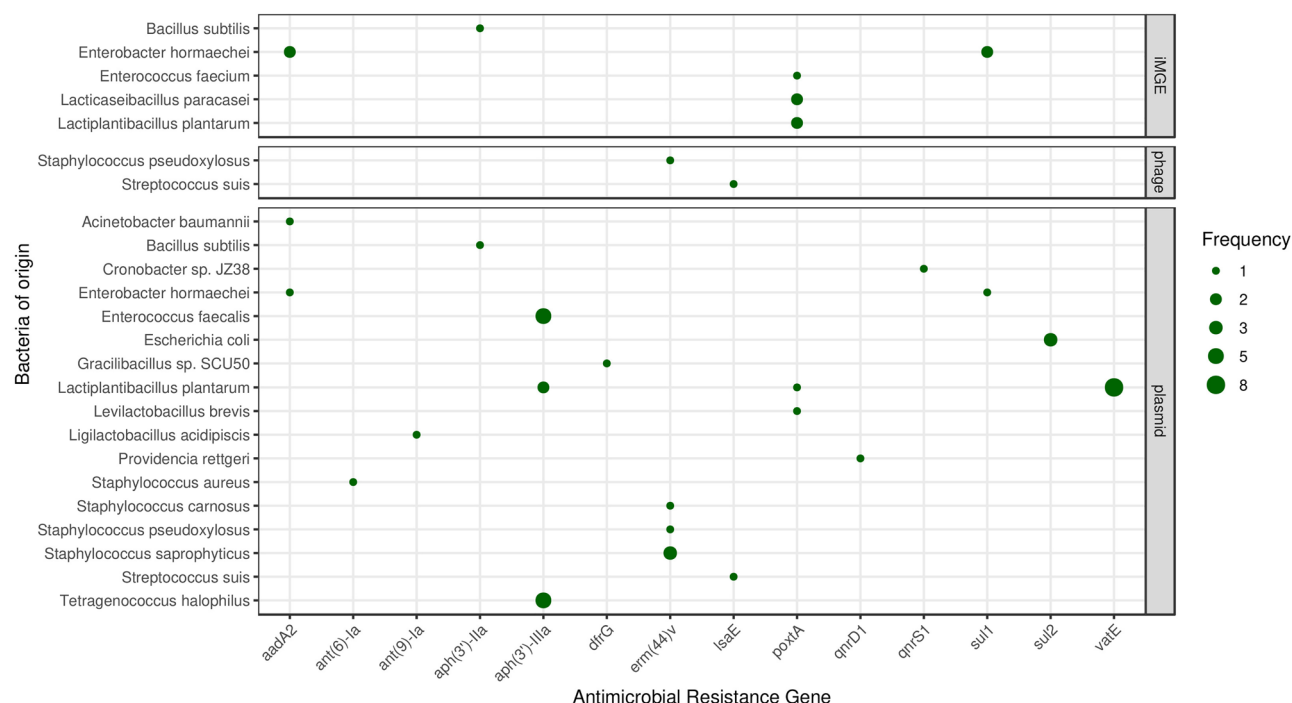


Figure 3. Mobile antimicrobial resistance gene frequency by bacteria of origin. The size of the dots indicates the occurrence frequency of the ARGs flanked by iMGE, positioned in a plasmid or a phage.

for this high ARG level. However, one possible cause might be that the PRJNA764355 samples were sequenced deeper and thus contained approximately 1.3 times more reads than the PRJNA495415 samples. It is known from previous studies that deeper sequencing leads to the generation of more complete genes by the de novo assembly^{5,7}.

In the following, our results will be interpreted from a perspective of bacteriological significance, genomic relevance and furthermore, antimicrobial stewardship and possible clinical aspects.

Taking the microbiome into consideration, bacteria that were predicted to harbor the identified ARGs can be classified according to their presence in silage. In the literature, the following bacteria are mentioned to be characteristic for silage: *B. subtilis*⁸, *E. faecium*⁹, *E. coli*¹⁰, *L. plantarum*¹¹, *L. lactis*¹², *L. brevis*^{13–15}, *L. acidiphilic*¹², *W. paramesenteroides*¹⁶. The genera of these species dominate the bacteriome of the samples. The identified *Cronobacter* sp. JZ38¹⁷ may be of plant origin. However, it can be assumed that other species may be present as contaminants of the silage: *A. amylophilus*, *E. hormaechei*, *E. faecalis*, *Gracilibacillus* sp. SCU50, *L. manihotivorans*, *L. paracasei*, *P. rettgeri*, *S. aureus*, *S. carnosus*, *S. pseudoxylus*, *S. saprophyticus*, *S. suis*, *T. halophilus*. Nevertheless, some of these bacteria are members of the *Lactobacillaceae* family, the *Leuconostoc* or *Enterobacter* genera. Numerous species of these groups are typical for fermented food and feed components.

From a genomic point of view, the following was found in the literature regarding the co-occurrence of the ARGs identified in our study and the bacteria carrying them. *AadA2* encoding an aminoglycoside nucleotidyltransferase has been described in *A. baumannii* in former publications^{18,19}. *ant(6)-Ia*, that is an aminoglycoside nucleotidyltransferase gene, appears in many species, including *Lactobacillus* spp.²⁰. Its species-specific association with *A. amylophilus* has not been described in any former publications. *aph(3')-IIa*, an aminoglycoside phosphotransferase²¹, to our knowledge, has not been detected in *B. subtilis* up until now. *QnrS1* encoding a quinolone resistance protein was originally identified in *Shigella flexneri*²². In line with our results, this gene has recently been mentioned to appear in *Cronobacter* spp. in a case report²³. *E. hormaechei* deriving *aadA2* and *sul1*, a sulfonamide resistant dihydropteroate synthase gene that is described to appear in Gram-negative bacteria²¹ have been reported to appear in the genom of *Enterobacter* spp. and *E. hormaechei*, respectively in former publications as well^{24,25}. Within the *Enterococcus* genus, two perfect ARG matches were identified, namely *aph(3')-IIIa* in *E. faecalis* and *poxA* in *E. faecium*. *aph(3')-IIIa* is an aminoglycoside phosphotransferase that normally appears in *S. aureus*²¹ and *Enterococcus* spp.²⁶, while *poxA* is a gene encoding an ABC-F subfamily (ATP-binding cassette-F) protein that facilitates resistance to tetracycline, phenicol, and oxazolidinone via modification of the bacterial ribosome. First detection of *poxA* took place in a methicillin-resistant *S. aureus* strain²¹, followed by other bacterial species, including *E. faecium*²⁷. *Sul2*, a sulfonamide resistant dihydropteroate synthase of Gram-negative bacteria is commonly described in *E. coli*^{21,28}. *DfrG* is a plasmid-encoded dihydrofolate reductase²¹ that, to our knowledge, has not been described in *Gracilibacillus* spp. up until now, but has already appeared in the *Bacillaceae* family²⁹. *ant(6)-Ia*, an aminoglycoside nucleotidyltransferase gene appears in many species, including *Lactobacillus* spp.²⁰. Its species-specific association with *L. manihotivorans* has not been described in any publications. *PoxA* that was detected in *L. paracasei*, *L. plantarum* and *L. brevis* in the silage samples, has been described to appear in *Lactobacillaceae*, namely *L. acidiphilus*, but not in these very species³⁰. Another

species that was detected harboring *aph(3')-IIIa* in the silage samples was *L. plantarum*. This finding is in line with the ARG-species match results mentioned in former publications³¹. Furthermore, *L. plantarum* was also associated with *vatE* that encodes an acetyltransferase conferring resistance against streptogramins²¹. *VatE* was originally found in *E. faecium*²¹ and has since then been identified in *Lactobacillaceae*³², but not specifically in *L. plantarum*. *L. acidipiscis ant(9)-Ia*, an aminoglycoside nucleotidyltransferase gene²¹ was associated with this genus for first within this study. Gene *qnrD1* encoding a quinolone resistance protein that is normally detected in *Salmonella enterica*²¹, has already been found in *Providencia* spp.³³ and was attached to *P. rettgeri* in our study as well. *S. aureus* could have been associated with two ARGs, *ant(6)-Ia* and *tetK* encoding a tetracycline efflux protein, that are both common findings in *Staphylococcus* spp.^{34,35}. Although, *erm(44)v* was first detected in the *S. saprophyticus*³⁶, no literature could be found about the appearance of this gene in *S. carnosus* or in *S. pseudoxylus* species. *IsaE* encoding another ABC-F subfamily protein conferring resistance to pleuromutilin, lincosamide, and streptogramin A is a common finding in *Streptococcus* spp.³⁷ and has also been associated with *S. suis* in previous publications³⁸. Besides the bacterial species mentioned above, *aph(3')-IIIa* was also detected in *T. halophilus*. This ARG is often appears in *Enterococcaceae*²¹ but has not yet been written down in this species. Furthermore, to our knowledge, *W. paramesenteroides* associated *ant(6)-Ia* has first been detected in this study.

Throughout our study, several ARGs were predicted to be co-occurring with genetic attributes facilitating mobility. The bioinformatic analysis of the mobility characteristics relied upon the identification of three major mobility determination groups, namely iMGEs, phages and plasmids. We found *aph(3')-IIa* linked to an integrated mobile genetic element in *B. subtilis* that is in line with similar findings of *E. coli*.²¹ While *aadA2* and *sul1* have both been described to appear on plasmids in *E. hormaechei*³⁹, we found them associated with iMGEs. Our finding on iMGE flanked *poxtA* in *E. faecium* is in line with the current literature⁴⁰. We found the same co-occurrence, namely *poxtA* and an iMGE, in *L. paracasei*. This phenomenon has not been published in that species to the best of our knowledge. Gene *erm(44)v* and *IsaE* were associated with prophages in *S. pseudoxylus* and *S. suis*. While a similar linkage can be found in the literature in connection with *erm(44)v*⁴¹, no details of mobility characteristics are mentioned in a recent report of the latter gene³⁸. All other mobile ARGs were detected on contigs that were predicted to derive from plasmids. In case of *aadA2* in *A. baumannii*²¹; *aadA2* and *sul1* in *E. hormaechei*²¹; *aph(3')-IIIa* in *E. faecalis*²¹; *sul2* in *E. coli*²¹; *aph(3')-IIIa* in *L. plantarum*⁴²; *qnrD1* in *P. rettgeri*²¹; *ant(6)-Ia* in *S. aureus*²¹ and *erm(44)v* in *S. saprophyticus*⁴¹ plasmid associations have been formerly described in the literature. To our knowledge, no publications have yet been released on the plasmid occurrence of *aph(3')-IIa* in *B. subtilis*, *dfrG* in *Gracilibacillus* sp. SCU50, *ant(9)-Ia* in *L. acidipiscis*, *IsaE* in *S. suis*, *qnrS1* in *Cronobacter* sp. JZ38, *erm(44)v* in *S. carnosus* and *S. pseudoxylus*. Hao et al. described *poxtA* embedded in a multi-resistance plasmid with mobile elements flanking in *E. fecalis*. This gene has been found in a number of Gram-positive bacteria, including enterococci as well, but it has neither been identified in *L. plantarum* nor *L. brevis*⁴³. Previous findings confirm the occurrence of *vat(E)* on plasmids⁴⁴. Nevertheless, in spite of its frequent presence in enterococci⁴⁵ there is no evidence of its former plasmid-associated appearance in *L. plantarum*. We found that gene *aph(3')-IIIa* of *T. halophilus* was encoded on a plasmid that is consistent with the fact that *aph(3')-IIIa* is often identified on high molecular weight plasmids and chromosomes of the enterococcal species⁴⁶. Nonetheless, to the best of our knowledge, a description of the *aph(3')-IIIa* gene in *T. halophilus* is a pioneer finding.

The mobility characteristics of the ARGs may not only provide us with information regarding the public health risk that may be associated with the samples, but also point to the possible origins of the genes. Regardless of human intervention, ARGs are present in the microbial communities⁴⁷. However, antimicrobial use and abuse intensifies the horizontal transfer of ARGs and thus contributes to the spread of AMR. In the animal production sector, the use of antibiotics is common, thus bacteria appearing in the feces and in the surroundings of the animals (e.g. in farm air, on tools, vehicles or other settings related to animals) often harbor bacteria with an advanced ARG set. Silage may get in direct physical contact with these bacteria at the farms and thus get contaminated with a few ARGs. Consequently, the presence of ARGs in the silage samples was well-expected, but the abundance of resistance genes and MGEs may increase due to the application of antibiotics.

Examining further aspects of antimicrobial stewardship and possible clinical relevance, phenotypical manifestations and public health considerations associated with the detected ARGs are both important. Intense antimicrobial use (AMU) can be associated with the headway of AMR, as antibiotic pressure selects for bacteria carrying ARGs that facilitate bacterial survival. Quantitatively, the majority of AMU around the globe occurs in agricultural settings^{48,49}. Intensive farming, that serves to fulfill the high global demand for animal proteins relies on an antibiotic infrastructure to treat and prevent disease and occasionally, to increase feed efficacy. In order to maximize economic gains, few countries still apply regulations that facilitate the use of low doses of antibiotics as growth promoters⁵⁰, while other regions, like the U.S. or Europe, have banned this practice. Nevertheless, besides the treatment of symptomatic infectious diseases, antibiotics are still widely used in the livestock sector for metaphylactic and prophylactic purposes in higher doses^{51,52}. Even though, compared to the poultry and pig production sector, average antibiotic usage has relatively lower rates by cattle⁵³, antimicrobial compounds are often chosen in this species as well. In cattle farming, mastitis is the most predominant reason for the administration of antibiotics by adult cattle, while enteritis and pneumonia is the most common reason for calves^{54,55}. According to various reports and studies from around the world^{54,56,57} tetracyclines are of inevitable significance in the medication of cattle, while beta-lactams, macrolides, sulfonamides, lincosamides and ionophore antibiotics are also very widely used. Of the European Medicines Agency (EMA) Highest-Priority Critically Important Antibiotics (HPCIA), namely third and fourth generation cephalosporins, fluoroquinolones and polymyxins, polymyxins and fluoroquinolones are the most applied, although their sales rates are still far below the most frequently administered antibiotic groups by livestock species⁵⁷. In our samples *E. faecium*, *L. paracasei*, *L. plantarum*, *L. brevis*, *S. aureus* and *S. suis* harbored genes, namely *poxtA* and *IsaE* that may confer resistance against multiple antibiotic groups, including tetracyclines. Moreover, *poxtA* was detected in the proximity of iMGEs in *L. paracasei* and *E. faecium* and harbored on a plasmid in *L. brevis*. In line with our findings, *Enterococcus* species related

to cattle were heavily associated with tetracycline resistance by other authors too⁵⁸. At some species *poxA* and *lsaE* were even predicted to co-occur with more than one MGE type. In the genome of *L. plantarum* *poxA* was predicted to be positioned on a plasmid and associated with an iMGE, while *S. suis* associated *lsaE* was located on a plasmid attached to a phage. Such genetic features may contribute to the horizontal transfer of ARGs among bacteria which is of outstanding clinical relevance in the case of such a commonly applied antibiotic group in cattle medicine, as tetracyclines. Perfect matches of genes conferring resistance against other clinically significant antibiotic groups, such as macrolides and sulfonamides were also identified in the genome of *E. hormaechei*, *E. faecium*, *E. coli*, *L. paracasei*, *L. plantarum*, *L. lactis*, *L. brevis*, *S. carnosus*, *S. pseudoxylus*, *S. saprophyticus* and *S. suis*. Of these genes, *lsaE*, *sul1*, *sul2* and *poxA* were even predicted to have enhanced mobility due to their association with multiple MGE groups. The only perfect match for an ARG against HPClAs, *qnrS1*, that can confirm resistance against fluoroquinolones, has been detected in a *Cronobacter* spp. The presence of several ARGs presumably associated with iMGEs in the feed of dairy cows harbors the potential to affect the resident microbiota of the animals. As *B. subtilis* and *E. faecium* frequently appear in probiotics for cattle^{59,60} it is possible that some microorganisms colonize niches in the foregut and proliferate the ARGs they possess. However, even if they cannot reproduce in the ruminal environment, ARGs can still be disseminated through horizontal gene transfer, especially in the presence of antibiotic therapy. Furthermore, ARGs can possibly spread further, to lower gastrointestinal (GI) regions. Fecal microbiota transfer administered to the stomach could restore the microbial population of the colon in human patients⁶¹, indicating a high volume of viable bacteria reaching the distal regions. Similar results were found in cattle with rumen microbiota transplantation affecting the microbial population of the hindgut⁶². If ARGs spread all around the GI tract, serious animal and public health concerns could be raised. Among enteric diseases, salmonellosis is the major indication of antibiotic therapy in dairy cattle. Enhanced antibiotic resistance of these bacteria could contribute to the economic loss from the disease as many strains already exhibit resistance to several antibiotics⁶³. Furthermore, during pathological conditions, like ruminal acidosis, bacteria can translocate to distant locations in the host's body. Interestingly bacterial translocation was even described in the absence of GI diseases in case of specific microorganisms in humans and rodents^{64,65}.

As a consequence of the colonization and possible ARG proliferation processes, pathologies caused by phenotypically resistant bacteria can induce animal welfare and economic issues. Animals harboring ARGs in their gut can contaminate their environment with ARGs through fecal matter as well as farm workers who get in direct contact with the animals, even consumers of dairy products can be affected, as farm animal-borne bacteria that harbor potentially mobile ARGs^{66–68} might be distributed by products intended for human consumption. For instance, we have previously found ARGs in raw milk samples provided for human consumption⁶⁹. Fecal contamination during milking⁷⁰ is a possible way of ARG transfer into raw milk, however other routes are also possible. In humans and in rodents for instance, maternal mononuclear cells transfer microorganisms to milk during lactation^{66,67}. The possibility for this phenomenon was described in cattle as well⁶⁸.

ARGs that are transferred to the human body through these routes might decrease the efficacy of antibiotic therapy. In order to gain a deeper insight into the exact role of silage in possible ARG transmission processes, many points still need to be examined and clarified. It would be essential to analyse the colonization success of ARG harboring silage-borne bacteria that enter the body of animals and the extent of ARG transfer of invasive donor bacteria to recipient bacteria living in the gastrointestinal tract. The silage involved in the study is of *Medicago* origin, and our results are based on data from only two projects. Hence, it would also be necessary to investigate the ARG content of other alfalfa and corn silages.

Antimicrobial resistance is an emerging global threat to public health that both affects agriculture and the healthcare sector. The usage of antibiotics in livestock species exceeds the rate of human applications⁷¹. Antibiotic use in food animal medicine is also considered a risk as it may provide an indirect transfer route of antibiotic residual⁷² ARGs via the food chain⁷³. Even though antimicrobials administered for veterinary use, may exert an undesired effect on the food chain, the presence of ARGs in dairy cattle nutrition research is still under-represented in the literature. According to our results, microbial mass contained in fermented feeds have other medical risks than transmitting contagious diseases, like listeriosis⁷⁴. The bacterial content of these mass feeds, that is either, required for the fermentation processes or collected from various sources of contamination on the farms, could play an essential role in the ARG shift through the food chain.

Materials and methods

Data. We searched appropriate datasets in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository. In December 2021, only two shotgun metagenomic BioProjects (PRJNA495415⁷⁵, PRJNA764355) could have been found that had adequate depth for the de novo assembly that our study is based on. The median read count (interquartile range, IQR) of the samples was 26.5×10^6 (3.0×10^6) and 34.7×10^6 (1.5×10^6) in datasets PRJNA495415 and PRJNA764355, respectively. There is limited metadata available of the samples in the NCBI SRA database (Table 2). Nevertheless, it can be assumed from the metadata that the samples of PRJNA495415 were taken at different fermentation periods. Samples were taken on days 0, 7, 14 and 28 were classified in groups A, B, C and D, respectively. Based on metadata of PRJNA764355 samples, no such stratification was possible, so all samples were classified as group E.

Bioinformatic analysis. Quality based filtering and trimming of the raw short reads was performed with TrimGalore (v0.6.6, <https://github.com/FelixKrueger/TrimGalore>), setting 20 as a quality threshold. Only reads longer than 50 bp were retained and taxonomically classified using Kraken2 (v2.1.1)⁷⁶ and a database created (24/03/2021) from the NCBI RefSeq complete archaeal, bacterial, viral and plant genomes. For this taxon assignment the -confidence 0.5 parameter was used to obtain more precise species level hits. The taxon classification data was managed in R⁷⁷ using functions of the packages phyloseq⁷⁸ and microbiome⁷⁹. The preprocessed reads

BioProject				PRJNA495415				PRJNA764355	
Group	A		B		C		D		E
Id	Run	Id	Run	Id	Run	Id	Run	Id	Run
1	SRR7990583	11	SRR7990582	21	SRR7990580	31	SRR7990581	41	SRR16036389
2	SRR7990587	12	SRR7990586	22	SRR7990585	32	SRR7990584	42	SRR16036390
3	SRR7990591	13	SRR7990590	23	SRR7990589	33	SRR7990588	43	SRR16036391
4	SRR7990592	14	SRR7990593	24	SRR7990594	34	SRR7990595	44	SRR16036392
5	SRR7990598	15	SRR7990599	25	SRR7990596	35	SRR7990597	45	SRR16036393
6	SRR7990604	16	SRR7990605	26	SRR7990600	36	SRR7990601	46	SRR16036394
7	SRR7990608	17	SRR7990609	27	SRR7990602	37	SRR7990603	47	SRR16036395
8	SRR7990610	18	SRR7990611	28	SRR7990606	38	SRR7990607	48	SRR16036396
9	SRR7990612	19	SRR7990613	29	SRR7990614	39	SRR7990615	49	SRR16036397
10	SRR7990616	20	SRR7990617	30	SRR7990618	40	SRR7990619	50	SRR16036398
								51	SRR16036399
								52	SRR16036400

Table 2. Analyzed samples. The samples of dataset PRJNA495415 were taken on days 0, 7, 14 and 28 were classified in groups A, B, C and D, respectively. All samples from BioProject PRJNA764355 are assigned to group E. Column Run contains the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) run identifiers.

were assembled to contigs with MEGAHIT (v1.2.9)⁸⁰ using default settings. The contigs were also classified taxonomically with Kraken2 with the same database as above. From the contigs all possible open reading frames (ORFs) were gathered with Prodigal (v2.6.3)⁸¹. The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v3.1.3)^{21,82} by Resistance Gene Identifier (RGI, v5.2.0) with Diamond⁸³. ORFs having a perfect match against the CARD database were exclusively kept for further analysis. Integrative mobile genetic element (iMGE) content of contigs harboring ARG was analyzed with MobileElementFinder (v1.0.3) and its database (v1.0.2)⁶. Following the distance concept of Johansson et al.⁶ for each bacterial species, only those with a distance threshold defined within iMGEs and ARGs were considered associated. In the MobileElementFinder database (v1.0.2) for *E. hormaechei*, the longest composite transposon (cTn) was the *Tn3000*. In case of this species, its length (11,823 bp) was taken as the cut-off value. For *E. faecium*, this threshold was the length of the *Tn6246* transposon, namely 5,147 bp. As the database neither contains species-level, nor genus-level cTn data for *Bacillus*, *Lactiplantibacillus* and *Lactocaseibacillus* species, a general cut-off value was chosen for the contigs of these species. This value was declared as the median of the longest cTns per species in the database (10,098 bp). The plasmid origin probability of the contigs was estimated by PlasFlow (v1.1)⁸⁴. The phage content of the assembled contigs was predicted by VirSorter2 (v2.2.3)⁸⁵. The findings were filtered for dsDNAPhages and ssDNAs. All data management procedures, analyses and plots were performed in R environment (v4.1.0)⁷⁷.

Data availability

The datasets analysed in the current study are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository and can be accessed through the PRJNA495415 and PRJNA764355 BioProject identifiers.

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Author contributions

N.S. takes responsibility for the integrity of the data and the accuracy of the data analysis. A.G.T., M.P., N.S. and S.Á.N. conceived the concept of the study. A.G.T., M.P., N.S. and S.Á.N. participated in the bioinformatic analysis. A.G.T., K.S., M.P., N.S. and S.Á.N. participated in the drafting of the manuscript. A.G.T., K.S., M.P., N.S. and S.Á.N. carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Antimicrobial resistance gene lack in tick-borne pathogenic bacteria

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Tick-borne infections, including those of bacterial origin, are significant public health issues. Antimicrobial resistance (AMR), which is one of the most pressing health challenges of our time, is driven by specific genetic determinants, primarily by the antimicrobial resistance genes (ARGs) of bacteria. In our work, we investigated the occurrence of ARGs in the genomes of tick-borne bacterial species that can cause human infections. For this purpose, we processed short/long reads of 1550 bacterial isolates of the genera *Anaplasma* (n = 20), *Bartonella* (n = 131), *Borrelia* (n = 311), *Coxiella* (n = 73), *Ehrlichia* (n = 13), *Francisella* (n = 959) and *Rickettsia* (n = 43) generated by second/third generation sequencing that have been freely accessible at the NCBI SRA repository. From *Francisella tularensis*, 98.9% of the samples contained the FTU-1 beta-lactamase gene. However, it is part of the *F. tularensis* representative genome as well. Furthermore, 16.3% of them contained additional ARGs. Only 2.2% of isolates from other genera (*Bartonella*: 2, *Coxiella*: 8, *Ehrlichia*: 1, *Rickettsia*: 2) contained any ARG. We found that the odds of ARG occurrence in *Coxiella* samples were significantly higher in isolates related to farm animals than from other sources. Our results describe a surprising lack of ARGs in these bacteria and suggest that *Coxiella* species in farm animal settings could play a role in the spread of AMR.

Diseases of bacterial origin are form a significant portion of public health issues. Vector-borne pathogens are gaining further importance as climate change, urbanization, and globalization affect the population size and geographical distribution of various pathogen vectors¹. Furthermore, antimicrobial resistance (AMR) of bacteria is often referred to as one of the main challenges of the 21st century². Several studies prove the widespread dissemination of antimicrobial resistance genes (ARGs) in various environmental or alimentary sample types^{3–8}. Vector-borne infections are an important and widespread aspect of microbial diseases. Indeed, around 80% of the human population of the Earth is estimated to be at risk of one or more vector-borne pathogens (VBPs)⁹. Since many of these VBPs are bacterial, treating a set of vector-borne infections relies upon the efficacy of antibiotics. Despite the potential and relatively dynamic changes expected in the spread of both AMR and VBPs in the coming decades, the associations of these global health issues are less studied.

Besides such significant arthropod pathogen vectors as mosquitoes, sandflies, blackflies, fleas, lice, tsetse flies, or triatome bugs, soft and hard ticks also serve as vectors for pathogenic microorganisms of human and veterinary medical significance^{9,10}. Considering that the spectrum of tick-borne bacterial pathogens is relatively wide, the assessment of the antimicrobial resistance status of tick-borne pathogens, such as the members of the genera *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella* and *Rickettsia* is an anticipatory public health step^{11–17}.

Our study aimed to obtain detailed knowledge of the ARG spectrum of the bacteria that could potentially cause the primary tick-borne infections. Therefore, whole-genome sequencing (WGS) data of bacterial isolates belonging to the genera of *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, *Rickettsia* were processed using a uniform bioinformatics pipeline⁸.

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Materials and methods

Samples. The available samples and corresponding sequencing data for each genus were retrieved and downloaded from the SRA database on the following days: *Anaplasma* 2022 Aug 16, *Bartonella* 2022 Aug 19, *Borrelia* 2022 Aug 21, *Coxiella* 2022 Aug 27, *Ehrlichia* 2022 Aug 12, *Francisella* 2022 Aug 12, *Rickettsia* 2022 Aug 12. Of the downloaded samples, the number of WGS samples per genus was as follows: *Anaplasma*: 40, *Bartonella*: 251, *Borrelia*: 707, *Coxiella*: 155, *Ehrlichia*: 16, *Francisella*: 1088, *Rickettsia*: 128.

For further analysis, the raw sequencing data from Illumina, Nanopore or PacBio platforms were considered only. Moreover, the analysis was restricted to samples from species of potential tick-borne spread and potentially associated human diseases. Due to the limited resources on the pathogenic potential of each bacterial species examined in the analysis, we have considered them as potentially pathogenic if information was found that they were isolated from humans. De novo assembly was performed on the sequencing data of these samples, followed by the prediction of the ARG content of the generated contigs. Only samples with an available representative genome at the NCBI database were included with the condition that sequencing coverage reached at least 95% of the genome. After the selection steps, a total of 1550 samples (*Anaplasma*: 20, *Bartonella*: 131, *Borrelia*: 311, *Coxiella*: 73, *Ehrlichia*: 13, *Francisella*: 959, *Rickettsia*: 43) were left for analysis (BioSample IDs of the samples at each genus considered for the analysis can be found in Supplementary File 1 and 8).

Bioinformatic analysis. For Illumina sequenced data, the quality-based filtering and trimming of the raw short reads was performed by TrimGalore (v0.6.6, <https://github.com/FelixKrueger/TrimGalore>), setting 20 as the quality threshold. Only reads longer than 50 bp were retained. Nanopore sequenced reads were adapter trimmed and quality-based filtered by Porechop (v0.2.4, <https://github.com/rrwick/Porechop>) and Nanofilt (v2.6.0)¹⁸, respectively. The long reads from the PacBio sequencing platform were prepared for assembly using the pbclip (<https://github.com/fenderglass/pbclip>) tool.

The Illumina reads were assembled to contigs by MEGAHIT (v1.2.9)¹⁹ using default settings. The assembly of the long read sequencing data was performed with Flye (v2.9-b1779)²⁰, and 3x round polishing was applied with Racon (v1.4.3)²¹ on the resulting contigs. In the case of hybrid sequencing, the sequenced reads were assembled by MaSuRCA (v4.0.9)²².

All possible open reading frames (ORFs) were predicted by Prodigal (v2.6.3)²³ on each contig. The protein-translated ORFs were searched for ARG sequences against the Comprehensive Antibiotic Resistance Database (CARD, v3.2.3)^{24,25} by the Resistance Gene Identifier (RGI, v5.2.0), running with Diamond²⁶ as the aligner. To capture ARGs with high-confidence rates for the following steps of the analysis, ORFs classified as perfect or strict matches were further filtered by 90% identity and 90% coverage thresholds.

ARGs predicted by RGI were further screened for potential associated mobile genetic elements to assess their potential for participation in Horizontal Gene Transfer (HGT) events. Contigs, throughout which ARGs were identified, were screened for the probability of plasmid origin by PlasFlow (v1.1)²⁷. The prediction of integrative mobile genetic elements (iMGE) on the contigs harboring ARGs were assessed by the MobileElementFinder (v1.0.3) and its database (MGEdb, v1.0.2)²⁸. An ARG was considered to be associated with an iMGE if it was within the distance of the median for the longest composite transposon stored for each bacterial species in the MGEdb database (distance threshold: 10098 bp). Bacteriophage sequences were predicted with VirSorter2 (v2.2.3)²⁹ and were filtered to dsDNA phage and ssDNA sequences only. ARGs within phage sequences at their whole length were considered to be associated with bacteriophages.

To mitigate the effect of the database and tool used for the ARG prediction on our results, we have further analysed them with two additional pipelines. AMRFinderPlus (v3.10.36)³⁰ is an alignment-based tool similar to RGI. We used AMRFinderPlus with a database (National Database of Antibiotic Resistant Organisms, NDARO, version 2022-05-26.1) without the SPSVERBc1 option to retain as much comparability as possible between its results and those from RGI and CARD. Similarly, the determined genes were filtered by 90% identity and 90% coverage thresholds. KmerResistance³¹ aligns raw reads to a user-defined database with the aid of the KMA aligner³² while also handling potential contamination. We have searched only the Illumina sequencing data from the samples considered in our analysis (for the samples included in the KmerResistance analysis, see Supplementary File 1) with KmerResistance (v2.0) against the CARD (v3.2.3) and NDARO (v2022-05-26.1) databases. Therefore, the number of samples analysed with KmerResistance as well was 1492. From the CARD database, only genes under the protein homolog models were used for the KmerResistance analysis. Results were filtered by 90% template coverage and 90% query identity thresholds. As the AMRFinderPlus analysis was run without the SPSVERBc1 option, virulence genes and genes conferring resistance to metal- and biocide compounds only were filtered from the KmerResistance results when run against the NDARO database. Data management and the figure generation were performed in the R-environment (v4.1.2)³³.

The association of the origin of the samples and the ARG positivity was analysed based on the metadata available for each biosample at the SRA database. The origin of samples was determined by the sample_origin and sample_host metadata columns. Based on this metadata information, the samples were manually sorted into the following categories: companion animal, environment, farm animal, human, vector, wild animal and NA when no information was available. Also, an NA category was used in the case of indicated hosts of frequently used laboratory animal species, as in those cases, we couldn't determine if the host and the sample were experimentally manipulated. Additionally, if any other metadata indicated the laboratory origin of the strain, the NA category was used for the sample origin.

Results

The ARG content of available WGS samples of human-pathogenic bacteria with possible tick-borne origin from the SRA database was analysed. After selecting samples with raw data of sufficient quality (for details, see [Materials and Methods](#)), 1550 samples remained for the ARG analysis. Samples represent species from 7 bacterial genera (*Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella* and *Rickettsia*), originating from at least 33 different countries. Even though most samples were supplemented with metadata of their country of origin, there were still 142 samples with this information missing. Countries with at least one sample included in our analysis are shown in Fig. 1. Predicted ARG content was analysed with RGI and CARD, a robust pipeline often employed to survey antimicrobial resistance genes in different environments^{3–7}. However, as resistance gene presence was rather scarce in the samples analysed in this study, we have included two additional methods for resistance gene prediction to augment our results. Even though we utilized these additional methodologies, we base our findings on the widely used and accepted RGI pipeline. Further information about the samples can be found in Supplementary File 1.

Resistance genes predicted by RGI and CARD. Even though we could include a large number of samples in our analysis, a surprisingly low number of resistance genes were predicted from the ORFs. Furthermore, the ARGs were distributed among only a handful of samples. The percentage of ARG positive samples was 0% for the *Anaplasma* and *Borrelia* genera, 1.5% for the *Bartonella* genus, 11.0% for the *Coxiella* genus, 7.7% for the *Ehrlichia* genus and 4.7% for the *Rickettsia* genus. The exception was the *Francisella* genus, where almost all samples carried at least one ARG (98.9%). The resistance genes predicted for each sample are shown in Supplementary File 2. Detected ARGs for each species are summarized in Table 1.

Although there is a high number of ARG-positive samples from the *Francisella* genus, a large part of them (791 samples) was predicted to contain only one ARG. Furthermore, all of these samples had the *FTU-1* gene as the one predicted resistance gene. *FTU-1* was very prevalent in the analysed *Francisella* samples as all positive ones harboured this gene (948 samples out of the total 959). This gene is a class A β -lactamase gene found in almost every *Francisella tularensis* genomes³⁴, including the reference genome of the species (based on the analysis with the same pipeline of the *F. tularensis* reference genome assembly available at NCBI: https://www.ncbi.nlm.nih.gov/assembly/GCF_000008985.1/). As a consequence of the high prevalence of this gene, we have additionally calculated the ratio for the *Francisella* samples containing ARGs without taking *FTU-1* gene into consideration. The percentage of positive samples without this gene is 16.3%, which is still the highest ratio found in our analysis. The number of ARG positive and negative samples can be found in Table 2. Supplementary File 2 shows all the resistance genes predicted by RGI for each positive biosample included in our analysis.

Mobility analysis. Contigs, where ARGs were predicted by RGI, were further analysed to determine if these genes could be associated with any mobile genetic elements. An ARG was considered potentially mobile if the contig it was found on was predicted to be of plasmid origin, if it was within a predicted phage sequence or if it was found within 10098 bp distance from the closest detected iMGE (for details on the mobile element determination and the selection of the cut-off value see [Materials and Methods](#)). The number of potentially mobile and

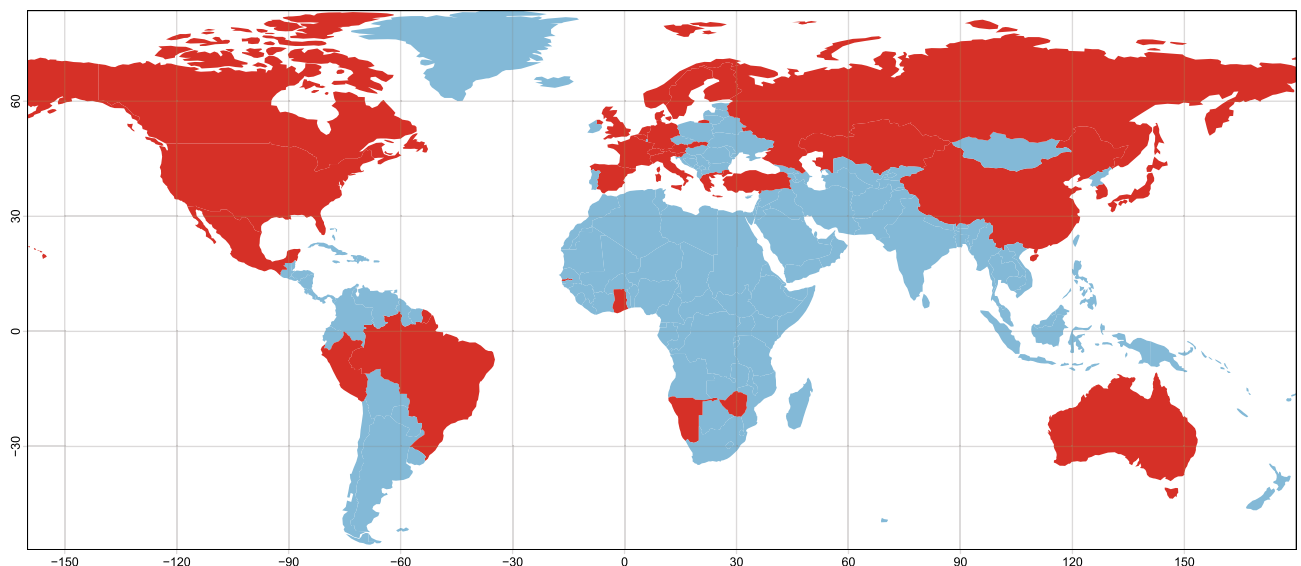


Figure 1. World map indicating the countries with at least one sample included in the analysis (red area). The countries with samples are the following: Australia, Austria, Belgium, Brazil, Canada, China, Denmark, Finland, France, Gambia, Germany, Ghana, Greece, Italy, Japan, Kazakhstan, Liechtenstein, Mexico, Namibia, Netherlands, Norway, Peru, Russia, Slovakia, Slovenia, South Korea, Spain, Sweden, Switzerland, Turkey, UK, USA, Zimbabwe. There were 142 samples without a designation on their country of origin.

Species	ARG
<i>Bartonella henselae</i>	PC1 β -lactamase (<i>blaZ</i>)
<i>Bartonella quintana</i>	APH(3')-IIIa, SAT-4
<i>Coxiella burnetii</i>	<i>AcrE</i> , <i>AcrS</i> , APH(3'')-Ib, APH(6)-Id, <i>ArmR</i> , <i>bacA</i> , <i>bcr-1</i> , CARB-16, <i>cpxA</i> , <i>emrA</i> , <i>emrB</i> , <i>emrK</i> , <i>emrY</i> , <i>ErmC</i> , <i>Escherichia coli</i> AcrAB-TolC with <i>MarR</i> mutations conferring resistance to ciprofloxacin and tetracycline, <i>E. coli</i> EF-Tu mutants conferring resistance to pulvomycin, <i>E. coli</i> <i>emrE</i> , <i>E. coli</i> <i>mdfA</i> , <i>E. coli</i> <i>soxR</i> with mutation conferring antibiotic resistance, <i>E. coli</i> <i>soxS</i> with mutation conferring antibiotic resistance, <i>E. coli</i> <i>UhpT</i> with mutation conferring resistance to fosfomycin, <i>evgA</i> , <i>FosA</i> , <i>gadW</i> , <i>gadX</i> , <i>marA</i> , <i>mdtE</i> , <i>mdtG</i> , <i>mdtP</i> , <i>MexI</i> , OXA-488, <i>Pseudomonas aeruginosa</i> <i>emrE</i> , <i>P. aeruginosa</i> <i>soxR</i> , TEM-116, <i>tet(44)</i> , <i>tet(H)</i> , TolC, <i>ugd</i>
<i>Ehrlichia ruminantium</i>	APH(3'')-Ib, APH(6)-Id
<i>Francisella tularensis</i> subsp. <i>holarctica</i>	AAC(6'')-II, <i>aad(6)</i> , <i>acrB</i> , <i>acrD</i> , <i>AcrE</i> , <i>AcrF</i> , <i>AcrS</i> , APH(3'')-IIIa, <i>bacA</i> , <i>baeR</i> , <i>baeS</i> , <i>cpxA</i> , <i>efmA</i> , <i>emrA</i> , <i>emrB</i> , <i>emrK</i> , <i>emrY</i> , <i>Enterococcus faecium</i> chloramphenicol acetyltransferase, <i>eptA</i> , <i>ErmB</i> , <i>E. coli</i> <i>acrA</i> , <i>E. coli</i> AcrAB-TolC with <i>AcrR</i> mutation conferring resistance to ciprofloxacin, tetracycline, and ceftazidime, <i>E. coli</i> AcrAB-TolC with <i>MarR</i> mutations conferring resistance to ciprofloxacin and tetracycline, <i>E. coli</i> <i>ampC</i> β -lactamase, <i>E. coli</i> EF-Tu mutants conferring resistance to pulvomycin, <i>E. coli</i> <i>emrE</i> , <i>E. coli</i> <i>gyrA</i> conferring resistance to fluoroquinolones, <i>E. coli</i> <i>mdfA</i> , <i>E. coli</i> <i>soxR</i> with mutation conferring antibiotic resistance, <i>E. coli</i> <i>soxS</i> with mutation conferring antibiotic resistance, <i>evgA</i> , FTU-1, <i>gadW</i> , <i>gadX</i> , <i>kdpE</i> , <i>Klebsiella pneumoniae</i> <i>KpnH</i> , <i>marA</i> , <i>mdtA</i> , <i>mdtB</i> , <i>mdtC</i> , <i>mdtE</i> , <i>mdtF</i> , <i>mdtG</i> , <i>mdtH</i> , <i>mdtM</i> , <i>mdtN</i> , <i>mdtO</i> , <i>mdtP</i> , <i>msbA</i> , <i>msrC</i> , <i>PmrF</i> , SAT-4, <i>tet(L)</i> , <i>tet(M)</i> , TolC, <i>ugd</i> , <i>vanA</i> , <i>vanH</i> gene in <i>vanA</i> cluster, <i>vanR</i> gene in <i>vanA</i> cluster, <i>vanS</i> gene in <i>vanA</i> cluster, <i>vanX</i> gene in <i>vanA</i> cluster, <i>vanY</i> gene in <i>vanA</i> cluster, <i>vanZ</i> gene in <i>vanA</i> cluster, <i>YojI</i>
<i>Francisella tularensis</i>	FTU-1
<i>Francisella tularensis</i> subsp. <i>mediasiatica</i>	FTU-1
<i>Francisella tularensis</i> subsp. <i>novicida</i>	FTU-1
<i>Francisella tularensis</i> subsp. <i>tularensis</i>	FTU-1, TEM-1, TEM-116
<i>Rickettsia conorii</i>	OXA-192
<i>Rickettsia japonica</i>	APH(3')-Ia

Table 1. Resistance genes detected for each species by RGI and CARD. In case of *Francisella tularensis*, the samples were listed at the subspecies level, where that information was available, otherwise, they were summarized at the species level.

Genus	Number of negative samples	Number of positive samples	Number of all samples
<i>Anaplasma</i>	20	0	20
<i>Bartonella</i>	129	2	131
<i>Borrelia</i>	311	0	311
<i>Coxiella</i>	65	8	73
<i>Ehrlichia</i>	12	1	13
<i>Francisella</i>	11	948	959
<i>Francisella</i> (without FTU-1)	803	156	959
<i>Rickettsia</i>	41	2	43

Table 2. ARG positive and negative samples by the genus of origin.

Genus	Number of mobile ARGs	Number of non-mobile ARGs
<i>Anaplasma</i>	0	0
<i>Bartonella</i>	1	2
<i>Borrelia</i>	0	0
<i>Coxiella</i>	24	31
<i>Ehrlichia</i>	2	0
<i>Francisella</i>	327	1359
<i>Francisella</i> (without FTU-1)	303	434
<i>Rickettsia</i>	2	0

Table 3. Number of mobile and immobile ARGs for each genus analysed.

immobile ARGs can be found for each genus in Table 3. Further details of mobile elements associated with each predicted ARG are presented in Supplementary File 2.

Comparing species and environment of origin between ARG positive and negative samples. Understanding the ecological environment where the samples included originated from is useful to gain a deeper insight into the potential factors underlying our results. The background of the samples, either positive or negative considering their ARG content, was assessed based on the origin of samples and species of the isolates. Samples were enrolled into origin categories based on their metadata (for details, see [Materials and Methods](#)). The ARG positive and negative sample distribution among the categories of origin are summarized in Table 4.

Interestingly, only samples originating from farm animals were positive to ARGs in the case of *Coxiella burnetii*, while such high dependence wasn't revealed for any other genus. To compare the association of positive samples to farm animal origins at *Coxiella* genus and all the remaining samples (excluding *Francisella*), Fisher's exact tests were performed on the contingency tables from Fig. 2. A significant difference was revealed in the case of *Coxiella* (OR: Inf, 95% CI 3.6–Inf, $p < 0.001$), however, such differences could not have been found in case of the rest of the samples (OR: Inf, 95% CI 0.01–Inf, $p > 0.9999$).

The species of origin of ARG positive and negative samples are also of a key importance to understand the presence of genotypic resistance among microorganisms from different ecological environments. The number of ARG positive and ARG negative samples from each species can be found in Supplementary File 3.

Genus	Companion animal	Environment	Farm animal	Human	Vector	Wild animal	NA
<i>Anaplasma</i>	0/2	0/0	0/9	0/6	0/1	0/0	0/2
<i>Bartonella</i>	1/11	0/0	0/0	1/86	0/0	0/2	0/30
<i>Borrelia</i>	0/0	0/0	0/0	0/47	0/254	0/1	0/9
<i>Coxiella</i>	0/0	0/1	8/19	0/6	0/15	0/1	0/23
<i>Ehrlichia</i>	0/0	0/0	0/0	0/7	1/1	0/0	0/4
<i>Francisella</i>	4/0	8/0	0/0	112/1	37/1	314/2	473/7
<i>Francisella</i> (without FTU-1)	0/4	0/8	0/0	2/111	0/38	0/316	154/326
<i>Rickettsia</i>	0/0	0/0	0/0	1/16	1/20	0/0	0/5

Table 4. Number of ARG positive/negative samples in different origin categories.

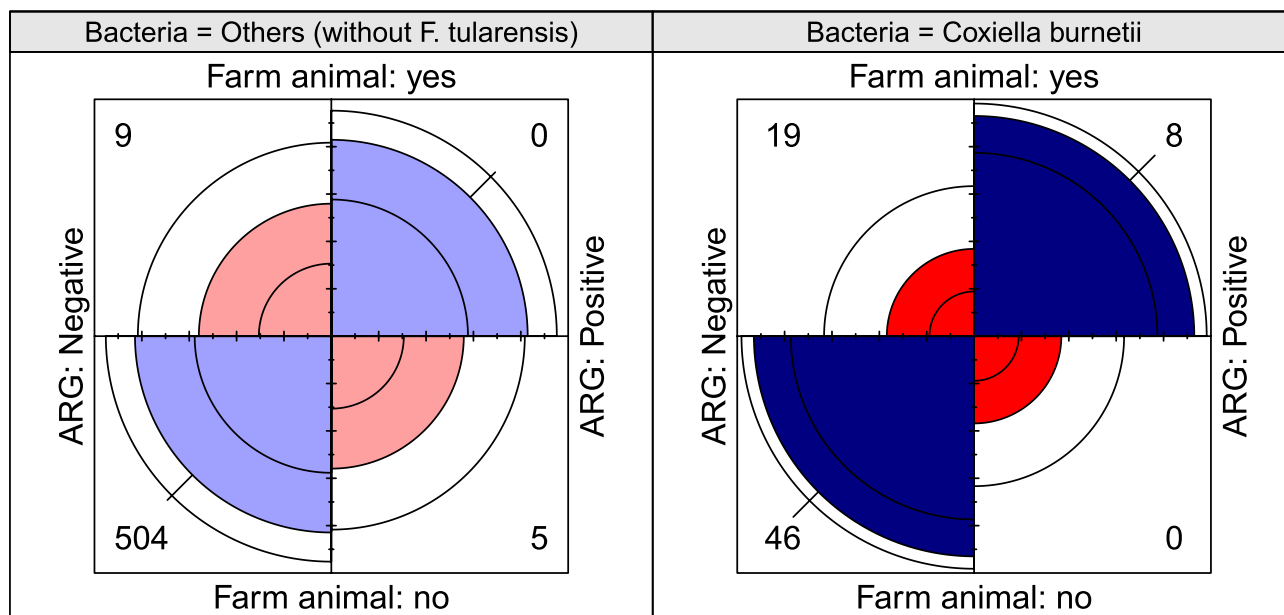


Figure 2. Fourfold displays showing the difference between ARG positivity and farm animal origin, in the case of *Coxiella burnetii* (right-hand side) and the rest of the samples (without *Francisella* genus, left-hand side). The plot is visualising the contingency tables for these categories³⁵. Numbers in quadrants correspond to the number of observed cases in that category. Quarter circles are proportional to the ratio of the counts associated with the diagonal frequencies after standardisation. A significant imbalance is apparent in the case of *Coxiella* based on the 95% confidence bands around the quadrants, as the Fisher's exact tests showed ($p < 0.001$) also (see the [Results](#) section for details).

Comparison between different methods. To understand the effect of the applied methods on our observations, we have complemented the analysis by including two additional methods. A similar approach to RGI is the NCBI's AMRFinderPlus pipeline which uses the NDARO database for ARG annotation. This tool utilizes a separate (the so-called plus database) for virulence-, metal- and biocide resistance genes. Even though CARD stores information regarding disinfectants, we have not used the plus genes in our analysis with AMRFinderPlus to keep as much consistency between the databases as possible. Furthermore, to compare the results from a pipeline with a fundamentally different approach, KmerResistance was also employed on the raw data with both the CARD and NDARO databases. For consistency to the analysis with AMRFinderPlus, plus genes were removed from the results of the KmerResistance analysis with the NDARO database. (Further details on the analysis pipelines are presented in the [Materials and Methods](#) section.).

Due to the inherent differences between databases and methods, their results are not directly comparable in a study like ours. However, to still augment the confidence of our findings, we compared the ARG positive samples among the four methods. Venn diagrams of ARG-positive samples from the so-called methodologies for each genus incorporated in the analysis can be found in Supplementary Fig. 1–8 (Supplementary File 4). Detailed ARG predictions from each method can be found in Supplementary Files 5–7. Results for *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia* and *Rickettsia* genera were in line by each employed method, with differences affecting only a few samples (Supplementary Fig. 1, 3–5 and 8 in Supplementary File 4). In the case of the genus *Bartonella*, KmerResistance with CARD and NDARO databases has predicted an additional 13 and 11 ARG positive samples compared to RGI, respectively (Supplementary Fig. 2 in Supplementary File 4). The short read-based approach predicted more positive samples in the case *Francisella* genus as well (when FTU-1 gene wasn't considered), where KmerResistance with CARD found 60 and KmerResistance with NDARO identified 34 ARG-positives more (Supplementary Fig. 7 in Supplementary File 4).

Discussion

We have revealed a lack of ARGs in the available and high-quality WGS samples of human-pathogenic and potentially tick-borne bacteria. *Francisella* showed the highest ratio of samples with at least one ARG (16.3%), however, ARG-positivity was generally very low in the rest of the analysed genera (only 2.2 %). This finding is surprising at first, considering the vast amount of antibiotic pollution and ARG prevalence of the natural and artificial environments^{3–7,36–38}. The effect of natural environments on the spread of antimicrobial resistance (AMR) is well represented by the fact that wild animals were found to harbour different ARGs^{39,40}, even though it might correlate with anthropogenic influence^{41,42}. Furthermore, it is well known that antibiotic resistance is not only dependent on human activity as its origins root long before the onset of clinical antibiotic usage^{43–45}. Considering this widespread occurrence of resistance, we believe that the potential factors influencing our findings deserve a closer look.

Our analysis has included data generated by second and third-generation sequencing technologies. Even though these methods have proven useful in numerous research areas, including antimicrobial resistance, they also have certain limitations. For example, if some genomic elements (e.g. ARGs) are absent from the sample, as in our case, only with sufficient sequencing depth can one conclude that true biological variation is the reason for the missingness⁴⁶. To prevent such artefacts in our results, we have limited our analysis to samples with at least 95% coverage to their respective representative genome. Furthermore, we have used a 90% coverage and identity threshold for filtering the raw ARG predictions for limiting potential false positive calls. Another potential source of bias in genome-based resistance gene surveillance studies is that the results can only depend on the currently available resistance gene resources and tools, that might have many differences among them⁴⁷. To mitigate these effects on our results, the samples included in our analysis were analysed with two additional tools and one additional database. NCBI's AMRFinderPlus tool with the NDARO database and the short read-based KmerResistance tool with both the CARD and NDARO databases were employed, besides the RGI with CARD database approach. These results indicate that RGI was highly concordant with the additional methods used for the analysis, even though KmerResistance has predicted more positive samples for the *Bartonella* and *Francisella* genera. It can be assumed that short-read technologies are more sensitive, which could explain why more positive samples were found with KmerResistance; however, these methods have not been systematically compared yet. Furthermore, it is worth noting that many of the analysed genera are intracellular bacteria, which might enhance the risk of contamination during sample collection and isolation procedures. Even though it might not be possible to exactly determine if a gene is originating from contamination, it would further highlight the lack of ARGs in the analysed bacteria.

Phenotypic resistance to various antibiotics has been described in the bacteria we have studied, but to the best of our knowledge, multi-drug resistant strains have not yet been found. Interestingly, the first choice for treatment of infections caused by the majority of bacteria included in our study is doxycycline^{14,48–56}. For *F. tularensis* aminoglycosides and fluoroquinolones are also commonly recommended⁵⁷. Resistance to fluoroquinolone antibiotics has been identified in *Bartonella*, *Borrelia* and *Ehrlichia* species^{50,55,56}. Nevertheless, fluoroquinolone resistance in *Ehrlichia* spp. is probably due to intrinsic effects⁵⁸. In *Ehrlichia*, resistance to macrolides, aminoglycosides and certain β -lactams has also been detected^{50,59}, which may be consistent with the aminoglycoside resistance genes we found in *E. ruminantium* (Table 1). In contrast to the lack of ARGs in *Borrelia* species involved in our study, β -lactam and rifampicin resistance have already been described for them^{54,55} (Table 1). However, it should be noted that using the two KmerResistance-based approaches, we did find an aminoglycoside resistance gene and an efflux pump in this group (*APH(3')-Ia*, *E. coli* *emrE*, Supplementary Files 6, 7). *C. burnetii* and *F. tularensis* samples were found to encode a wide range of resistance genes in our analysis (Table 1). While phenotypically manifested ciprofloxacin and chloramphenicol resistance have already been described in *C. burnetii*⁵², furthermore, resistance to penicillin has also been described⁶⁰, several strains appear

to show susceptibility to ampicillin⁵². Moreover, in *F. tularensis*, resistance to β -lactam, macrolides, linezolid and clindamycin was described previously^{61–63}.

However, it is important to note that the resistance gene content of the genome does not necessarily correlate with phenotypic resistance⁶⁴, and in any case, our results will be limited by the capacity of databases and tools^{47,65–67}. Furthermore, there are many factors affecting the in vivo susceptibility of a microorganism, compared to what was discovered in vitro^{56,68}. In addition, it should be noted that of the 156 ARG positive *F. tularensis* samples in our analysis (when the *FTU-1* gene was excluded), 153 were from BioProject PRJNA669398. In this BioProject, samples derived from the live vaccine strain of *F. tularensis* subsp. *holartica* (LVS) and were tested for the presence of evolutionary mutations under the pressure of doxycycline and ciprofloxacin antibiotics⁶⁹. However, it is interesting to note that of the strains included in our study and identified as LVS based on the available metadata, 43.7% (153/350) of the strains belonging to the BioProject PRJNA669398 were found to be ARG positive (without *FTU-1*), while 0% (0/49) of the LVS strains included in other BioProjects were positive. These aspects raise the question of the reliability of the samples from this BioProject.

The general consensus is that de novo resistance develops in response to selective pressure from antibiotics or potentially other stressors (e.g. heavy metals), which is then associated with resistance mutations or the uptake of acquired ARGs via horizontal gene transfer^{70–72}. Consequently, it is important to consider the ecological environment of microorganisms to investigate the underlying effects of the resistance gene deficiency we have observed. Most microorganisms we studied are obligate intracellular bacteria, strictly bound to vectors and hosts^{49,56,73–79}. Exceptions are *Borrelia* species, which despite being strongly associated with hosts- and vectors are not intracellular pathogens⁸⁰, and the *Francisella* and *Coxiella* genera, where transmission pathways outside of vectors are also important^{81,82}. Consequently, there is a limited medium in which selective pressure and the presence of ARGs would co-occur. A tick or possibly another arthropod vector could theoretically be subjected to more or less antibiotic exposure in its environment, for example, when it takes up fluid from plants or soil, but the extent to which this effect could represent a true selective force is questionable. In addition, the presence of available ARGs might also be limited, as it has been described that the ticks' own bacteriome limits the colonisation of microorganisms from the environment⁸³.

Bacteria in hosts can be subjected to more pronounced antibiotic pressure. Of course, this is not primarily in wild animals, but when bacteria are introduced into domestic animals or even humans. It is assumed that a significant proportion of human infections are treated with antibiotics, and even if this were to involve the uptake of ARGs of these strains, it is unlikely that the bacteria that have acquired resistance genes would be re-introduced into a tick. Domestic animals may be of much greater importance in this respect. In their case, antibiotic treatment is also significant, or perhaps even more significant, while a vector may also go undetected more easily. Farm animals may be particularly noteworthy in this respect. However, it is interesting that we found a strong concordance between ARG positivity and the farm animal origin of the samples only in the case of *C. burnetii* (Fig. 2). One could hypothesize that the majority of *C. burnetii* samples from farm animals were from intensive keeping conditions, whereas the opposite was true for samples from other genera. Cattle can be considered as the classic intensively kept animal in contrast to goats, sheep and horses. For *C. burnetii* almost half of the 27 samples from farm animals were derived from cattle (14), of which 5 were positive, and there were 3 additional positive samples from goats (Supplementary Files 1, 2). It is important to note, however, that one positive sample in the genus *Ehrlichia* was from a tick collected directly from a sheep (BioSample: SAMN04335506, Supplementary File 1). Nevertheless, this was classified of vector origin in our analysis as it is not known if the tick was infected a priori. It should also be noted that the *Anaplasma* genus also included 9 samples of farm animal origin, 6 of which were of cattle origin, 1 of equine origin and 2 of sheep origin, but none of these was found to be positive (Supplementary Files 1, 2). Our reasoning is, of course, limited by the availability of metadata, but it does not appear that the confounding effect of cattle, as traditionally intensively kept animals, is the cause of the phenomenon observed in the case of *Coxiella*. A difference between the *Anaplasma* and *Coxiella* genera may be due to their life cycle. Namely, the extracellular form of *Coxiella* also plays an important role in its spread, and consequently, it is not entirely bounded to vectors⁸¹. Of course, the ability of the environmental form of *C. burnetii* to take up genes from the environment and the extent to which these might be necessary for its survival in the presence of antibiotics is questionable. Furthermore, it is important to note that our analysis is limited by the bias that the use of public data might cause and further research is needed to determine the generalizability of these findings.

Francisella tularensis is similar to *C. burnetii* in some respects. The role of vectors in its spread is not necessarily exclusive⁸², and it can occur in the infected hosts in an extracellular state^{84,85}. Several ARG positive samples of *F. tularensis* were found, but these were almost exclusively from an experiment in which artificial antibiotic pressure was applied to the bacteria, making these results difficult to interpret (see above). Furthermore, it is, of course, possible that the microorganisms studied do acquire resistance genes. However, these were impossible to detect because they were dropped by the bacteria. This may be because it is assumed that the tick's gut provides a fundamentally different microenvironment for the bacteria than the circulation of the host animal, including the existing selection pressure. The continuous activation of resistance mechanisms is an energy-demanding process, so without therapeutic pressure, these costs exceed the benefits. Thus, the bacteria try to get rid of them, which may explain the lack of ARGs. This effect has been described for ARGs; however, the fitness cost might depend on the resistance mechanism^{70,86,87}.

Our results show that the potentially tick-borne and human pathogenic microorganism strains we investigated lack antimicrobial resistance genes. Comprehending the processes underlying this phenomenon may be an important aspect in understanding the ecology of these species and, through this, in assessing the risk of phenotypic antibiotic resistance in clinical settings. However, an exception to the above statement of the tick-borne human pathogens is *Coxiella burnetii*, where resistance is a potential veterinary medical and public health threat when farm animals and their products are considered.

Data availability

Datasets analysed in the present study are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA, <https://www.ncbi.nlm.nih.gov/sra>). For more information on the BioSample IDs and the corresponding BioProject of each analysed samples, please see the Supplementary File 8.

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Author contributions

N.S. takes responsibility for the data's integrity and the data analysis's accuracy. M.P. and N.S. conceived the concept of the study. M.P., N.S. and S.Á.N. participated in the bioinformatic analysis. M.P. and N.S. participated

in the drafting of the manuscript. A.G.T., GV, L.M., MP, N.S., R.F. and S.Á.N. carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

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Article

Canine Saliva as a Possible Source of Antimicrobial Resistance Genes

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Abstract: While the One Health issues of intensive animal farming are commonly discussed, keeping companion animals is less associated with the interspecies headway of antimicrobial resistance. With the constant advance in veterinary standards, antibiotics are regularly applied in companion animal medicine. Due to the close coexistence of dogs and humans, dog bites and other casual encounters with dog saliva (e.g., licking the owner) are common. According to our metagenome study, based on 26 new generation sequencing canine saliva datasets from 2020 and 2021 reposit in NCBI SRA by The 10,000 Dog Genome Consortium and the Broad Institute within Darwin's Ark project, canine saliva is rich in bacteria with predictably transferable antimicrobial resistance genes (ARGs). In the genome of potentially pathogenic *Bacteroides*, *Capnocytophaga*, *Corynebacterium*, *Fusobacterium*, *Pasteurella*, *Porphyromonas*, *Staphylococcus* and *Streptococcus* species, which are some of the most relevant bacteria in dog bite infections, ARGs against aminoglycosides, carbapenems, cephalosporins, glycylicyclines, lincosamides, macrolides, oxazolidinone, penams, phenicols, pleuromutilins, streptogramins, sulfonamides and tetracyclines could be identified. Several ARGs, including ones against amoxicillin–clavulanate, the most commonly applied antimicrobial agent for dog bites, were predicted to be potentially transferable based on their association with mobile genetic elements (e.g., plasmids, prophages and integrated mobile genetic elements). According to our findings, canine saliva may be a source of transfer for ARG-rich bacteria that can either colonize the human body or transport ARGs to the host bacteriota, and thus can be considered as a risk in the spread of antimicrobial resistance.

Keywords: antimicrobial resistance; bacteriome; resistome; mobilome; dog saliva

1. Introduction

Antimicrobial resistance (AMR) is a threat of utmost significance that constantly raises medical challenges all around the globe. It is a fact that the development of AMR and the increase in its prevalence are due to the use or misuse of antimicrobials. Moreover,

according to the concept of One Health, the effects of antimicrobial use in human, animal and environmental sectors are interconnected, and thus interdependent. In the case of AMR, the relatedness of human and animal dimensions described by the One Health approach can be the best referred to by considering that most antimicrobial classes are co-used in both sectors. Human health antimicrobial use has been overshadowed for years by farm animal mass medication, although this tendency has recently changed in some parts of the world [1]. While the appearance and advance of AMR and, as an underlying cause, the enrichment and transmission of antimicrobial resistance genes (ARGs) in antibiotic-dense environments such as intensive animal production farms, is a well-examined phenomenon [2,3], the spread of AMR may also derive from other animal-borne routes.

Over the past decades, the number of companion animals has been tendentially and steadily rising [4]. Between 2000 and 2017, the number of dogs in the United States escalated from 68 million to 89.7 million [5]. In total, 67.9% of all households in the U.S. were associated with the ownership of various pet species and 48% of all with dogs in 2016 [4]. In the years 2019–2020, 50% of the U.S. population owned a dog [6]. The coronavirus disease (COVID-19) pandemic outbreak has resulted in elevated companion animal acquisition rates, albeit often followed by retention or replacement [7,8]. In addition to the popularity of keeping small animals, the quality of human–pet bonds has also changed. According to the survey of the American Veterinary Medical Association, 70% of pet owners consider their pets as family members, 17% as companions and 3% as property [9]. The role of pets can principally be defined as social companionship. Nowadays, having physical proximity is very common for pet–owner co-existences; pets often sleep together with their owners and lick their face or wounds [10]. Unfortunately and unsurprisingly, with such high dog numbers, the occurrence of dog bites is also common. Between 2001 and 2003, approximately 4.5 million dog bites were registered yearly in the United States, 19% of which necessitated medical intervention [11]. In the years 2005–2013, an average of 337,103 dog bite injuries were treated at U.S. emergency departments [12], although dog bites in general are under-reported [13]. In English hospitals, the number of dog bite admissions rose from 6.34 (95%CI 6.12–6.56) in 1998 to 14.99 (95%CI 14.67–15.31) admissions per 100,000 inhabitants in 2018 [14]. In parallel, a study on the database of The International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10) reveals that dog attack fatalities (excluding deaths caused by postattack infectious complications) have been constantly rising between 1995 and 2016. Incidence rates reached 0.009 per 100 000 inhabitants in Europe, 0.011 in the U.S., 0.007 in Canada and 0.004 in Australia [15]. Interestingly, 3 of 5 bites are executed by family dogs, which is more common than attacks by strays [16].

The modern mindset of providing regular veterinary healthcare services to our pets and keeping them in our closest surroundings may contribute to the interspecies transmission of AMR. Several studies have already turned their attention to the role of companion animals in the headway of AMR [17–22]. Nevertheless, the significance of the direct pet-borne AMR spread route has been given less attention when compared with the rather indirect, mostly food-transmitted farm-animal-associated route. The routes of ARG transmission can be assessed by analyzing the genetic surroundings of ARGs. Genetic elements that facilitate horizontal gene transfer (HGT) including plasmids, bacteriophages and integrative mobile genetic elements (iMGes) may contribute to three different HGT processes, namely conjugation, transduction or transformation. By conjugation, genes are delivered to a recipient cell on plasmids if bacterial cells are physically binded, while by transduction, direct cell-to-cell contact is disclaimed due to the presence of bacteriophages as means of gene conduit. Transformation negates the need for particular delivery processes. In this case, bacteria take up genetic fragments from their environment [23]. After dog bites or close encounters with saliva from dogs that undergo veterinary treatments, and thus may carry bacteria with a possibly enriched ARG content, resistant bacteria may be introduced to the human body, and later the HGT of antimicrobial resistance determinants may be

exchanged with the host bacteriota. Our study aimed to reveal the ARG content of canine saliva samples, attach the ARGs with the bacterial species that they derive from and report the ARGs' spreading capabilities to weigh the above-mentioned phenomenon. For this purpose, freely accessible next-generation sequencing (NGS) shotgun datasets were downloaded and bioinformatically analyzed using an advanced metagenomic pipeline.

2. Materials and Methods

Deep-sequenced canine saliva datasets were searched in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository. In May 2021, two shotgun metagenomic BioProjects (PRJNA648123 [24]—The 10,000 Dog Genome Consortium and PRJNA683923 [25]—Broad Institute, Darwin's Ark project) with more than 100,000,000 paired-end reads per sample were identified (Table 1). Both projects collected and sequenced dog saliva samples to investigate polymorphisms in the dog genome from which the samples were derived. The median read count (interquartile range, IQR) of the samples was 177.7×10^6 (26.6×10^6) and 417.7×10^6 (90.1×10^6) in datasets PRJNA648123 and PRJNA683923, respectively.

Table 1. The list of analyzed samples was obtained from the National Center for Biotechnology Information Sequence Read Archive. Column Run contains the NCBI SRA run identifiers. Bacterial read count represents the number of reads that were classified taxonomically to any bacteria.

ID	BioProject	Run	Bacterial Read Count
1	PRJNA648123	SRR12330029	2,900,387
2		SRR12330041	16,153,172
3		SRR12330042	13,072,781
4		SRR12330043	13,774,332
5		SRR12330044	6,123,646
6		SRR12330045	16,707,766
7		SRR12330098	18,826,266
8		SRR12330104	27,598,592
9		SRR12330220	9,938,948
10		SRR12330260	17,642,933
11		SRR12330298	17,277,697
12		SRR12330356	13,988,719
13		SRR12330364	17,378,513
14		SRR12330377	12,155,726
15		SRR12330378	34,183,357
16		SRR12330382	22,353,314
17		SRR12330383	22,886,951
18		SRR12330384	18,328,656
19		SRR12330385	6,631,504
20	PRJNA683923	SRR13340534	0
21		SRR13340535	6,752,169
22		SRR13340537	8,245,374
23		SRR13340538	41,212,470
24		SRR13340539	13,028,655
25		SRR13340540	6,964,460
26		SRR13340541	6,279,921

Bioinformatic Analysis

Quality-based filtering and trimming of the raw short reads was performed by Trim-Galore (v0.6.6, <https://github.com/FelixKrueger/TrimGalore>, accessed on 24 September 2022), setting 20 as a quality threshold. Only reads longer than 50 bp were retained and taxonomically classified using Kraken2 (v2.1.1) [26] and a database created (24 March 2021) from the NCBI RefSeq complete archaeal, bacterial, viral and plant genomes. For this taxon assignment, the `-confidence 0.5` parameter was used to obtain more precise species-level hits. The taxon classification data were managed in R [27] using functions of the packages phyloseq (v1.36.0) [28] and microbiome (v1.14.0) [29]. Reads classified as origin of bacteria were assembled to contigs by MEGAHIT (v1.2.9) [30] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. All possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3) [31] from the contigs. The protein-translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v3.1.3) [32,33] by Resistance Gene Identifier (RGI, v5.2.0) with Diamond [34]. The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. All nudged hits were excluded. The iMGE content of the ARG-harboring contigs was analyzed by MobileElementFinder (v1.0.3) and its database (v1.0.2) [35]. Following the distance concept of Johansson et al. [35] for each bacterial species, only those with a distance threshold defined within iMGes and ARGs were considered associated. In the MobileElementFinder database (v1.0.2) for *Bacteroides*, the longest composite transposon (cTn) was the *Tn6186*. In the case of this genus, its length (8505 bp) was taken as the cut-off value. For the genera *Enterococcus* and *Klebsiella*, *Tn6246* (5147 bp) and *Tn125* (10,098 bp) provided the thresholds, respectively. In the case of *Escherichia coli*, this limit was the length of the *Tn1681* transposon, namely 24,488 bp, while for *Pseudomonas aeruginosa* *Tn6060* (25,440 bp). As the database neither contains species-level, nor genus-level cTn data for the rest of the species, a general cut-off value was chosen for the contigs of these species. This value was declared as the median of the longest cTns per species in the database (10,098 bp). The plasmid origin probability of the contigs was estimated by PlasFlow (v1.1) [36]. The prophage content of the assembled contigs was predicted by VirSorter2 (v2.2.3) [37]. The findings were filtered for dsDNA phages and ssDNAs. All data management procedures, analyses and plottings were performed in R environment (v4.1.0) [27].

3. Results

After presenting the bacteriome and the identified AGRs (resistome), predictions regarding the mobility potential of ARGs were also resumed based on genetic characteristics that may play a significant role in HGT.

3.1. Bacteriome

By taxon classification, the number of reads aligning to bacterial genomes differed in the various samples. In the saliva, median bacterial read count of the samples was 4.3×10^6 (IQR: 3.4×10^6). A total of 16 major bacterial genera were detected within the saliva samples, out of which several aerobic and anaerobic genera often become isolated from infected dog bite wounds. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the saliva samples is shown in Figure 1. In the saliva samples, the dominant genera (with mean prevalence) in descending order were *Porphyromonas* (49%), *Prevotella* (15%), *Pasteurella* (12%), *Neisseria* (10%), *Capnocytophaga* (9%), *Conchiformibius* (7%), *Frederiksenia* (7%), *Cutibacterium* (6%), *Actinomyces* (5%), *Campylobacter* (4%), *Desulfomicrobium* (4%), *Bacteroides* (3%), *Fusobacterium* (3%), *Mycoplasma* (3%), *Treponema* (3%) and *Streptococcus* (2%). In the sample No. 20, no reads were classified to bacteria.

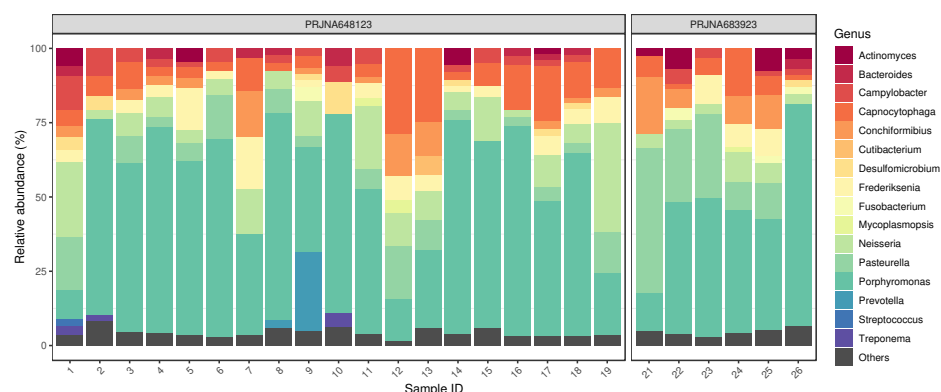


Figure 1. Saliva core bacteriome. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples. In the sample No. 20, no reads were classified to bacteria.

3.2. Resistome

Applying the above-mentioned filtering conditions, we identified 69 ARGs that are presented together with their prevalence rates within the samples and the drug classes that these ARGs affect adversely in Table 2. These ARGs per sample with their coverage and the sequence identity rate are shown in Figure 2. As a result of the taxon classification on the contigs harboring the ARGs, it was possible to predict the bacterial species of putative origin for all but ten genes (Table 3). The dominant mechanisms of identified ARGs were antibiotic inactivation (47.69%), antibiotic target protection (23.41%), antibiotic target alteration (15.90%), antibiotic efflux (7.80%) and antibiotic target replacement (5.20%).

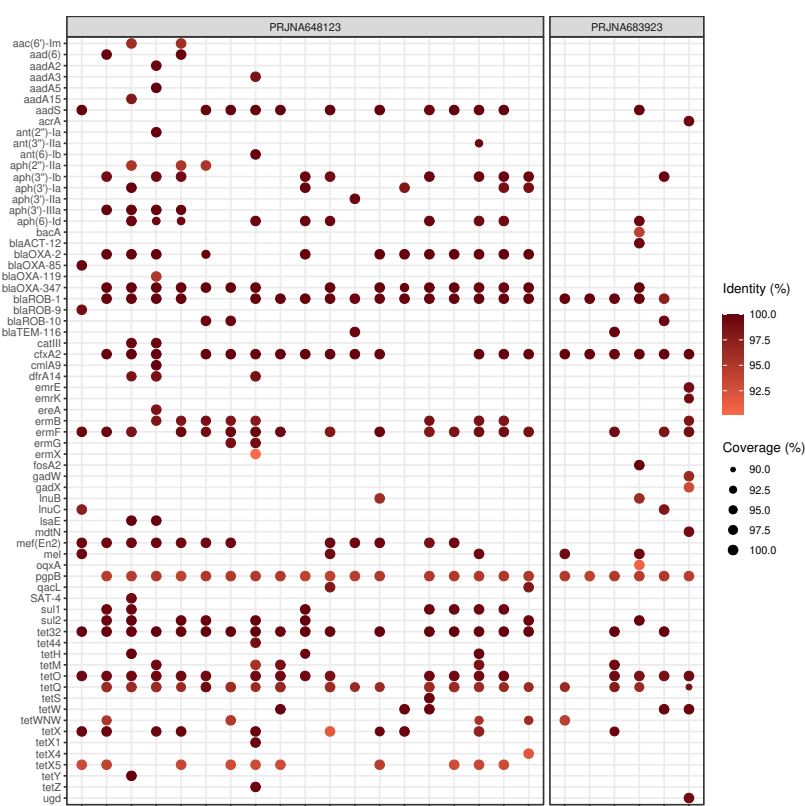


Figure 2. Identified ARGs by samples. For each sample–ARG combination, only the best finding is plotted. The size and the color of the dots correspond to the coverage and the sequence identity of hits on reference genes, respectively. In sample No. 20, there was no identifiable ARG. The gene names that are too long are abbreviated (*acrA*: *E. coli* *acrA*; *emrE*: *E. coli* *emrE*).

Table 2. Identified ARGs and the drug classes affected by them. The frequency columns show how many samples the genes occurred in.

ARG(s)	Frequency n	%	Drug Class
<i>aac(6')-Im</i>	2	7.7	aminoglycoside
<i>aad(6)</i>	2	7.7	aminoglycoside
<i>aadA2</i>	1	3.8	aminoglycoside
<i>aadA3</i>	1	3.8	aminoglycoside
<i>aadA5</i>	1	3.8	aminoglycoside
<i>aadA15</i>	1	3.8	aminoglycoside
<i>aadS</i>	12	46.2	aminoglycoside
<i>acrA</i>	1	3.8	cephalosporin, fluoroquinolone, glycylicline, penam, phenicol, rifamycin, tetracycline, triclosan
<i>ant(2'')-Ia</i>	1	3.8	aminoglycoside
<i>ant(3'')-IIa</i>	1	3.8	aminoglycoside
<i>ant(6)-Ib</i>	1	3.8	aminoglycoside
<i>aph(2'')-IIa</i>	3	11.5	aminoglycoside
<i>aph(3'')-Ib</i>	10	38.5	aminoglycoside
<i>aph(3')-Ia</i>	5	19.2	aminoglycoside
<i>aph(3')-IIa</i>	1	3.8	aminoglycoside
<i>aph(3')-IIIa</i>	4	15.4	aminoglycoside
<i>aph(6)-Id</i>	10	38.5	aminoglycoside
<i>bacA</i>	1	3.8	peptide
<i>blaACT-12</i>	1	3.8	carbapenem, cephalosporin, cephamycin, penam
<i>blaOXA-2</i>	12	46.2	carbapenem, cephalosporin, penam
<i>blaOXA-85</i>	1	3.8	carbapenem, cephalosporin, penam
<i>blaOXA-119</i>	1	3.8	carbapenem, cephalosporin, penam
<i>blaOXA-347</i>	16	61.5	carbapenem, cephalosporin, penam
<i>blaROB-1</i>	21	80.8	cephalosporin, penam
<i>blaROB-9</i>	1	3.8	cephalosporin, penam
<i>blaROB-10</i>	3	11.5	cephalosporin, penam
<i>blaTEM-116</i>	2	7.7	cephalosporin, monobactam, penam, penem
<i>catIII</i>	2	7.7	phenicol
<i>cfxA2</i>	20	76.9	cephamycin
<i>cmlA9</i>	1	3.8	phenicol
<i>dfrA14</i>	3	11.5	diaminopyrimidine
<i>emrE</i>	1	3.8	macrolide
<i>emrK</i>	1	3.8	tetracycline
<i>ereA</i>	1	3.8	macrolide
<i>ermB</i>	9	34.6	lincosamide, macrolide, streptogramin
<i>ermF</i>	18	69.2	lincosamide, macrolide, streptogramin
<i>ermG</i>	2	7.7	lincosamide, macrolide, streptogramin
<i>ermX</i>	1	3.8	lincosamide, macrolide, streptogramin
<i>fosA2</i>	1	3.8	fosfomycin
<i>gadW</i>	1	3.8	fluoroquinolone, macrolide, penam
<i>gadX</i>	1	3.8	fluoroquinolone, macrolide, penam
<i>lnuB</i>	2	7.7	lincosamide
<i>lnuC</i>	2	7.7	lincosamide
<i>lsaE</i>	2	7.7	lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>mdtN</i>	1	3.8	acridine dye, disinfecting agents and intercalating dyes, nucleoside
<i>mef(En2)</i>	12	46.2	macrolide
<i>mel</i>	5	19.2	lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
<i>oqxA</i>	1	3.8	diaminopyrimidine, fluoroquinolone, glycylicline, nitrofurantoin, tetracycline
<i>pgpB</i>	23	88.5	peptide
<i>qacL</i>	2	7.7	disinfecting agents and intercalating dyes
<i>SAT-4</i>	1	3.8	nucleoside
<i>sul1</i>	7	26.9	sulfonamide
<i>sul2</i>	8	30.8	sulfonamide
<i>tet32</i>	19	73.1	tetracycline
<i>tet44</i>	1	3.8	tetracycline
<i>tetH</i>	3	11.5	tetracycline
<i>tetM</i>	5	19.2	tetracycline
<i>tetO</i>	18	69.2	tetracycline
<i>tetQ</i>	20	76.9	tetracycline
<i>tetS</i>	1	3.8	tetracycline
<i>tetW</i>	5	19.2	tetracycline
<i>tetWNV</i>	5	19.2	tetracycline
<i>tetX</i>	10	38.5	glycylicline, tetracycline
<i>tetX1</i>	1	3.8	tetracycline
<i>tetX4</i>	1	3.8	glycylicline, tetracycline
<i>tetX5</i>	10	38.5	tetracycline
<i>tetY</i>	1	3.8	tetracycline
<i>tetZ</i>	1	3.8	tetracycline
<i>ugd</i>	1	3.8	peptide

Table 3. Identified ARGs and the predicted bacterial species of origin. For ten genes (*aadA5*, *aadA15*, *ant(2'')-Ia*, *bacA*, *blaACT-12*, *cmIA9*, *fosA2*, *oqxA*, *tetX1* and *tetY*), no species-level prediction was obtained as to which bacterium the contig carrying the gene might have originated from.

ARG(s)	Bacteria of Origin
<i>aac(6')-Im</i>	<i>Clostridioides difficile</i>
<i>aad(6)</i>	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i>
<i>aadA2</i>	<i>Acinetobacter baumannii</i>
<i>aadA3</i>	<i>Neisseria animaloris</i>
<i>aadS</i>	<i>Bacteroides fragilis</i> , <i>Capnocytophaga</i> sp. H2931, <i>Capnocytophaga</i> sp. H4358, <i>Capnocytophaga stomatis</i> , <i>Chryseobacterium indologenes</i> , <i>Riemerella anatipestifer</i>
<i>acrA</i>	<i>E. coli</i>
<i>ant(3'')-IIa</i>	<i>Aeromonas hydrophila</i>
<i>ant(6)-Ib</i>	<i>Amedibacterium intestinale</i>
<i>aph(2'')-IIa</i>	<i>C. difficile</i>
<i>aph(3'')-Ib</i>	<i>Corynebacterium</i> sp. 1959, <i>Haemophilus parahaemolyticus</i> , <i>Klebsiella michiganensis</i> , <i>Moraxella bovis</i> , <i>Variovorax</i> sp. SRS16
<i>aph(3')-Ia</i>	<i>Corynebacterium</i> sp. 1959, <i>E. coli</i> , <i>Klebsiella quasipneumoniae</i> , <i>Variovorax</i> sp. PAMC28562
<i>aph(3')-IIa</i>	<i>E. coli</i>
<i>aph(3')-IIIa</i>	<i>E. faecium</i> , <i>S. aureus</i> , <i>Streptococcus agalactiae</i>
<i>aph(6)-Id</i>	<i>Corynebacterium</i> sp. 1959, <i>K. michiganensis</i> , <i>Neisseria shayegani</i> , <i>Providencia rettgeri</i> , <i>Variovorax</i> sp. SRS16
<i>blaOXA-2</i>	<i>A. baumannii</i> , <i>A. hydrophila</i> , <i>P. aeruginosa</i>
<i>blaOXA-85</i>	<i>Fusobacterium ulcerans</i>
<i>blaOXA-119</i>	<i>Geobacter sulfurreducens</i>
<i>blaOXA-347</i>	<i>Alistipes shahii</i> , <i>B. fragilis</i> , <i>Bacteroides heparinolyticus</i> , <i>Capnocytophaga</i> sp. H2931, <i>Capnocytophaga</i> sp. H4358, <i>C. stomatis</i> , <i>Chryseobacterium</i> sp. POL2, <i>Elizabethkingia anophelis</i> , <i>Empedobacter brevis</i> , <i>Myroides odoratimimus</i> , <i>R. anatipestifer</i>
<i>blaROB-1</i>	<i>Actinobacillus pleuropneumoniae</i> , <i>Conchiformibius steedae</i> , <i>Glaesserella parasuis</i> , <i>Haemophilus haemolyticus</i>
<i>blaROB-9</i>	<i>G. parasuis</i>
<i>blaROB-10</i>	<i>Bibersteinia trehalosi</i>
<i>blaTEM-116</i>	<i>E. coli</i>
<i>catIII</i>	<i>K. michiganensis</i>
<i>cfxA2</i>	<i>Capnocytophaga cynodegmi</i> , <i>Parabacteroides distasonis</i> , <i>Porphyromonas cangingivalis</i> , <i>Porphyromonas crevioricanis</i> , <i>Porphyromonas gingivalis</i> , <i>Tannerella forsythia</i>
<i>dfrA14</i>	<i>K. michiganensis</i> , <i>Ochrobactrum anthropi</i>
<i>emrE</i>	<i>E. coli</i>
<i>emrK</i>	<i>E. coli</i>
<i>ereA</i>	<i>Geobacter daltonii</i>
<i>ermB</i>	<i>Enterococcus gilvus</i> , <i>Enterococcus</i> sp. FDAARGOS_375, <i>S. agalactiae</i> , <i>Streptococcus suis</i>
<i>ermF</i>	<i>A. shahii</i> , <i>B. fragilis</i> , <i>C. stomatis</i> , <i>C. indologenes</i> , <i>P. distasonis</i> , <i>P. cangingivalis</i> , <i>Prevotella intermedia</i> , <i>R. anatipestifer</i>
<i>ermG</i>	<i>C. difficile</i>
<i>ermX</i>	<i>Trueperella pyogenes</i>
<i>gadW</i>	<i>E. coli</i>
<i>gadX</i>	<i>E. coli</i>
<i>lnuB</i>	<i>S. suis</i>
<i>lnuC</i>	<i>Streptococcus equi</i> , <i>Streptococcus gwangjuense</i>
<i>lsaE</i>	<i>S. suis</i>
<i>mdtN</i>	<i>E. coli</i>
<i>mef(En2)</i>	<i>B. fragilis</i> , <i>P. cangingivalis</i> , <i>P. gingivalis</i> , <i>P. intermedia</i>
<i>mel</i>	<i>Streptococcus pluranimalium</i>
<i>pgpB</i>	<i>P. gingivalis</i>
<i>qacL</i>	<i>P. aeruginosa</i>
<i>SAT-4</i>	<i>S. aureus</i>
<i>sul1</i>	<i>A. hydrophila</i> , <i>P. aeruginosa</i>
<i>sul2</i>	<i>Corynebacterium</i> sp. 1959, <i>H. parahaemolyticus</i> , <i>K. michiganensis</i> , <i>M. bovis</i> , <i>Pasteurella multocida</i> , <i>P. rettgeri</i>
<i>tet32</i>	<i>Blautia hansenii</i> , <i>Bulleidia</i> sp. zg-1006, <i>C. difficile</i> , <i>Clostridium cellulovorans</i> , <i>Eubacterium maltosivorans</i> , <i>Eubacterium</i> sp. NSJ-61, <i>Faecalibacterium prausnitzii</i> , <i>Lachnoanaerobaculum umeaense</i> , <i>Peptoclostridium acidaminophilum</i> , <i>Roseburia intestinalis</i> , <i>Streptococcus anginosus</i> , <i>Streptococcus constellatus</i> , <i>S. equi</i>
<i>tet44</i>	<i>A. intestinale</i>
<i>tetH</i>	<i>Proteus vulgaris</i> , <i>Pseudomonas putida</i>
<i>tetM</i>	<i>C. difficile</i> , <i>Enterococcus faecalis</i> , <i>Mogibacterium pumilum</i> , <i>Streptococcus</i> sp. FDAARGOS_521
<i>tetO</i>	<i>C. difficile</i> , <i>Enterococcus hirae</i> , <i>Murdochella vaginalis</i> , <i>Streptococcus acidominimus</i> , <i>S. anginosus</i> , <i>S. constellatus</i> , <i>S. equi</i> , <i>S. suis</i>
<i>tetQ</i>	<i>Alistipes indistinctus</i> , <i>Bacteroides dorei</i> , <i>B. heparinolyticus</i> , <i>Bacteroides ovatus</i> , <i>Bacteroides</i> sp. HF-5287, <i>Phocaecicola coprophilus</i> , <i>P. crevioricanis</i> , <i>Prevotella fusca</i> , <i>P. intermedia</i>
<i>tetS</i>	<i>Streptococcus parauberis</i>
<i>tetW</i>	<i>Enterocloster boltea</i> , <i>F. prausnitzii</i> , <i>Megasphaera stantonii</i> , <i>S. suis</i>
<i>tetWNW</i>	<i>Filifactor alocis</i> , <i>M. pumilum</i>
<i>tetX</i>	<i>B. fragilis</i> , <i>P. distasonis</i> , <i>P. intermedia</i> , <i>R. anatipestifer</i>
<i>tetX4</i>	<i>R. anatipestifer</i>
<i>tetX5</i>	<i>B. fragilis</i> , <i>C. stomatis</i> , <i>R. anatipestifer</i>
<i>tetZ</i>	<i>Rothia nasimurium</i>
<i>ugd</i>	<i>E. coli</i>

3.3. Mobilome

Many of the identified ARGs are harbored by iMGEs, prophages or plasmids. The frequencies of iMGEs, prophages and plasmids associated with ARGs by bacteria of origin are summarized in Figure 3. Some genes could have been attached to two of the above-mentioned mobility groups in the genome of one species, including the iMGE and prophage co-appearance of aminoglycoside resistance encoding *aad(6)* and *aph(3')-IIIa* in *E. faecium*; the iMGE and plasmid co-appearance of *aph(3')-Ia* in *Corynebacterium* sp. 1959 and *K. quasipneumoniae*; *aph(3')-Ia* and *aph(6)-Id* in *Variovorax* sp. SRS16; *aph(3'')-Ib* in *Variovorax* sp. PAMC28562; tetracycline resistance encoding *tetM* in *E. faecalis*; and prophage and plasmid co-appearance of macrolide, lincosamide and streptogramin resistance encoding *ermB* in *Enterococcus* sp. FDAARGOS_375. The *blaOXA-2*, *blaOXA-347* and *blaTEM-116* genes associated with amoxicillin–clavulanate resistance all appeared in plasmids in various species; moreover, *blaOXA-2* was associated with both an iMGE and a plasmid in the genome of *P. aeruginosa*.

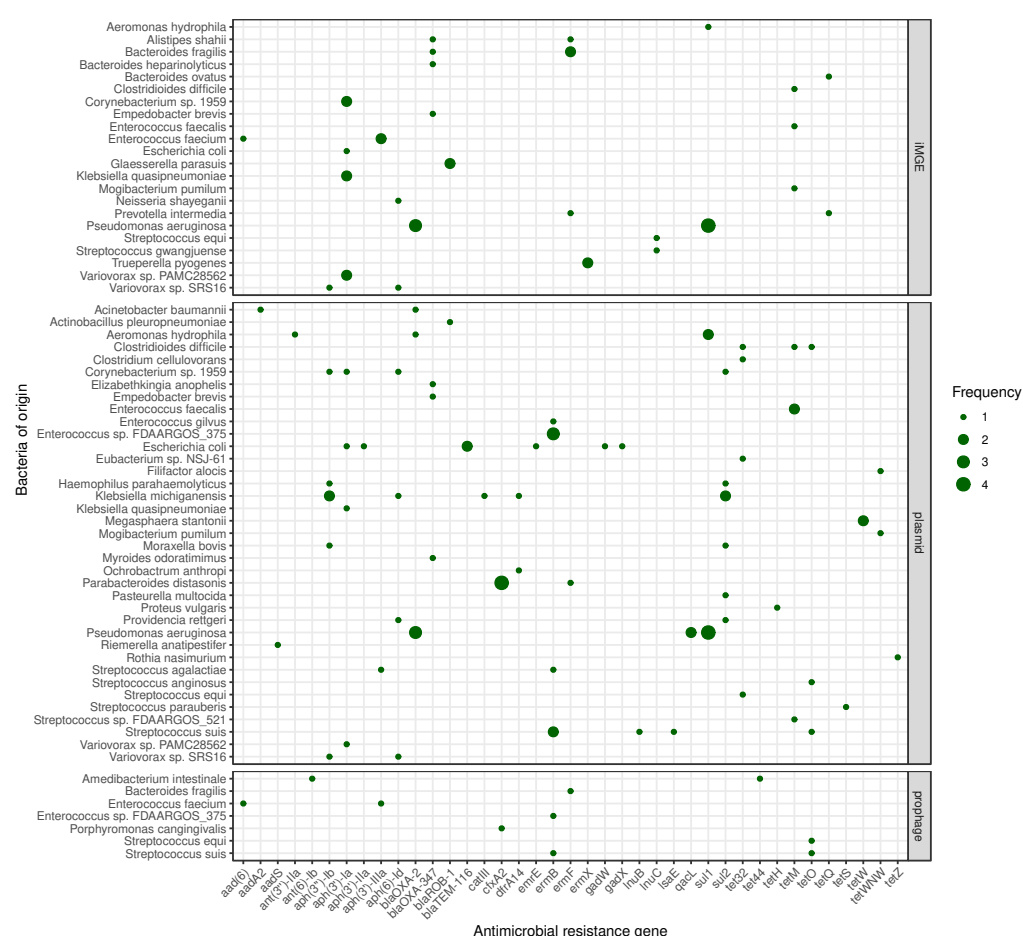


Figure 3. Mobile ARG frequency by bacteria of origin. The size of the dots indicates the occurrence frequency of the given gene flanked by iMGE, positioned in plasmid or prophage.

4. Discussion

During the bacteriome, resistome and mobilome analysis of the canine saliva samples, a large set of results was obtained that can be examined from a One Health point of view, merging the small animal veterinary sector with the perspective of the human healthcare system.

ARGs were identified in all but one sample (No. 20). No reads of bacterial origin were found in this sample. We speculate that this may be since only those reads generated from sequencing were uploaded to the SRA mapped to the dog genome.

Many of the detected aerobic and anaerobic bacterial genera often become isolated from infected dog bite wounds. Dog bite infections are normally polymicrobial, and the bite wound bacteriota consist of bacteria from the animals' oral cavity, the recipients' skin and the environment. The most common pathogens in dog bites are *Pasteurella* spp. (*P. canis* and *P. multocida*), *Staphylococcus* spp., *Streptococcus* spp. and *Capnocytophaga* spp., *Porphyromonas* spp., *Bacteroides* spp., *Fusobacterium* spp. and *Corynebacterium* spp. [38], which all appeared in the analyzed saliva samples. Some other bacterial groups of a relatively higher clinical significance that were detected in the saliva samples, including *Enterococcus* spp., *Moraxella* spp., *Neisseria* spp., *Prevotella* spp. and *Pseudomonas* spp., are also often isolated from dog bite wound infections. The vast majority of other genera isolated in the samples have been mentioned to appear in dog saliva in previous publications with variable abundance rates [13,39]. Even though members of *Clostridium* spp. were detected in the samples, genome fragments of *C. tetani*, the bacterium responsible for tetanus, were not identified. The number of detected ARGs was relatively high in the salivary bacteriome. Examining eight genera (*Pasteurella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Capnocytophaga* spp., *Porphyromonas* spp., *Bacteroides* spp., *Fusobacterium* spp. and *Corynebacterium* spp.) that were indicated to be the most relevant ones in dog bite infections by other authors [38,39], we could identify genes that confer resistance against aminoglycosides, carbapenems, cephalosporins, glycolcyclines, lincosamides, macrolides, oxazolidinone, penams, phenicols, pleuromutins, streptogramins, sulfonamides and tetracyclines, while other antimicrobial groups including fluoroquinolones appeared in *E. coli*, one of the six leading pathogens responsible for the deaths associated with resistance in 2019 worldwide [40].

Such a great number and broad spectrum of ARGs and potentially affected antimicrobial groups associated with the canine saliva samples may be related to the use of antibiotics at small-animal veterinary practices. Antibiotic consumption rates in the companion animal sector are rather difficult to evaluate. However, some systems exist for the surveillance of magnitude of companion animal antibiotic consumption, such as the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) [41], VetCompass [42] or the Small Animal Veterinary Surveillance Network (SAVSNET) [43], these rates are still less well-documented. Moreover, in many countries, antimicrobial use is often just estimated by rough sales data [21]. Nevertheless, according to the two UK-based surveillance systems (VetCompass from Royal Veterinary College, and SAVSNET from Liverpool University) and one EU report (ESVAC), antibiotics are rather frequently prescribed at small-animal clinics. A study states 1 in 4 UK dogs (25.2%, 95% CI: 25.1–25.3%) were treated with antibiotics in a two-year period [44]. Even though the vast majority of veterinarians are aware of the fact that improper AMU contributes to selection for AMR, and that it is a significant problem according to nationwide surveys [45,46], there are many factors that influence the antibiotic prescription preferences of veterinarians in addition to the perspectives of antimicrobial stewardship [47–52].

Broad-spectrum amoxicillin–clavulanate is the flagship of antimicrobial agents applied in dogs in many countries, while first-generation cephalosporins are also routinely used [21,53,54]. Lincosamides (clindamycin), macrolides, tetracyclines (doxycycline), nitroimidazoles and trimethoprim/sulphonamides have also been reported to be frequently used in small-animal practices [21]. Third- and fourth-generation cephalosporins, fluoroquinolones and polymyxins that belong to category B, 'last resort', or highest-priority Critically Important Antibiotics (HPCIA) according to the European Medicines Agency [41] should be avoided unless sensitivity testing is conducted and no other antibiotics would be effective. Nevertheless, HPCIA have been estimated to be prescribed in around 5–6% of total antimicrobial agent usage events. Of the HPCIA category, fluoroquinolones are the most common in dogs, constituting ~4 to 5% of total antibiotic prescriptions [55].

Nevertheless, AMR determinants against the above-mentioned antimicrobial compound groups have been detected in and associated with many bacterial species of the examined canine saliva samples. Some of these ARG-associated bacteria can also exert pathogenic effects and are often isolated by dog bite infections. ARGs against cephalosporins were identified in many, often clinically significant bacteria, including but not limited to *Bacteroides* spp., *Capnocytophaga* spp., *E. coli*, *F. ulcerans*, *Porphyromonas* spp. and *P. aeruginosa*. Likewise, ARGs against lincosamides appeared in *Bacteroides* spp., *C. stomatis*, *Enterococcus* spp., *P. cangingivalis*, *P. intermedia* and *Streptococcus* spp.; ARGs against macrolides in *Bacteroides* spp., *C. stomatis*, *Enterococcus* spp., *Porphyromonas* spp., *P. intermedia* and *Streptococcus* spp.; ARGs against tetracyclines in *Bacteroides* spp., *C. stomatis*, *Enterococcus* spp., *P. crevioricanis*, *Prevotella* spp., *P. putida* and *Streptococcus* spp.; ARGs against sulfonamides in *Corynebacterium* spp., *M. bovis*, *P. multocida* and *P. aeruginosa*; and ARGs against fluoroquinolones in *E. coli* (non-exhaustive lists of detected ARG-bacteria associations in the samples, with an emphasis on clinically relevant bacteria). Bacterial associations and the significance of AMR determinants affecting amoxicillin–clavulanate, the most commonly prescribed antibiotic in veterinary medicine, are discussed later on. Even though nitroimidazoles (e.g., metronidazole) are described to be often used in small-animal veterinary practices, no ARGs could be detected in the canine saliva samples against this antibiotic group.

In the current literature, human infections associated to dog bites are better and more frequently documented than the transmission route of licking. Three to thirty percent of dog bites lead to infection [13]. The management of animal bites rests on two pillars: local wound care and adequately applied systemic treatment. Essentials of local therapy include inspection, debridement of the wound accompanied by the removal of possible foreign bodies, e.g., teeth, and irrigation with saline solution. As for the systematic therapy, tetanus boosters (if none given in the past year) and rabies prophylaxis should always be considered. In our study, genome fragments of *C. tetani*, the causative agent of tetanus, were not detected in any of the examined saliva samples. No consensus has yet been found in the use of antibiotics for animal bite wound care. Prophylactic antibiotics should be considered unless the wound is very superficial and clean. Explicit indications for antibiotic prophylaxis or therapy include presentation at least 8 h after the bite, clear signs of superinfection, moderate or severe wounds with crush injuries or devitalized tissues requiring surgery, deep puncture wounds (exceeding the layer of epidermis), wounds close to joints, diabetes mellitus, asplenic or immunocompromised state, alcohol abuse, or involvement of the genital area, face or hand [56–60]. In the absence of the above reasons, antibiotic therapy may not be necessary. Interestingly, injuries are normally located on the head, neck and face in children and on the hand or upper extremity in adults due to height ratios with the attacking dog [13,61]. An adequately chosen antibiotic agent is expected to be effective against anaerobe bacteria (*Bacteroides* spp., *Fusobacterium* spp., *Porphyromonas* spp., *Prevotella* spp. etc.), in addition to the *Staphylococcus*, *Streptococcus* and *Pasteurella* species. Prophylactic treatment is normally 3 to 5 days long, while medication for 10 days or longer is recommended if the wound is infected. The first-line choice for oral therapy is amoxicillin–clavulanate, accompanied with a first dose of intravenous antibiotic (e.g., ampicillin/sulbactam, ticarcillin–clavulanate, piperacillin–tazobactam, or a carbapenem) in high-risk patients. Amoxicillin–clavulanate is often combined with metronidazole or clindamycin and is also sometimes replaced with cephalosporins, e.g., cefuroxime, cefotaxime, ceftriaxone or amoxicillin, fluoroquinolones, sulfamethoxazole and trimethoprim, and alternatively, although less effective, azithromycin or doxycycline in this combination [58,60]. Due to high resistance rates, flucloxacillin, erythromycin and cephalosporins are often ineffective in *Pasteurella* infections, and thus should rather be avoided [57]. In our case, no genes conferring resistance to these agent groups could be identified in *Pasteurella* spp.

Data on the outcome of antibiotic prophylaxis in animal bite management by humans is limited and rather controversial and conflicting. While a meta-analysis of eight randomized trials indicated a benefit of antibiotic prophylaxis [62], some studies concluded that antibiotic prophylaxis does not result in a statistically significant difference in the frequency of wound infections among treated and untreated patient groups, except for wounds to the hand [63]. Based on other publications, antibiotic prophylaxis should be recommended for high-risk patient groups only [64,65].

Based on antibiotic prescription data from human and veterinary medical practices described above, amoxicillin–clavulanate and cephalosporins are the most commonly used agents in the treatment of animal patients and dog bite infections, while lincosamides (mostly clindamycin), sulfonamides (mostly potentiated sulfonamides) and fluoroquinolones also appear in both sectors [21,53–55,57,58,61].

Amoxicillin–clavulanate, the most commonly used antibiotic in small-animal medicine, and the first choice for canine bite wounds, is a member of broad-spectrum penicillins that have been a frequently consumed key antibiotic group in the high-income super-region between 2000 and 2018 according to a global study [66]. All in all, six ARG types were detected in the dog saliva samples that may confer resistance against amoxicillin–clavulanate, which were either the members of the *bla*TEM or OXA family [67,68]. *bla*TEM-116 was identified in *E. coli*, while various members of the OXA family appeared in many genera, including *A. baumannii*, *Bacteroides* spp., *Capnocytophaga* spp., *F. ulcerans* and *Pseudomonas* spp., which can have a high clinical relevance in dog bite infections. Moreover, plasmid-borne *bla*OXA-2, *bla*OXA-347 and *bla*TEM-116 genes in different bacteria, and OXA-2 associated with both an iMGE and a plasmid from *P. aeruginosa*, all confer resistance against amoxicillin–clavulanate and have a higher potential to spread from bacteria to bacteria. The accumulation of various mobility factors around the genes may increase the chance of the horizontal transfer of the given ARG. The canine saliva-borne transmission of bacteria harboring mobile ARGs may hamper antimicrobial use in human clinical settings and can also contribute to the spread of AMR among the bacteria derived from pets to the bacteriome appearing in humans.

Cephalosporins, which are also commonly used both in companion animals medicine [21,54] and human medicine, including in protocols for dog bite infections [58,61], have been associated with all in all twenty-four bacterial species harboring related ARGs in the canine saliva samples. These bacteria include pathogens such as *A. baumannii*, *Bacteroides* spp., *Capnocytophaga* spp., *F. ulcerans* and *P. aeruginosa* that contain *bla*OXA-2, *bla*OXA-347, *bla*OXA-347, *bla*OXA-85 and *bla*OXA-2, respectively. Importantly, *P. aeruginosa*-associated *bla*OXA-2, which was associated with both an iMGE and a plasmid, has a high potential for HGT and can be considered as a major public health concern.

Lincosamides, including clindamycin, are also significant in both veterinary medicine [21,54] and the treatment of dog bite cases in humans [58,61]. Genes affecting lincosamides appeared in seventeen bacterial species, many of which, e.g., *B. fragilis* (*ErmF*), *C. stomatis* (*ErmF*), *Porphyromonas* spp. (*ErmF*) and *Streptococcus* spp. (*ErmB*, *lnuB* and *lnuC*), are potentially pathogenic. While the above-mentioned species, which are often associated with dog bite infections, contained no genetic element around these ARGs that could facilitate their transfer, an *Enterococcus* species from the canine saliva samples is linked to carrying *ermB* with prophage and plasmid co-appearance. Thus, the possibility of the transfer of this gene to other bacteria with higher clinical significance is given in the case of lincosamides as well.

Sulfonamide resistance genes appeared in relatively fewer, namely eight, bacterial species, including *P. multocida* and *P. aeruginosa* harboring *sul2* and *sul1*, respectively. In addition to the low ARG counts, none of the sulfonamide resistance genes appeared to be mobile, while, in contrast, HGT has been found to be highly characteristic for these two genes in other publications [69].

In the case of fluoroquinolones, only *E. coli* harbored related ARGs, namely the non-mobile *acrA*, *gadW* and *gadX*. Interestingly, while both *gadW* and *gadX* are *AraC* family regulators that promote *mdtEF* expression to confer multidrug resistance, when they co-occur, *gadW* inhibits *gadX*-dependent activation by repressing *gadX* (CARD).

Furthermore, fosfomycin and tygecyclin, which are often used as last-resort antimicrobial agents [70,71] and are involved in the list of Critically Important Antibiotics for Human Medicine by WHO [72], also appeared among the affected antibiotic groups, namely due to the presence of *fosA2* and *tetX4*, *tetX5*, respectively. Nonetheless, while the members of the *tet(X)* family are often plasmid-associated [73–76], their plasmid relatedness was not predicted within this study.

While the above-mentioned findings may raise awareness of the potential public health risk associated with canine saliva, this material has been used to promote rapid healing and to reduce bacterial contamination in the past according to the reports of ethnoveterinary and ethnomedicinal practices [77,78]. Antimicrobial and anti-inflammatory activity of canine saliva induced by thiocyanate, lysozyme and, indirectly, nitrate, among others [79,80], can even appear at low concentrations [81]. However, according to our findings, canine saliva can also be associated with public health risks, since salivary bacteria may contaminate the surroundings of people and may also colonize human skin and mucous membranes. Thus, ARG-rich bacteria present in and around humans do not even necessarily need to transfer their ARGs to potentially cause severe harm to various groups of people with weaknesses of the immune system, e.g., extremities in age or diseased state.

As a common trend among many nations, veterinary use of antibiotics is gradually declining [49,55,81–83]. In human medicine, antibiotic sales elevated by 65% in low- and middle-income countries and decreased slightly by 4% in high-income countries between 2000 and 2015, which adds up to a rise in global antibiotic consumption rates [66,84]. As a presumable conclusion, several genes conferring resistance against clinically important antimicrobial groups are present in the salivary bacteriome of dogs that may drift to the genome of bacteria in humans. Encounters with dog saliva and dog bites may serve as an interspecies platform for the migration of bacteria and ARGs. Transmitted bacteria may cause clinical symptoms, and ARGs that they harbor may confer resistance against antimicrobial agents of a clinical relevance.

Author Contributions: N.S. takes responsibility for the integrity of the data and the accuracy of the data analysis. A.G.T., L.M. and S.N. conceived the concept of the study. E.G.K. and N.S. participated in the sample selection. A.G.T. and N.S. participated in the bio-informatic analysis. A.G.T., B.R., I.T. and N.S. participated in the drafting of the manuscript. A.D., A.G.T., Á.V.P., B.R., I.T., L.M., N.S., S.K. and T.N. carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Clinical considerations on antimicrobial resistance potential of complex microbiological samples

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ABSTRACT

Antimicrobial resistance (AMR) is one of our greatest public health challenges. Targeted use of antibiotics (ABs) can reduce the occurrence and spread of AMR and boost the effectiveness of treatment. This requires knowledge of the AB susceptibility of the pathogens involved in the disease. Therapeutic recommendations based on classical AB susceptibility testing (AST) are based on the analysis of only a fraction of the bacteria present in the disease process. Next and third generation sequencing technologies allow the identification of antimicrobial resistance genes (ARGs) present in a bacterial community. Using this metagenomic approach, we can map the antimicrobial resistance potential (AMRP) of a complex, multi-bacterial microbial sample. To understand the interpretiveness of AMRP, the concordance between phenotypic AMR properties and ARGs was investigated by analyzing data from 574 *Escherichia coli* strains of five different studies. The overall results show that for 44% of the studied ABs, phenotypically resistant strains are genotypically associated with a 90% probability of resistance, while for 92% of the ABs, the phenotypically susceptible strains are genotypically susceptible with a 90% probability. ARG detection showed a phenotypic prediction with at least 90% confidence in 67% of ABs. The probability of detecting a phenotypically susceptible strain as resistant based on genotype is below 5% for 92% of ABs. While the probability of detecting a phenotypically resistant strain as susceptible based on genotype is below 5% for 44% of ABs. We can assume that these strain-by-strain concordance results are also true for bacteria in complex microbial samples, and conclude that AMRP obtained from metagenomic ARG analysis can help choose efficient ABs. This is illustrated using AMRP by a canine external otitis sample.

Subjects Genomics, Microbiology, Veterinary Medicine, Infectious Diseases

Keywords Antimicrobial resistance, Genomics, Complex microbial sample, Clinical metagenomics

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Additional Information and
Declarations can be found on
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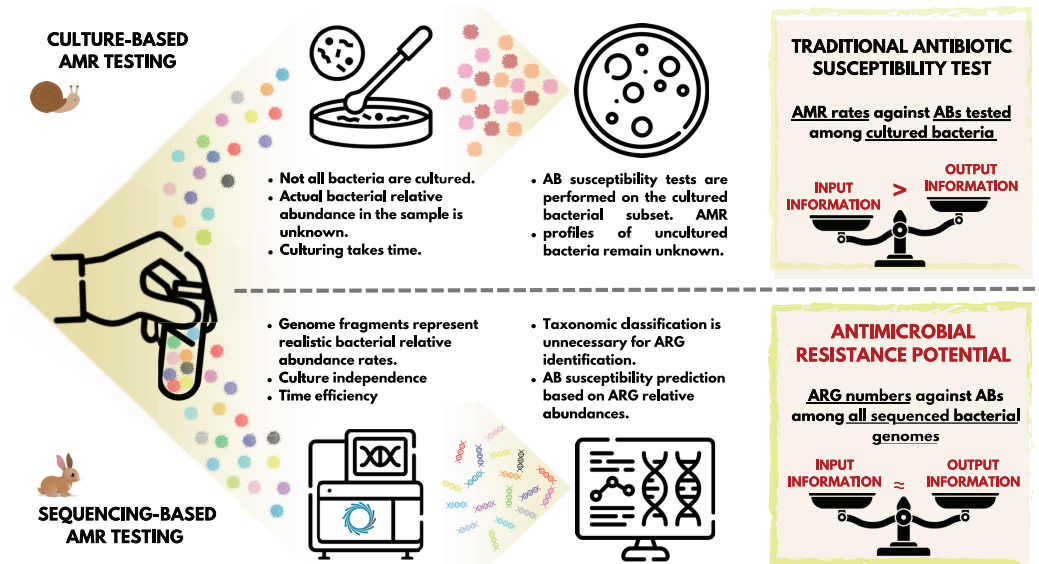
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GRAPHICAL ABSTRACT



INTRODUCTION

The appearance and use of antibiotics (ABs) has brought an unprecedented advance in the treatment of bacterial infections (*Muteeb et al., 2023; Solymosi et al., 2024*). The general consensus is that the case-specific, targeted use of ABs is favorable in clinical settings. This means using AB compounds that are supposed to be effective against the bacteria involved in the disease process to be treated (*Magnusson, 2020*). The classic standard is to take a sample from the patient for microbiological testing, and perform the bacterial culturing and the AB susceptibility testing (AST) of it (*Motro & Moran-Gilad, 2017*). Based on these tests, we obtain information regarding the set of ABs that the bacteria in the culture showed susceptibility to, and the set of compounds to which they are moderately or not at all susceptible (*Khan, Siddiqui & Park, 2019*). The general procedure for this widely-used routine bacteriology technique is to first obtain a mixed culture from the sample, then select certain colonies for pure culture (*Boolchandani, D'Souza & Dantas, 2019*), and perform disk diffusion or microbroth dilution AST (*Boolchandani, D'Souza & Dantas, 2019*). Importantly, even in a mixed bacterial culture, only a subset of the microorganisms and bacteria involved in the pathological process are represented. One partial explanation for this is that a significant proportion of the bacteria that have been identified to date are challenging to culture (*Steen et al., 2019*). Furthermore, even in case of cultivable species, providing the same culture conditions (e.g., type of medium) favors the growth of some taxa over others (*Stewart, 2012*). The latter issue can be avoided by creating mixed cultures in parallel, providing different culture environments (*Van Belkum et al., 2013*). Nevertheless, AB susceptibility testing is performed on an unpredictably small subset of bacterial strains. Additionally, several phases of traditional microbiological testing are time-consuming processes that take at least a few days to complete. Furthermore, the entire conventional diagnostic workflow is rather labour-intensive (*Motro & Moran-Gilad, 2017*).

At the end of the diagnostic process, the phenotypic AMR profile delivered to clinicians refers to a fraction of the bacteria present in the disease process.

At the same time, next-generation sequencing facilitates the identification of a wide variety of genes, including antimicrobial resistance genes (ARGs). Moreover, the identification of the ARGs in microbiological samples can be performed in a considerably shorter time, regardless of whether the bacteria carrying them have been cultured or not yet, or whether they are considered pathogenic or not (Motro & Moran-Gilad, 2017). While the classical approach outlined above yields a phenotypic AB susceptibility profile, ARG detection yields a genotypic AMR profile (Boolchandani, D'Souza & Dantas, 2019). The AB resistance detected in phenotypic settings reflect the activity and functionality of the genetic background (Chibucos et al., 2014), whether or not the genes or polymorphisms that contribute to the generation of the expressed effect, AMR, are known. In the case of a genotypic resistance profile, we do not know whether the gene is expressed or not. However, we can assume that there is a possibility for the expression of the ARGs present that can be interpreted as antimicrobial resistance potential (AMRP).

For the clinical evaluation of AMRP, it is essential to know the extent to which phenotypic AMR equals with the presence or absence of ARGs. As outlined above, the assessment of this from complex microbiological samples is significantly biased. However, by examining pure bacterial cultures, we can get an idea of the correspondence between AMRP and phenotypic AMR. If AMRP and phenotypic AMR exhibit a high degree of overlap for a given set of strains, it can be reasonably assumed that culturing all strains in a complex microbiological sample would yield comparable results. In such cases, the metagenomic ARG analysis of these complex samples may result in AMRP profiles that could contribute to the selection of ABs in the following way. Based on the AMRP, those ABs could be prioritised that are potentially impeded with the lowest probability by the ARG-harboursing bacteria involved in the pathogenesis. Following this approach, the concordance of the AMR phenotype with the presence of ARGs was initially analysed in 574 strains of *Escherichia coli* from five distinct BioProjects. Subsequently, a metagenomic ARG analysis was conducted on a complex microbiological sample from a case of chronic, canine otitis externa, with the objective of describing the AMRP of the complex microbiological sample.

METHODS

Portions of this text were previously published as part of a preprint (<https://www.biorxiv.org/content/10.1101/2024.05.14.594174v1>). To investigate phenotypic and genotypic AMR, antibiograms and Illumina sequencing data of *E. coli* strains used by Pataki et al. (2020) were downloaded from the European Nucleotide Archive (ENA) at EMBL-EBI repository (<https://www.ebi.ac.uk/ena/browser/home>, accessed on 2/5/2024). Of these *E. coli* datasets, BioProjects that contained at least 10 samples and an antibiogram involving more than one AB compound were included in the analyses. In the antibiograms, the resistant (R), intermediate (I), susceptible (S) categories for each AB were defined by the authors publishing the datasets based on different AST standards and cut-off versions in each BioProject: PRJDB7087 (European Committee on Antimicrobial

Susceptibility Testing, EUCAST, 2018, number of records with R phenotype: 1154, I: 143, S: 1667), [PRJEB14086](#) (EUCAST, 2013/652/EU, R: 231, I: 0, S: 467), [PRJEB21880](#) (EUCAST, 2015, R: 92, I: 0, S: 1294), [PRJEB21997](#) (EUCAST, 2017, R: 806, I: 0, S: 748), [PRJNA266657](#) (Clinical and Laboratory Standards Institute, CLSI, 2015, R: 245, I: 97, S: 570). Raw FASTQ files were obtained from each BioProject. Bioinformatic analysis of the FASTQ files began with quality control, followed by the trimming and filtering of the raw short reads using TrimGalore (v.0.6.6, <https://github.com/FelixKrueger/TrimGalore>), setting a quality threshold of 20. More than 50 bp long reads were assembled to contigs by MEGAHIT (v1.2.9) ([Li et al., 2015](#)) using default settings.

The metagenomic sample was collected from the right external ear canal of a 4-year-old neutered male Belgian griffon before total ear canal ablation (TECA) or lateral bulla osteotomy (LBO) surgery. The dog was previously diagnosed with an aural mass submitted as an inflammatory polyp and end-stage external otitis. The detection of the symptoms took place around 6 weeks before the surgery. Multiple conservative treatment rounds with AB and anti-inflammatory agents had been performed previously with no success. No tumor tissues were identified *via* histopathology examination. The history of the dog did not include severe allergies. The skin of the ear canal was red, swollen, scaled, thickened, and covered with bloody, purulent ear discharge before the surgery. By the routine bacteriology testing *Pseudomonas aeruginosa* and, in small amounts, *Malassezia pachydermatis* were detected from the sample. Even though, the identified *Pseudomonas aeruginosa* strain appeared to be multi-resistant, ceftazidime, gentamicin, tobramycin, ciprofloxacin, marbofloxacin and polymyxin B were indicated to be potentially effective, if administered locally, in great concentrations. DNA extraction was performed with QIAamp PowerFecal Pro DNA Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. The concentrations of the extracted DNA solutions were evaluated with an Invitrogen Qubit 4 Fluorometer using the Qubit dsDNA HS (High Sensitivity) Assay Kit. The metagenomic long-read library was prepared by the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) from Oxford Nanopore Technologies (ONT). The sequencing was implemented with a MinION Mk1C sequencer using an R10.4.1 flow cell from ONT. The basecalling was performed using dorado (<https://github.com/nanoporetech/dorado>, v0.4.3) with model dna_r10.4.1_e8.2_400bps_fast@v4.2.0, based on the POD5 files converted from the FAST5 files generated by the sequencer. The raw reads were adapter trimmed and quality-based filtered by Porechop (v0.2.4, <https://github.com/rrwick/Porechop>) and Nanofilt (v2.6.0) ([De Coster et al., 2018](#)), respectively.

For the Illumina short read samples, the ARG content of the sequence in the contigs and for the ONT metagenome sample in the long reads was identified using ResFinder (v4.5.0) (minimum coverage 60%, minimum identity 90%) ([Bortolaia et al., 2020](#)).

In the agreement analysis between phenotypic AMR and AMR prediction based on ARG detection, we only used AST results that did not use AB combinations, as the genetic background of resistance to single ABs is better understood than ARG determination of resistance to AB combinations. From AST results, only resistant and susceptible results were used. As only two of the five BioProjects in the study included I AST results, only S and R AST data were used to present the analyses in a consistent manner. However, the

BioProjects that also included I results were re-evaluated separately. For these two BioProjects, the non-susceptible (I + R) phenotype was used in addition to the susceptible (S) phenotype. ABs negatively affected by the detected ARGs were recorded and collected by each sample.

Based on the AST and ARG results, 2×2 cross-tabulations (TP : true positive, TN : true negative, FP : false positive, FN : false negative) were generated by each AB compound. These were used to calculate metrics to describe the predictive goodness of the genome-based prediction of the phenotype. Phenotypic AST results were the reference (ground truth). Accordingly, the TP group refers to strains with both phenotypic and genotypic resistance to a given AB compound, TN to strains with both phenotypic and genotypic sensitivity, FP to strains with phenotypic sensitivity and genotypic resistance, and FN to strains with phenotypic resistance without the detection of any ARGs to a given drug. In addition to sensitivity ($SE = TP/(TP + FN)$), specificity ($SP = TN/(TN + FP)$), negative predictive value ($NPV = TN/(TN + FN)$) and positive predictive value ($PPV = TP/(TP + FP)$), point and 95% CI estimates were also calculated for false resistance (major error rate, $ME = FP/(TN + FP)$) and false susceptibility (very major rate, $VME = FN/(TP + FN)$).

Internationally accepted guidelines and standards are also available to assess the accuracy of the different AST methods in relation to standard reference methods, *i.e.*, to determine VME and ME values. In the United States, the FDA (Food and Drug Administration) regulations for the marketing of different AB susceptibility testing devices are quite strict. Thus, the ME of the test device examined must not exceed 3% when testing the efficacy of different bacterial species-drug combinations. In case of VME , the proposed statistical criteria include an upper 95% confidence limit for the VME of 7.5% at most, and a lower 95% confidence limit for the VME of 1.5% at most (FDA, US, 2009). An international standard has also been established for the evaluation of AB susceptibility testing devices, which proposes similar but not identical criteria as acceptable limits of accuracy (International Organization for Standardization, 2006). The above guidelines and standards have been tailored to compare different phenotypic techniques rather than phenotypic and genotypic methods, which have the potential for greater variation. Thus, we have used the 5% margin of error used in statistics.

RESULTS

In Figs. 1 and 2 we summarize the point and 95% CI estimates of the agreement, prediction goodness metrics for the phenotypic (ground truth) and genotype-predicted AMR, by ABs and BioProjects (Solymosi et al., 2024).

ABs for which the NPV was estimable and reached 90% in at least half of the BioProjects ($n = 21$): amikacin, azithromycin, ceftazidime, chloramphenicol, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, kanamycin, meropenem, nalidixic acid, streptomycin, sulfamethoxazole, sulfisoxazole, temocillin, tetracycline, tigecycline, tobramycin, and trimethoprim. ABs for which the PPV was estimable and reached 90% in at least half of the BioProjects ($n = 15$): ampicillin, cefepime, cefotaxime, cefoxitin, chloramphenicol, ciprofloxacin, colistin, fosfomycin, gentamicin, kanamycin, piperacillin,

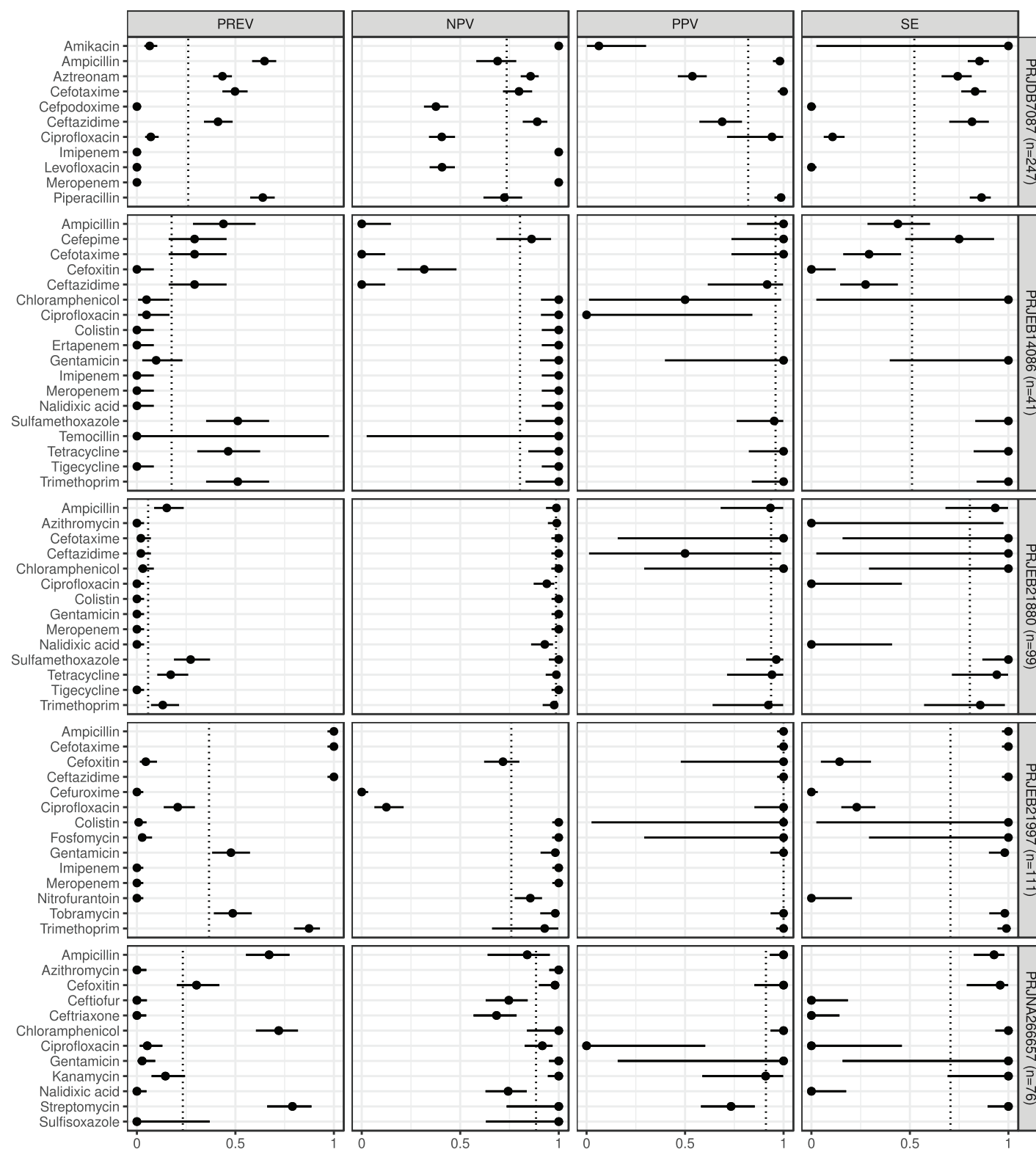


Figure 1 Predictive performance by BioProjects. Antimicrobial resistance gene-based prediction compared to the AB susceptibility test results using five BioProject datasets. Column PREV shows the prevalence (with 95% CI) of phenotypic antimicrobial resistance against certain ABs within the BioProjects. Point and 95% CI estimates for negative and positive predictive values, and sensitivity are presented in columns NPV, PPV, and SE. The dotted vertical lines represent the mean of the metrics within the given BioProject. [Full-size !\[\]\(fcc3264021d438d9732560e78099f674_img.jpg\) DOI: 10.7717/peerj.18802/fig-1](https://doi.org/10.7717/peerj.18802/fig-1)

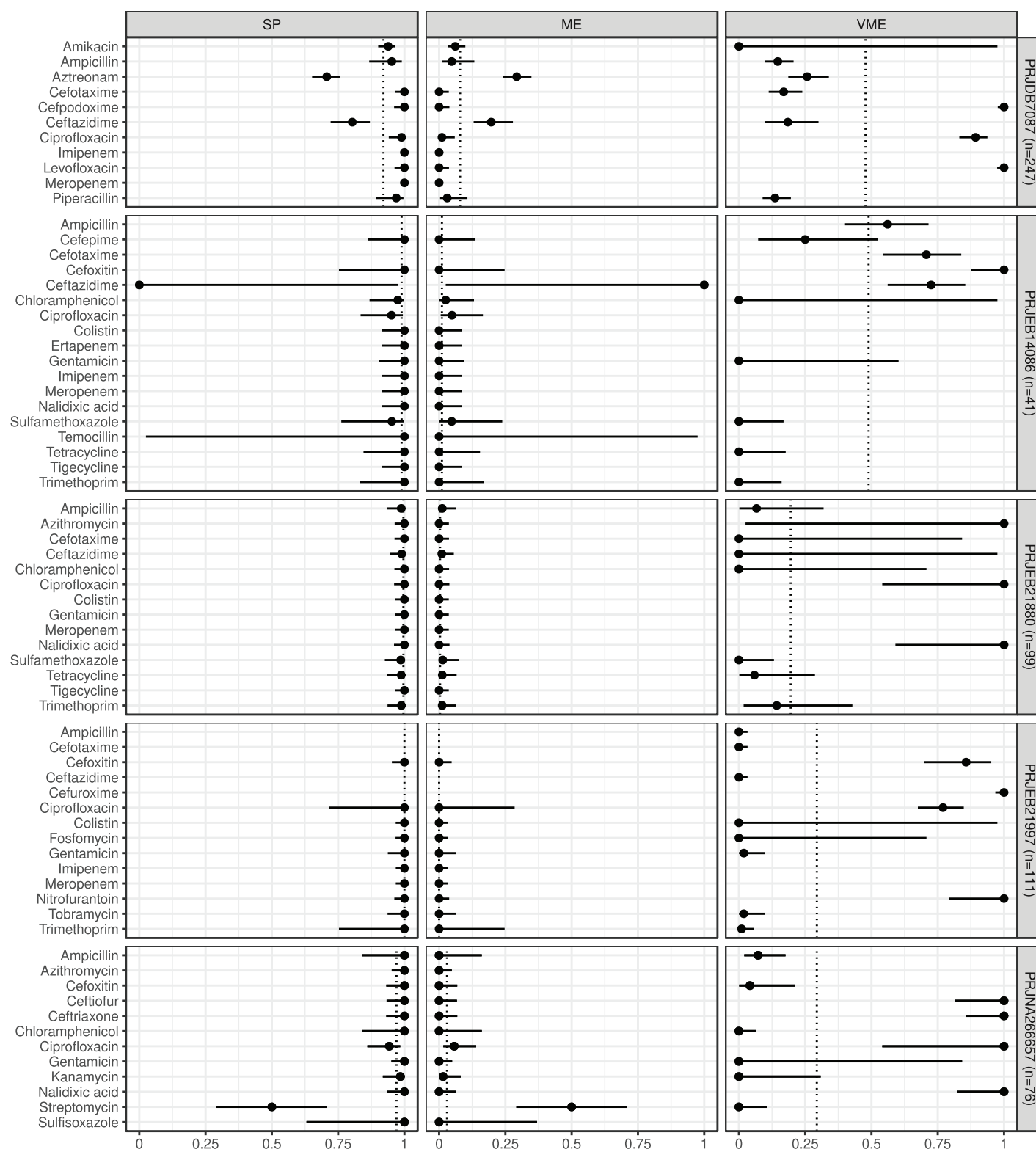


Figure 2 Predictive performance by BioProjects. Antimicrobial resistance gene-based prediction compared to the AB susceptibility test results using five BioProject datasets. Point and 95% CI estimates for specificity, major and very major error rates are presented in columns SP, ME, and VME. The dotted vertical lines represent the mean of the metrics within the given BioProject. [Full-size DOI: 10.7717/peerj.18802/fig-2](https://doi.org/10.7717/peerj.18802/fig-2)

sulfamethoxazole, tetracycline, tobramycin, and trimethoprim. ABs for which the *SE* was estimable and reached 90% in at least half of the BioProjects ($n = 13$): amikacin, ampicillin, ceftazidime, chloramphenicol, colistin, fosfomycin, gentamicin, kanamycin, streptomycin, sulfamethoxazole, tetracycline, tobramycin, and trimethoprim. ABs for which the *SP* was estimable and reached 90% in at least half of the BioProjects ($n = 29$): amikacin, ampicillin, azithromycin, cefepime, cefotaxime, cefoxitin, cefpodoxime, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, kanamycin, levofloxacin, meropenem, nalidixic acid, nitrofurantoin, piperacillin, sulfamethoxazole, sulfisoxazole, temocillin, tetracycline, tigecycline, tobramycin, and trimethoprim. ABs for which *ME* was estimable and did not exceed 5% in at least half of the BioProjects ($n = 28$): ampicillin, azithromycin, cefepime, cefotaxime, cefoxitin, cefpodoxime, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, kanamycin, levofloxacin, meropenem, nalidixic acid, nitrofurantoin, piperacillin, sulfamethoxazole, sulfisoxazole, temocillin, tetracycline, tigecycline, tobramycin, and trimethoprim. ABs for which *VME* was estimable and did not exceed 5% in at least half of the BioProjects ($n = 15$): amikacin, cefotaxime, ceftazidime, chloramphenicol, colistin, fosfomycin, gentamicin, kanamycin, nalidixic acid, nitrofurantoin, streptomycin, sulfamethoxazole, tetracycline, tobramycin, and trimethoprim. [Figure S1](#) shows the same metrics for the two BioProjects that also included the I AST phenotype.

The estimated metrics for ABs for which at least two BioProjects had identical cut-offs are shown in [Fig. 3](#). According to these overall estimations the proportion of the ABs for which the *NPV* was estimable and reached 90% was 67% ($n = 8$): chloramphenicol, ciprofloxacin b, colistin, gentamicin, imipenem, meropenem, tigecycline, and trimethoprim. The proportion of the ABs for which the *PPV* was estimable and reached 90% was 67% ($n = 6$): ampicillin, cefotaxime, ciprofloxacin a, colistin, gentamicin, and trimethoprim. The proportion of the ABs for which the *SE* was estimable and reached 90% was 44% ($n = 4$): chloramphenicol, colistin, gentamicin, and trimethoprim. The proportion of the ABs for which the *SP* was estimable and reached 90% was 92% ($n = 11$): ampicillin, cefotaxime, chloramphenicol, ciprofloxacin a, ciprofloxacin b, colistin, gentamicin, imipenem, meropenem, tigecycline, and trimethoprim. The proportion of the ABs for which *ME* was estimable and did not exceed 5% was 92% ($n = 11$): ampicillin, cefotaxime, chloramphenicol, ciprofloxacin a, ciprofloxacin b, colistin, gentamicin, imipenem, meropenem, tigecycline, and trimethoprim. The proportion of the ABs for which *VME* was estimable and did not exceed 5% was 44% ($n = 4$): chloramphenicol, colistin, gentamicin, and trimethoprim.

The ARG frequencies associated with the AB identified in the metagenome analysis are summarised in [Fig. 4](#), while [Fig. 5](#) shows the ABs for which no ARG was detected in the sample ([Solymosi et al., 2024](#)).

DISCUSSION

Based on the phenotypic and genotypic resistance concordance analyses in *E. coli* strains, the following conclusions can be drawn ([Solymosi et al., 2024](#)). For 39% of ABs, sensitivity

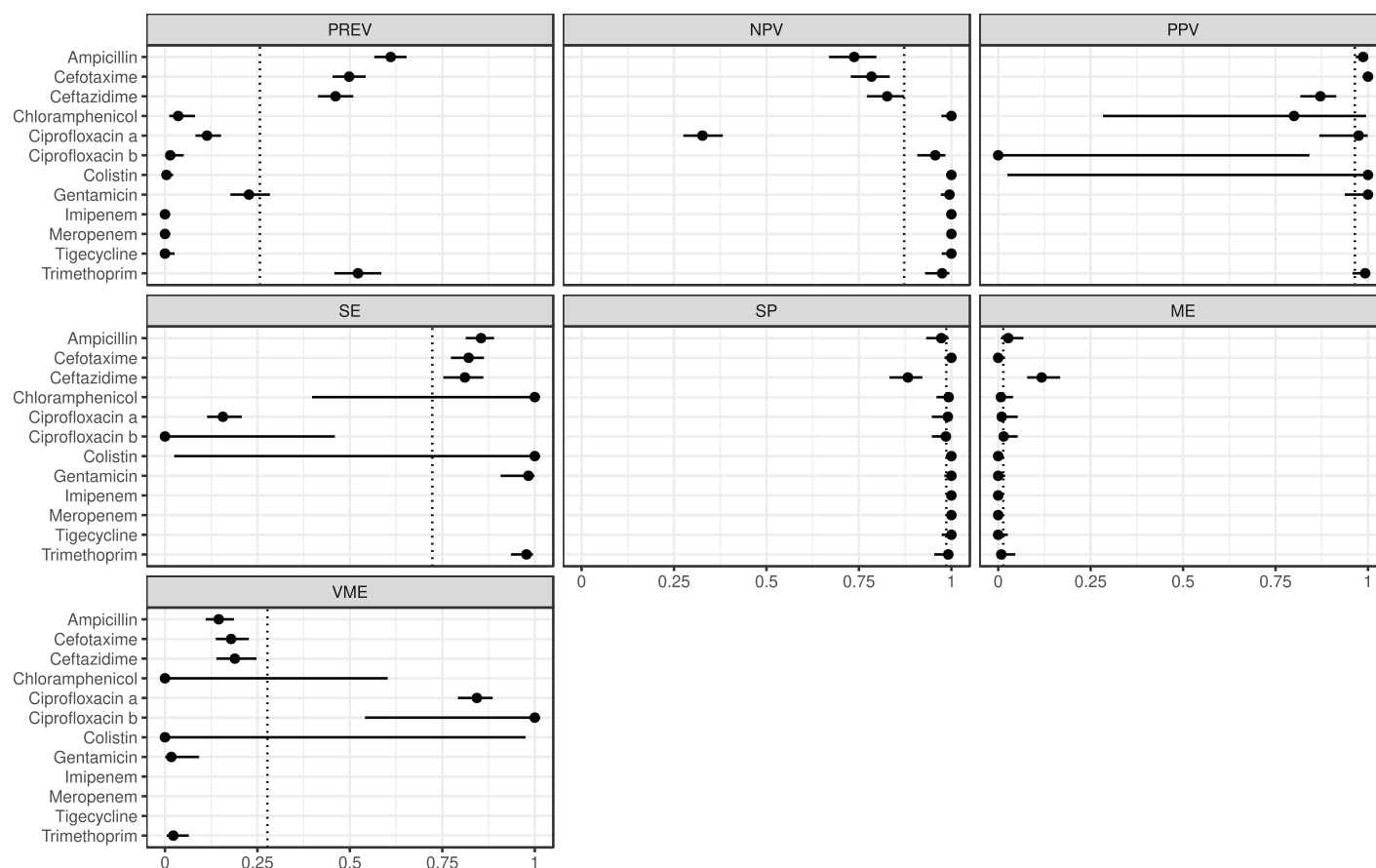


Figure 3 Overall predictive performance for ABs having the same minimum inhibitory concentration (MIC) breakpoints in at least two BioProjects. Column PREV shows the prevalence (with 95% CI) of phenotypic antimicrobial resistance against certain ABs within the BioProjects. Point and 95% CI estimates for negative and positive predictive values, sensitivity, specificity, major and very major error rates are presented in columns NPV, PPV, SE, SP, ME, and VME, respectively. In the case of ciprofloxacin there were two MIC breakpoint sets having data from two BioProjects. For ciprofloxacin a the MIC breakpoints were $S \leq 0.25$ and $R > 0.5$ mg/L, while for ciprofloxacin b $S \leq 0.5$ and $R > 1$ mg/L.

Full-size [DOI: 10.7717/peerj.18802/fig-3](https://doi.org/10.7717/peerj.18802/fig-3)

exceeded 90% in the evaluated BioProjects. In other words, for these ABs, phenotypically resistant strains are genotypically associated with a 90% probability of resistance. Based on the positive predictive values, a strain identified as genotypically resistant has at least a 90% probability of being phenotypically resistant for 45% of ABs. At 45% of ABs, there is less than a 5% probability that a phenotypically resistant strain will be identified as genotypically susceptible. By 88% of ABs, phenotypically susceptible strains are at least 90% likely to be susceptible based on genotype. Strains detected as genotypically sensitive are at least 90% likely to be phenotypically sensitive at 64% of the ABs tested. The probability of identifying a phenotypically sensitive strain as resistant based on genotype is less than 5% for 85% of ABs.

According to the overall estimations (Fig. 3) the 44% of the ABs, phenotypically resistant strains are genotypically associated with a 90% probability of resistance, while for the 92% of the ABs the phenotypically susceptible strains are genotypically susceptible with a 90%

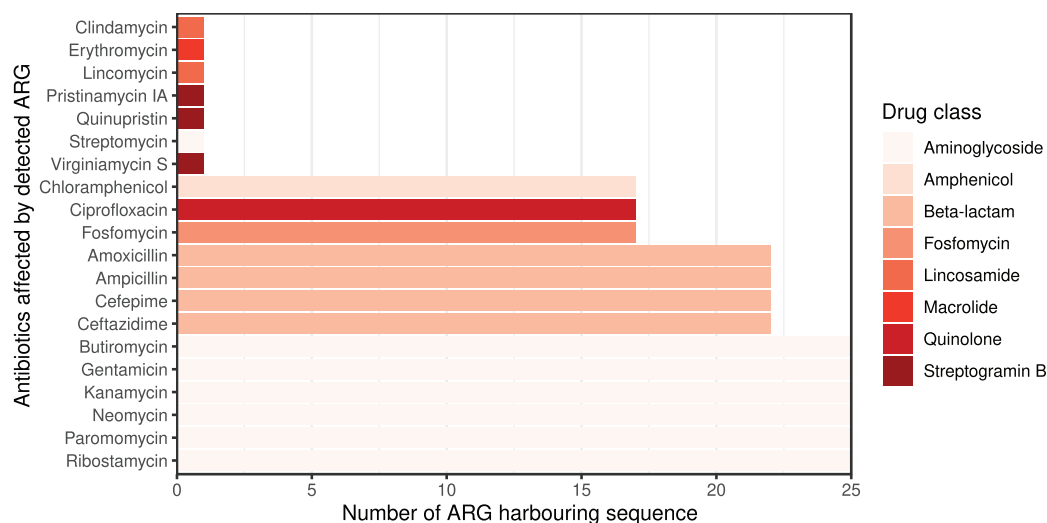


Figure 4 Detected ARGs in the metagenome of the canine external otitis sample. The number of sequences harboring ARGs by affected AB compounds shown horizontally. The color of the bars represents the drug class to which a given AB compound belongs.

Full-size [DOI: 10.7717/peerj.18802/fig-4](https://doi.org/10.7717/peerj.18802/fig-4)

probability. ARG detection showed a phenotypic prediction with at least 90% confidence in 67% of ABs. The probability of detecting a phenotypically susceptible strain as resistant based on genotype is below 5% for 92% of ABs. While the probability of detecting a phenotypically resistant strain as susceptible based on genotype is below 5% for 44% of ABs.

To have more detailed understanding of the agreement results, a further aspect of challenges related to AST should be considered. Even broth microdilution, which is the AST method proposed by EUCAST gives debatable results, and as do other classical, phenotype-based methods (e.g., disk diffusion). Dissimilarity in laboratory conditions, human resources, and discrepancies in laboratory techniques may lead to different results (Rebello et al., 2022).

A recent study including a machine learning-assisted ARG-based workflow for phenotypic AMR prediction indicates that the highest prediction accuracy for AMR against the drug classes tested appears by macrolides and sulfonamides. The highest uncertainty was found by beta-lactams that were also tested in combinations (Hu et al., 2024). From the demonstrated BioProjects, azithromycin, sulfamethoxazole, and sulfisoxazole fall into the above categories. In contrast to the above mentioned publication, only sulfixazole yielded above average results considering all the investigated factors (NPV, PPV, SE, SP, ME, VME). In line with the above-mentioned study, rather large deviations from the mean were found by beta-lactams. However, this finding be explained by the large number of beta-lactams. In our case 14 different compounds were tested in the above-mentioned drug class (ampicillin, aztreonam, cefepime, cefotaxime, cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, cefuroxime, ertapenem, imipenem, meropenem, piperacillin, temocillin). These ABs belong to separate subcategories within beta-lactams

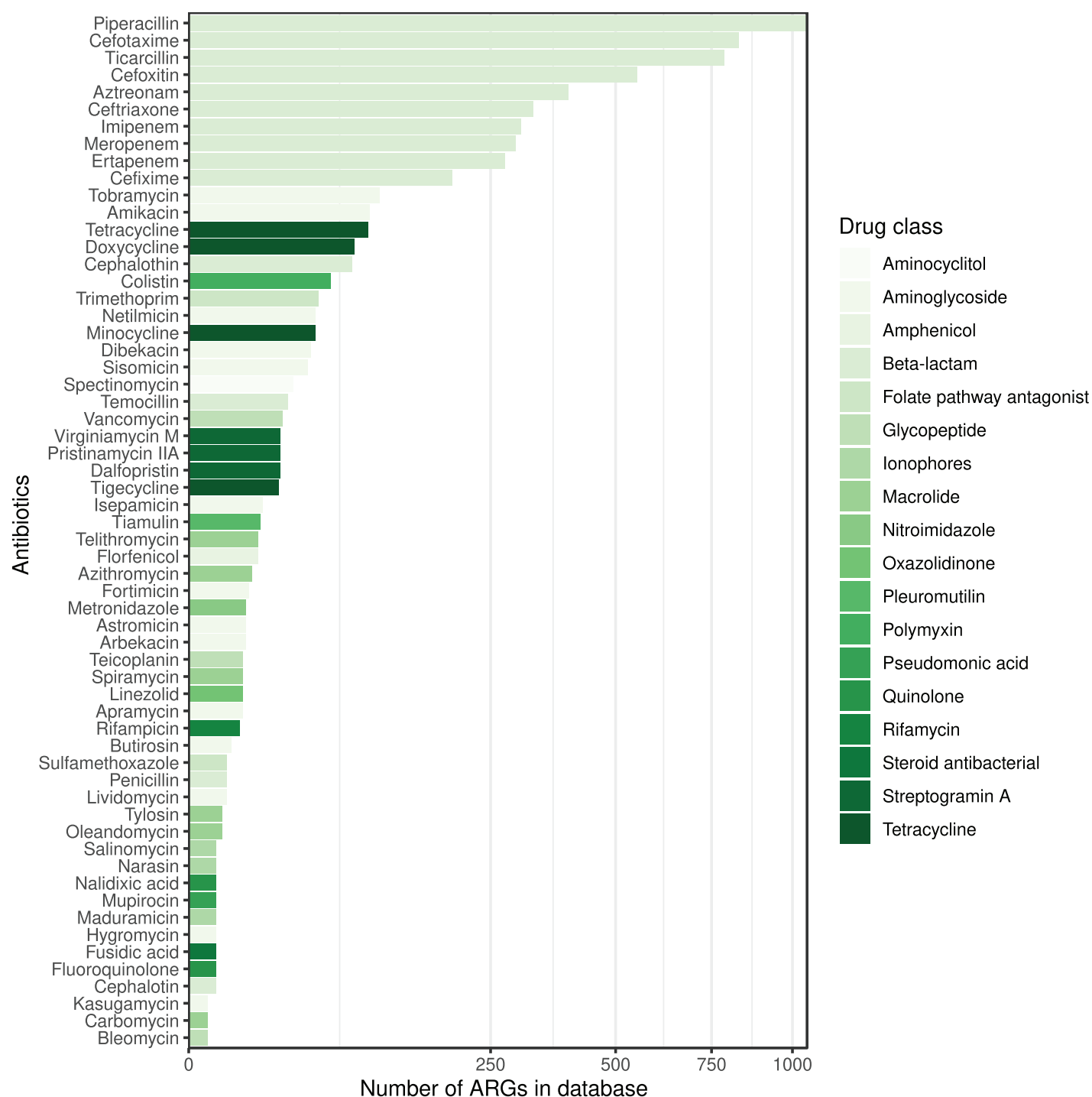


Figure 5 ABs for which no ARG was detected in the metagenomic sample are sorted by decreasing number of ARGs in the database affecting AB compounds. The color of the bars represents the drug class to which a given AB compound belongs.

Full-size DOI: 10.7717/peerj.18802/fig-5

(cephalosporins, penams, penems, monobactams) that have very different properties from each other or even within the subcategory (e.g., cephalosporin generations) ([Bush & Bradford, 2016](#)).

In contrast to single-strain cultures, similar comparisons using descriptive measures cannot theoretically be made with complex microbiological samples. One of the reasons for this is that only a small proportion of bacteria can be easily cultured and tested for phenotypic resistance ([Steen et al., 2019](#)). The recent advance of culturomics may partly address this aspect. Culturomics methods facilitates higher throughput, i.e., the identification of numerous difficult-to-culture bacteria, by applying several different growth conditions and media simultaneously ([Lagier et al., 2015](#)). Even though, the number of condition combinations required for the same throughput can be experimentally reduced ([Diakite et al., 2020](#)), not all culturomics combinations are used in routine diagnostics. Thus, several bacteria involved in disease processes caused by a complex microbial communities can remain hidden, even if they play a critical role. However, shotgun metagenomic studies offer the opportunity to obtain data on the relative abundance rates of the taxa and, of particular clinical relevance, the genes, including ARGs of complex microbiomes. The ARG prevalence data can then be used to gain insight into the AMRP of a given sample. Unfortunately, due to the limitations of bacteriological culturing, the concordance of AMRP and complex bacterial phenotypic resistance cannot currently be depicted. However, if we think of the complex microbiological samples as sets of bacterial single-strains, we can assume that the phenotypic and genotypic resistance/susceptibility matches for the strains may also be extended to them.

The fact that the study was carried out on *E. coli* strains may also be taken into account when evaluating the results. According to the publication by [Hu et al. \(2024\)](#), in which phenotypic AMR profile prediction was performed using genotype-based machine learning algorithms by various bacterial species, predictions for *E. coli* strains were less robust and reliable compared to other species such as *Campylobacter jejuni* and *Enterococcus faecium* ([Hu et al., 2024](#)). These differences in phenotypic AMR profile prediction at the bacterial species level may also be relevant in bacterial communities containing multiple taxonomic groups. Furthermore, in addition to AMR at the cellular level, there is an increasing focus on the so-called cooperative resistance of whole bacterial communities. The dynamics within microbial communities (regardless of being single-species populations or mixed populations) can influence the response to antimicrobial therapy ([Denk-Lobnig & Wood, 2023](#)). A bacterial cell that can inactivate ABs with an exoenzyme can confer immunity to a cell that does not have the enzyme ([Vega & Gore, 2014](#)). Such interspecies interactions are already known, e.g. *Stenotrophomonas maltophilia* can also degrade imipenem in an enzyme-mediated manner, thereby protecting susceptible *Pseudomonas aeruginosa* in cystic fibrosis ([Denk-Lobnig & Wood, 2023](#)). Cooperative resistance is present in all microbe-rich environments ([Vega & Gore, 2014](#)). Most clinical samples are microbe-rich and therefore using genotypic resistance testing in practice should be considered for its beneficial, additional value.

From a clinical point of view, the question is which ABs are effective against the bacteria involved in a complex microbial community that induces or sustains a disease process. From the presented studies of *E. coli* strains, phenotypic susceptibility can be predicted with high confidence for the vast majority of ABs tested based on the *ME*, *NPV*, *SP* metrics of the genotype. If we extend these approach to metagenomic datasets, its clinical applicability for the selection of therapeutic agents could be considered. Reflecting to the AMRP based on the metagenomic analysis presented above, we can conclude the following. Even infections caused by complex microbial communities can be associated with ABs that were not affected by any ARGs found, despite being associated with ARGs in the reference database. The number of ARGs for different ABs and drug classes may vary widely in the different databases, and it is important to note that each ARG database is incomplete. However, we can assume that ABs against which no ARGs can be detected in a sample are more likely to be clinically effective if the ARG reference databases include a relatively higher number of ARGs against them.

Conventional phenotypic AST procedures are still among the most significant methods for everyday routine bacteriological diagnostics. However, the adoption of ONT sequencing, followed by comprehensive bioinformatic analysis, allows for rapid detection of microorganisms, along with the assessment of their abundance rates, presence of various genes, including ARGs or even virulence genes, possibly all within a timeframe of 4 to 24 h (Ring et al., 2023). Consequently, clinical metagenomics based on ONT has the potential to enhance diagnostic microbiology and clinical practices significantly. Nonetheless, the innovative nature of this approach raises several questions that have been addressed within this study, including discrepancies observed between the identified ARGs and the expected phenotypic antimicrobial resistance (Yee, Dien Bard & Simner, 2021).

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Norbert Solymosi conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Adrienn Gréta Tóth conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Sára Ágnes Nagy conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- István Csabai conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Csongor Feczkó performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Tamás Reibling conceived and designed the experiments, authored or reviewed drafts of the article, sample collection, and approved the final draft.
- Tibor Németh conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving body and any reference numbers):

The sample we present is from a dog that was operated on with external otitis. In this operation, it is a routine procedure to collect specimens for microbiological control. This sampling is not an additional intervention; in this way, there is no requirement for research approval.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The data is available at NCBI BioProject: [PRJNA1045271](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1045271), [PRJDB7087](https://www.ncbi.nlm.nih.gov/bioproject/PRJDB7087), [PRJEB14086](https://www.ncbi.nlm.nih.gov/bioproject/PRJEB14086), [PRJEB21880](https://www.ncbi.nlm.nih.gov/bioproject/PRJEB21880), [PRJEB21997](https://www.ncbi.nlm.nih.gov/bioproject/PRJEB21997), [PRJNA266657](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA266657).

Data Availability

The following information was supplied regarding data availability:

The data is available at NCBI SRA: [SRR31306311](https://www.ncbi.nlm.nih.gov/sra/SRR31306311).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.18802#supplemental-information>.

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