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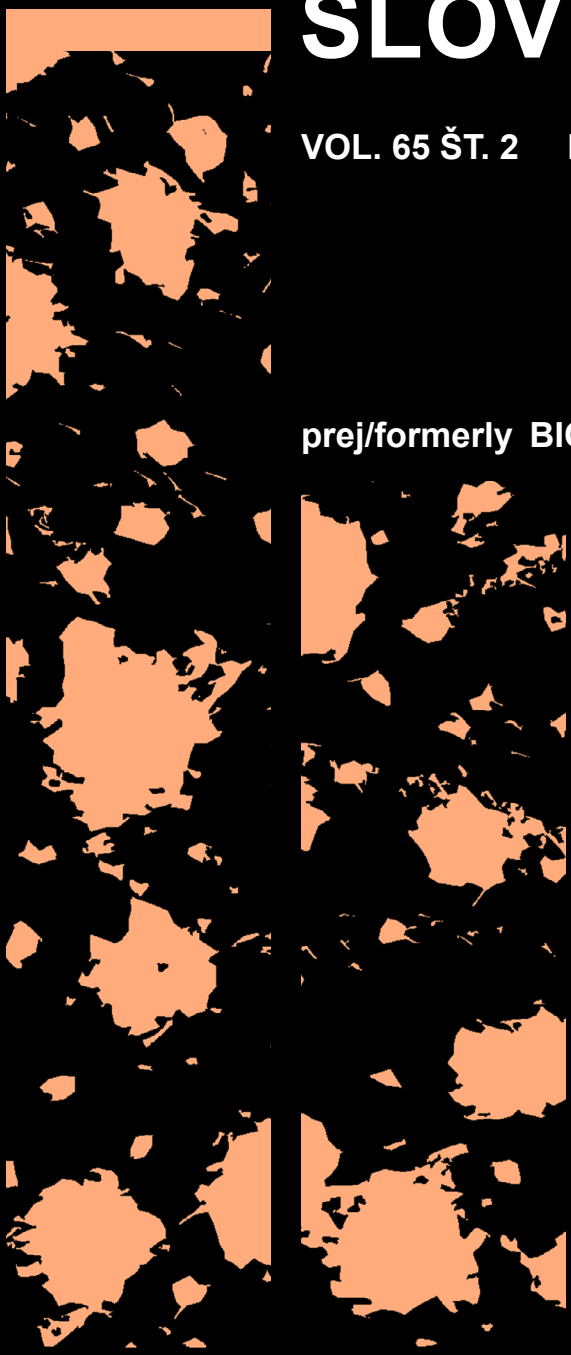

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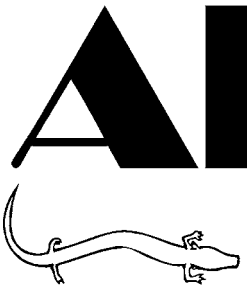

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Masna spektrometrija v raziskavah kačjih strupov

Mass spectrometry in snake venom research

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Izvleček: Masna spektrometrija omogoča hitro in zanesljivo identifikacijo in karakterizacijo proteinov in peptidov v kačjih strupih. Z vse večjo dostopnostjo transkiptomskih in genomskih podatkov se veča podatkovna baza proteinskih zaporedij, ki je ključna za identifikacijo proteinov. Kačje strupe analiziramo z večdimenzionalnim proteomskim pristopom, poimenovanim »venomika«. Proteine najprej med seboj ločimo z eno- ali dvo-dimenzionalno gelsko elektroforezo ali s hitro tekočinsko kromatografijo na obrnjenih fazah. Posamezne proteinske lise oziroma frakcije encimsko razgradimo in dobljene peptide analiziramo z masnim spektrometrom. Proteine identificiramo s primerjavo masnih spektrov peptidov s spektri v podatkovni bazi. Visoko zmogljivi masni spektrometri omogočajo analizo strupov tudi brez predhodnega ločevanja mešanice proteinov v strupu. Analizirali smo proteinski sestavi (proteoma) dveh evropskih, medicinsko najbolj zanimiv kačjih strupov, modrasovega (*Vipera a. ammodytes*) in gadovega (*Vipera b. berus*). Modras je najbolj strupena evropska kača. Njen ugriz je sicer redko smrten, pogosto pa zahteva bolnišnično opazovanje in zdravljenje s protistrupom. Gad je najbolj razširjena evropska strupenjača, katere ugriz v večini primerov izzove blažje simptome kot ugriz modrasa. S proteomsko raziskavo smo na molekulskem nivoju razložili opažene razlike v delovanju obeh strupov. Poleg tega smo analizirali tudi proteom strupa malega gada (*Vipera ursinii* ssp.), najbolj ogrožene evropske kačje vrste. Za človeka ne predstavlja nobene nevarnosti. V naravi se prehranjuje pretežno z insekti, medtem ko jih v ujetništvu hranijo z mišmi. Primerjava proteomske analize strupa kač iz naravnega okolja in strupa kač iz ujetništva je pokazala očitne razlike. Sestava kačjega strupa je torej pogojena z dieto. Masna spektrometrija je zelo uporabno orodje tudi pri karakterizaciji protistrupov (antivenomika), za določanje njihove specifičnosti in nevtralizacijske moči.

Ključne besede: antivenomika, kačji strup, mali gad, masna spektrometrija, modras, navadni gad, proteomika, venomika, *Vipera a. ammodytes*, *Vipera b. berus*, *Vipera ursinii*

Abstract: Mass spectrometry allows rapid and reliable identification and characterisation of proteins and peptides in snake venoms. With the increasing availability of transcriptomic and genomic data, there is a growing database of protein sequences that is essential for protein identification. Snake venoms are analysed using a multi-

dimensional proteomic approach known as ‚venomics'. Proteins are first separated by one- or two-dimensional gel electrophoresis or reversed-phase liquid chromatography. The individual protein spots or fractions are digested enzymatically and the resulting peptides are analysed by mass spectrometry. The proteins are identified by comparing the mass spectra of the peptides with those in the database. High-performance mass spectrometers allow the analysis of venoms even without prior separation of the protein mixture. We have analysed the protein composition (proteome) of two European snake venoms of greatest medical interest, the nose-horned viper (*Vipera a. ammodytes*) and the common adder (*Vipera b. berus*). The nose-horned viper is the most venomous European snake. Although its bite is rarely fatal, a human wictim often needs to be observed in hospital and treated with an antivenom. The adder is the most widespread European venomous snake and its bite causes milder symptoms than the bite of the nose-horned viper in most cases. To explain the observed differences in the effects of the two venoms at the molecular level, a proteomic study was performed. We also analysed the proteome of the venom of the meadow viper (*Vipera ursinii*), the most threatened snake species in Europe. It does not pose a threat to humans. In the wild, it feeds mainly on insects, while in captivity it is fed on mice. A comparison of the proteome of the venom of snakes in the wild and snakes in captivity showed clear differences. Thus, the composition of snake venom is diet-dependent. Mass spectrometry is also a very useful tool in the characterisation of antivenoms (antivenomics) to determine their specificity and neutralising power.

Keywords: antivenomics, common adder, mass spectrometry, meadow viper, nose-horned viper, proteomics, snake venom, venomics, *Vipera s. ammodytes*, *Vipera b. berus*, (*Vipera ursinii*)

Uvod

Strupene kače so skozi evolucijo razvile enega najbolj izpopolnjenih orožij v naravi. Njihov strup lahko povzroči prizadetost ali celo smrt, zato so se jih ljudje skozi zgodovino bali, jih častili in jim pripisovali nadnaravne moči. Raziskave kačjega strupa so v sodobnem času usmerjene predvsem v obvladovanje kačjih ugrizov, v razvoj diagnostike in protistrupov (antidotov) ter novih pristopov zdravljenja. Kačji strupi so zanimivi tudi kot potencialni vir visoko specifičnih farmakološko aktivnih snovi in spojin vodnic za razvoj inovativnih zdravil. Opisanih je že več kot 3000 vrst kač, od katerih je le ena petina strupenih (Uetz in sod. 2022). Slednje razvrščamo v štiri družine: Colubridae (goži), Elapidae (strupeni goži), Atractaspidae (zemeljski gadi) in Viperidae (gadi). Približno deset odstotkov vseh kač (376 vrst) pripada družini Viperidae. Ta se naprej deli na tri poddružine: *Azemiopinae* in *Crotalinae* (jamičarke) ter *Viperinae* (pravi gadi). Evolucijski izvor poddružine *Viperinae* (trenutno obsega 100 vrst) je še vedno nejasen, vendar sega v srednji

Okrajšave: 1DE, enodimenzionalna gelska elektroforeza; 2DE, dvodimenzionalna gelska elektroforeza; cDNA, komplementarna deoksiribonukleinska kislina; CRISP, s cisteinom bogati sekretorni proteini; CTL, lektini tipa C; DIS, disintegrini; DNA, deoksiribonukleinska kislina; ELISA, encimsko-imunski test; ESI, elektrosprej ionizacija (ang. *electrospay ionization*); LC, tekočinska kromatografija (ang. *liquid chromatography*); KUN, peptidi Kunitzovega tipa; LAO, *L*-aminokislinske oksidaze; MALDI, ionizacija z lasersko desorbcijo ob pomoči matrice (ang. *matrix--assisted laser desorption/ionization*); MPKS, metaloproteinaze iz kačjih strupov; MS, masna spektrometrija; MS/MS, tandemska masna spektrometrija; NaDS-PAGE, poliakrilamidna gelska elektroforeza v prisotnosti natrijevega dodecil sulfata; NCBI, Nacionalni center za biotehnološke informacije (ang. *National Center for Biotechnology Information*); RNA, ribonukleinska kislina; RP-HPLC, visokotlačna tekočinska kromatografija na obrnjenih fazah; $sPLA_2$, sekretorne fosfolipaze A_2; SPKS, serinske proteaze iz kačjih strupov; TOF, čas preleta ionov (ang. *time of flight*); WHO, Svetovna zdravstvena organizacija (ang. *World Health Organization*).

eocen in zgodnji miocen (42 do 34 milijonov let nazaj) (Alencar in sod. 2016). Najstarejši znani fosil, *Vipera antiqua*, pa so našli v srednji Evropi in je datiran v zgodnji miocen, pred približno 22,5 milijoni let (Šmíd in Tolley 2019). Odtlej so se *Viperinae* razvile v več linij in osvojile "Stari svet". Ob Evropi (z izjemo Irske in nekaj sredozemskih otokov), te kače najdemo na Bližnjem vzhodu, v Afriki (z izjemo Madagaskarja) in v Aziji, celo na skrajno vzhodnih otokih, Tajvanu in Sahalinu. V Sloveniji lahko srečamo modrasa (*Vipera ammodytes ammodytes*), navadnega gada (*Vipera berus berus*) in laškega gada (*Vipera aspis*, podvrsta *francisciredi*). Prvi je najbolj strupena, drugi pa najbolj razširjena evropska strupenjača.

Strupene kače so za onesposobitev ali celo usmrtitev plena razvile zelo zapleten strupni aparat (Mebs 2002). Na obeh straneh zgornje čeljusti se nahajajo posebne strupne žleze, ki razvojno izhajajo iz žlez slinavk in proizvajajo strupen izloček oziroma strup. Tega ob ugrizu kače vbrizgajo v plen skozi ostre strupnike, različno dolge votle zobe z majhno odprtinico na konici. Pri velikih kačah iz družine gadov, npr. puhnici (*Bitis arietans*), so strupniki daljši od 3 cm, pri slovenskih strupenjačah pa niso daljši od enega centimetra. Količino iztisnjenega strupa kača regulira s pritiskom mišic na žlezo. Tako v primeru obrambnega ugriza včasih celo ne pride do izločanja strupa. Takemu ugrizu pravimo suhi ugriz. Kače svoj plen pogoltnejo v celoti, zato mora strup hitro in učinkovito prizadeti vitalne telesne funkcije žrtve, npr. blokirati krčenje mišic ali pretok krvi. Delovanje strupa je odvisno od njegove sestave, ki je značilna za vsako kačjo družino. Strupi gožev in gadov najbolj zmotijo strjevanje krvi (hemotoksičnost), strupi zemeljskih gadov delovanje srca (kardiotoksičnost), strupi strupenih gožev pa poleg delovanja srca tudi delovanje živčevja (nevrotoksičnost).

Izvor in sestava kačjih strupov

Kačji strupi vsebujejo mešanico biološko aktivnih proteinov in peptidov (približno 90-95 % mase strupa) ter drugih neproteinskih sestavin, vključno z ogljikovimi hidrati, lipidi, amini in anorganskimi solmi (Villar-Briones in Aird 2018; Mebs 2002). Proteinske komponente izvirajo iz genov, ki sicer nosijo zapis za telesne proteine, po navadi tiste, ki sodelujejo v ključnih fizioloških procesih v telesu (npr. v hemostazi, prenosu živčnega signala …). V evoluciji je prišlo do podvajanja teh genov, prenosa ene od kopij v strupno žlezo in njenega razvoja v strupni žlezi, kjer je v procesu neofunkcionalizacije razvila nove funkcije za učinkovito delovanje strupne žleze (Barua in Mikheyev 2020). Da bi bili učinkoviti pri lovljenju plena in obrambi pred plenilci, se geni za toksine, v primerjavi z geni za netoksične proteine, razvijajo precej hitreje (Kini 2018). Predlaganih je bilo več različnih mehanizmov za razlago tega zanimivega pojava, kot so pogostejše mutacije v eksonih (delih gena, ki nosijo zapis za protein) v primerjavi z introni (delih gena, ki ne nosijo zapisa za protein) in nesinonimne zamenjave v eksonih (zamenjava nukleotida v DNA, ki povzroči spremembo v aminokislinskem zaporedju), visoka pogostost točkovnih mutacij, spremembe na meji med intronom in eksonom, izbris eksona in izguba/pridobitev domen z rekombinacijo ter hitro kopičenje mutacij, ki povzročajo spremembe na površini proteinske molekule. V multigenskih družinah toksinov se ohrani osnovno molekulsko ogrodje izvornega proteina (tridimenzionalna struktura), spreminjajo pa se aminokislinski ostanki izven ogrodnih, ki so ključni za funkcijo. Tako skozi evolucijo nastajajo izooblike osnovne molekule z novimi aktivnostmi (Fry in sod. 2009). Dejavniki, ki vplivajo na neofunkcionalizacijo so številni, nekateri še neznani, povzročajo pa variacije v sestavi strupa. Na krajši rok pa so za sestavo strupa pomembni dejavniki, ki vplivajo na izražanje strupnih genov. Tako sestava strupa variira glede na vrsto in geografsko podvrsto kače, njen spol, starost in velikost kot tudi prehrano in letni čas (Chippaux in sod. 1991; Lang Balija in sod. 2005).

Venomika kačjih strupov

V novem tisočletju je masna spektrometrija (MS) postala glavna analitska metoda v proteomiki, masovno in hitropretočno identifikacijo in karakterizacijo kompleksnih proteinskih zmesi, kakršne so tudi kačji strupi. Skokovit tehnični napredek na področju MS in proteomike vzporedno z nekaterimi

drugimi »omik«-tehnologijami, transkriptomiko in genomiko, toksinologom omogočajo bolj in bolj poglobljeno kvalitativno in kvantitativno analizo živalskih strupov, tako imenovano venomiko (Calvete in sod. 2007; Calvete in sod. 2009).

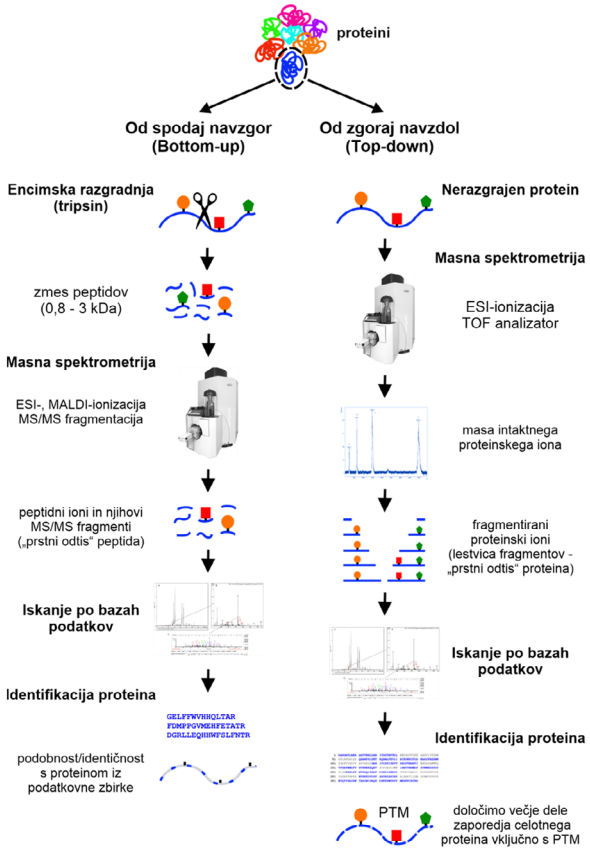
Masna spektrometrija–Proteomika

MS temelji na analizi ionov v plinastem stanju. Ti se v magnetnem polju ločijo glede na razmerje njihove mase in naboja (m/z). Glede na način ionizacije (npr. elektrosprej (ang. *electrospay ionization*, ESI); ionizacija z lasersko desorbcijo ob pomoči matrice (ang. matrix-assisted laser desorption/ionization, MALDI) in masnega analizatorja (npr. kvadrupolni, na ionsko past, na čas preleta ionov (ang. *time of flight*, TOF) ločimo več vrst masnih spektrometrov. Za določanje mase celih proteinskih molekul se uporablja kombinacija MALDI- ali ESI-TOF, medtem ko je kombinacija ESI in analizatorja z ionsko pastjo ali trojnega kvadrupolnega (ang. *quadrupole*, Q) analizatorja primerna za generiranje sekundarnih ionskih spektrov in s tem za »*de novo*« sekvenciranje peptidov. Identifikacijo proteinov z MS najpogosteje izvajamo s pristopoma »od spodaj navzgor« (ang. *bottom-up*) in »od zgoraj navzdol« (ang. *top-down*) (Sl. 1).

Proteomika »od spodaj navzgor« temelji na MS in MS/MS analizi peptidov, dobljenih z encimsko ali kemično razgradnjo proteinov. Peptidni oziroma prekurzorski ion ujamemo v kolizijski celici in izzovemo njegovo delno fragmentacijo. Tako se v prekurzorskem ionu delno cepijo le vezi v osnovni peptidni verigi, da nastanejo fragmenti - produktni ioni, ki se med seboj razlikujejo le za en aminokislinski ostanek. Produktne ione analiziramo, da dobimo MS/MS spekter, ki je specifičen za vsak peptid oziroma njegov prekurzorski ion. Odvisen je od njegovega aminokislinskega zaporedja in predstavlja njegov »prstni odtis«. Peptide identificiramo z bioinformatskimi analizami MS in MS/MS spektrov, ki uporabljajo različne algoritme za iskanje po podatkovnih bazah (npr. Mascot in Sequest) (Chen in sod. 2020). Slednje temelji na matematični primerjavi prekrivanja masnih spektrov analiziranih peptidov z bazo podatkov, v kateri so eksperimentalno določeni ali/in teoretično generirani peptidni MS spektri proteinov. Rezultat računalniške analize je najverjetnejša identiteta analiziranega proteina.

Pri proteomskem pristopu »od zgoraj navzdol« (Sl. 1) analiziramo cele proteinske molekule (Schaffer in sod. 2019). Z ESI ali MALDI ionizacijo se v plinski fazi pripravijo ioni celih proteinov, ki jih potem analiziramo s TOF analizatorjem. Prednost te metode je, da lahko med seboj ločimo vse oblike, v katerih določeni protein obstaja (proteoforme) - so sicer produkt istega gena, a rezultat specifičnih genetskih variacij, alternativnega spajanja in posttranslacijskih modifikacij. Proteinski ioni lahko fragmentirajo z disociacijo, povzročeno s trki, ali z metodama »mehke« disociacije z zajemanjem elektronov ali prenosom elektronov v masnem spektrometru. Pri tem razberemo molekulsko maso proteinskega iona in mase njegovih fragmentov - peptidov (»prstni odtis« proteina). Če je za identifikacijo potrebno, lahko večje peptide dodatno fragmentiramo in produkte analiziramo s tandemsko MS (MS/MS). V primeru MS analize čistih proteinov lahko določimo njihovo celotno primarno strukturo vključno s post-translacijskimi modifikacijami. Fragmentacija ionov celih proteinov z visoko molekulsko maso (večjo od 50-70 kDa) v plinski fazi je težavna, zato je za razločevanje razlik med velikimi molekularnimi ioni podobnih mas potreben instrument zelo visoke ločljivosti. Glavni izzivi, s katerimi se trenutno sooča proteomika »od zgoraj navzdol«, so še posebej omejena topnost proteinov, dinamično območje proteomov, kompleksnost proteomov in analiza kompleksnih podatkov (Melby in sod. 2021).

Glede na dopolnjujočo se naravo informacij, ki jih zagotavljata oba pristopa masne analize proteinov, se bosta v proteomiki še naprej uporabljala oba. Različne statistične metode in metode strojnega učenja, ki so bile razvite za izvajanje poglobljene analize v proteomskih študijah, pa nam omogočajo, da kvalitativne in kvantitativne podatke o proteinih uporabimo pri rekonstrukciji proteinskih interakcij in signalnih omrežij.



Slika 1: Pristopi v proteomiki, ki temeljijo na masni spektrometriji. S proteomiko »od spodaj navzgor« (ang. *bottom-up*) analiziramo manjše proteinske fragmente (peptide), pridobljene z encimsko (najpogosteje se uporablja tripsin) ali kemično razgradnjo proteinov. Posamezni peptidni ion v masnem spektrometru fragmentiramo, da dobimo tandemski masni spekter (MS/MS spekter), ki je značilen za vsak peptid oziroma njegov prekurzorski ion (»prstni odtis« peptida) posebej. Z uporabo računalniških algoritmov primerjamo izmerjene mase peptidnih ionov in/ali njihovih fragmentov z bazo podatkov, v kateri so teoretično generirani peptidni masni spektri (MS in MS/MS) proteinov. Rezultat te analize je najverjetnejša identiteta analiziranega proteina. S proteomiko »od zgoraj navzdol« (ang. *top-down*) dobimo informacijo o masi celih, intaktnih proteinskih molekul, vključno z njihovimi post-translacijskimi modifikacijami. Za identifikacijo cele proteine fragmentiramo v masnem spektrometru, da dobimo masno lestvico fragmentov, značilno za vsak protein (t.i. »prstni odtis« proteina).

Figure 1: Mass spectrometry-based approaches in proteomics. Bottom-up proteomics is used to analyse small protein fragments (peptides) obtained by enzymatic (usually trypsin) or chemical degradation of proteins. A single peptide ion is fragmented inside mass spectrometer to obtain a tandem mass spectrum (MS/MS spectrum), characteristic for each peptide i.e. its precursor ion (the peptide's »fingerprint«). Computer algorithms are used to compare the acquired masses of the peptide ions and/or their fragments with a database containing theoretically generated peptide mass spectra (MS and MS/MS) of proteins. The result of this analysis is the most probable identity of the analysed protein. Top-down proteomics provides information on the mass of whole, intact protein molecules, including their post-translational modifications. For identification, the proteins are fragmented in the mass spectrometer to obtain a ladder of fragment masses, characteristic for each protein (the protein's »fingerprint«).

Proteomski pristopi v venomiki

Ena sama analitična metoda ne zadostuje za razkritje kompleksnosti kačjega strupa, vsak pristop pa ima svoje prednosti in omejitve. Postopek se začne z odvzemom strupa, ki je preprost, vendar ključen korak, ki ima velik vpliv na nadaljnjo analizo in razlago podatkov. Pred tem morajo raziskovalci pridobiti uradna dovoljenja za terensko delo, ki so odvisna od kraja zbiranja in statusa ohranjenosti ciljne vrste. V Sloveniji so vse strupene kače razglašene za ogrožene vrste (Uradni list RS, 1993). Ročno odvzemanje strupa z »molžo« je daleč najpogostejša metoda za pridobivanje kačjega strupa. Pri tem žival ugrizne v s tanko membrano prekrito čisto stekleno posodo in vanjo sprosti strup. Tako zbrani strup lahko desetletja hranimo zamrznjenega pri temperaturah od -20 do -80 °C. Večinoma se strup hrani v liofilizirani obliki. Tako zagotovimo še dolgotrajnejšo stabilnost proteinskih komponent.

Calvete in sod. (2007) so predlagali postopek za analizo kačjih strupov, ki temelji na proteomiki »od spodaj navzgor« in je do danes ostal zlati standard venomike. Po ločevanju surovega strupa z visokotlačno tekočinsko kromatografijo na obrnjenih fazah (RP-HPLC) sledi analiza proteinskih frakcij z enodimenzionalno poliakrilamidno gelsko elektroforezo v prisotnosti detergenta natrijevega dodecil sulfata (1D NaDS-PAGE; 1DE). Odvisno od količine, proteine v gelu vizualiziramo z različnimi z MS kompatibilnimi barvili, kot npr. Coomassie modrim, Ponceau rdečim in koloidnim srebrom (Miller in sod. 2006). Obarvane proteinske lise izrežemo iz gela, proteine v gelu reduciramo, alkiliramo njihove proste SH-skupine (npr. karbamidometiliramo) in jih razgradimo s tripsinom. Nastale peptide ekstrahiramo iz gela, nato pa analiziramo in identificiramo s tandemsko MS. Relativno količino proteina lahko ocenimo glede na njegovo UV absorbcijo (površino vrha pri RP-HPLC analizi), intenzivnost proteinske lise na gelu ali glede na relativno intenzivnost MS spektrov treh najbolj intenzivnih ionov. V nekaterih proteomskih študijah so metodo RP-HPLC uspešno zamenjali z gelsko filtracijo, kombinacijo RP-HPLC/1DE pa z dvodimenzionalno gelsko elektroforezo (2DE) (Abd El-Aziz in sod. 2020). Slednja omogoča natančnejši vpogled v makromolekulsko organizacijo in v število proteoform posameznih toksinov v strupu.

Opisani pristop ima tudi nekaj pomanjkljivosti. Zahteva večje količine strupa ter dolgotrajnejšo manipulacijo vzorcev, kar povečuje možnost njihove kontaminacije. Poleg tega razgradnja vzorca s tripsinom pogosto preprečuje jasno identifikacijo številnih različic posameznih toksinov, izooblik ali kompleksnih multimernih struktur. Isti peptid pogosto identificiramo v več različnih proteoformah, kar vnaša dvom pri določanju identitete toksinov, posledično pa otežuje določanje skupnega števila in relativne vsebnosti proteoform, prisotnih v strupu.

Tem omejitvam se lahko izognemo tako, da nativne strupne proteine analiziramo s tandemsko MS v okviru proteomskega pristopa »od zgoraj navzdol« (Melani in sod. 2016; Ghezellou in sod. 2019). Surove vzorce strupa injiciramo v MS instrument neposredno preko sistema za tekočinsko kromatografijo, kar bistveno zmanjša potrebno količino strupa in skrajša operativni čas. Ta proteomski pristop pa zahteva tehnološko bolj zahtevne pristope, ki vključujejo visokoresolucijske MS instrumente z ustrezno računalniško in programsko podporo, ti pa so na voljo le v specializiranih laboratorijih (Melby in sod. 2021). Poleg tega kačji strupi pogosto vsebujejo veliko proteinov z visoko molekulsko maso, katerih analiza je, kot je omenjeno zgoraj, še vedno zahtevna in manj uspešna.

Ne glede na izbiro pristopa k analizi kačjega strupa je uspešnost identifikacije proteinov v največji meri odvisna od baze podatkov oziroma poznavanja proteoma, transkriptoma in genoma določene vrste kače. V proteinski knjižnici Nacionalnega centra za biotehnološke informacije (»National Center for Biotechnology Information«; NCBI) za taksonomsko skupino kače (Serpentes) najdemo že pol milijona vnosov, od tega za družino *Viperidae* 118.685 in poddružino *Viperinae* 6.550 vnosov.

Raznolikost in številčnost toksinov v proteomih kačjih strupov

Tasoulis in Isbister sta v svojem članku iz leta 2017 zbrala podatke o vseh 132 do takrat objavljenih proteomskih raziskavah kačjih strupov. V samo

štirih naslednjih letih je bilo analiziranih še dodatnih 79 novih proteomov (Tasoulis in sod. 2021). Ta trend se nadaljuje in v bazi Pubmed (https://pubmed.ncbi.nlm.nih.gov/) v zadnjem letu najdemo že 32 novih objav proteomov kačjih strupov. To je nedvomno spodbudila Svetovna zdravstvena organizacija (World Health Organization, WHO), ko je l. 2017 zastrupitvam s kačjimi strupi ponovno podelila status zanemarjene tropske bolezni, ki na vseh celinah povzroča ogromno trpljenja zaradi invalidnosti in prezgodnjih smrti (Chippaux 2017). Skupno so v proteomskih raziskavah odkrili toksine iz 63 encimskih in neencimskih družin proteinov in peptidov (Tasoulis in Isbister, 2017; Tasoulis in sod. 2021). Med njimi so tudi družine z majhnim številom proteinov, katerih funkcija ali biološki pomen ni znan. Encimske komponente so večinoma hidrolaze in *L*-aminokislinske oksidaze (LAO), ki sodelujejo pri usmrtitvi plena in pomagajo pri njegovi prebavi (Mebs, 2002). Med neencimskimi komponentami najdemo toksine, ki prizadenejo živčni sistem in celične membrane ter peptide z različnimi znanimi (npr. encimski inhibitorji) ali še neznanimi funkcijami. Glede na dosedanje proteomske raziskave so posamezni kačji strupi sestavljeni iz toksinov, ki jih lahko uvrstimo v le tri pa do celo 20 različnih proteinskih družin. V povprečju večino kačjih strupov sestavljajo toksini iz štirih proteinskih družin: triprstni proteini, sekretorne fosfolipaze A_2 ($sPLA_2$), serinske proteaze iz kačjih strupov (SPKS) in metaloproteinaze iz kačjih strupov (MPKS). Prvi dve družini prevladujeta pri strupenih gožih, zadnje tri pa pri gadih, v katerih so triprstni proteini zelo redko prisotni. Večina preostalih proteinov v kačjih strupih sodi med s cisteinom bogate sekretorne proteine (CRISP), peptide Kunitzovega tipa (KUN), LAO, natriuretične peptide, lektine tipa C (CTL) in disintegrine (DIS).

Skoraj dve tretjini vseh proteomskih raziskav se nanaša na strupe gadov. Njihovi strupi imajo bolj kompleksno sestavo od strupov strupenih gožev glede na število proteinskih družin, ki jih sestavljajo. Čeprav so strupi strupenih gožev sestavljeni iz manjšega števila proteinskih družin, pa je raznolikost toksinov znotraj njih zelo velika. Primer takega strupa je strup trakaste egiptovske kobre (*Naja annulifera*), ki ga povečini sestavljajo toksini iz ene same proteinske družine, to je triprstni proteini (78 %), vendar je znotraj te družine prisotnih 18 različnih izooblik (Tan in sod. 2020). V strupu vzhodne zelene mambe (*Dendroaspis polylepis*) so zabeležili celo 80 različnih triprstnih proteinov (Ainsworth in sod. 2018).

Proteomska slika strupov kač iz poddružine Viperinae

Celovito zbirko podatkov o proteomih strupov poddružine *Viperinae*, analitičnih metodah in postopkih najdemo v nedavno objavljenem članku Damm in sod. (2021), ki obravnava 54 objavljenih proteomskih študij, v katerih je bilo analiziranih 89 strupov iz 37 različnih vrst kač, ki pripadajo 11 rodovom. Primerjave njihovih proteomov so pokazale izjemne razlike na znotrajvrstni in medvrstni ravni med rodovi s poudarkom na regionalnih razlikah. Največ raziskav je posvečenih medicinsko najbolj pomembnim vrstam iz rodov *Bitis*, *Echis,* in *Daboia*, ki povzročijo največ smrti v ruralnih predelih Afrike, Indije in Srednjega Vzhoda (Gutiérrez in sod. 2010; Amr in sod. 2020; Pintor in sod. 2021). Veliko raziskav se po drugi strani posveča tudi medicinsko pomembnim evropskim strupenjačam iz rodu *Vipera* (Di Nicola in sod. 2021). V večini raziskav je bila uporabljena proteomika »od spodaj navzgor«, proteini v strupih pa so bili predhodno ločeni z eno izmed tekočinskih (gelska filtracija, ionsko-izmenjevalna, RP-HPLC) in/ali gelskih kromatografij (1DE, 2DE). Le v šestih raziskavah so uporabili proteomiko »od zgoraj navzdol«, od tega v štirih primerih za analizo strupov kač iz rodu *Vipera* (*V. ammodytes*, *V. anatolica*, *V. kaznakovi* in *V. transcaucasiana*) (Göçmen in sod. 2015; Hempel in sod. 2018; Petras in sod. 2019; Gopcevic in sod. 2021).

V strupih kač poddružine *Viperinae* prevladujejo štiri glavne družine toksinov, MPKS, $sPLA_2$, SPKS in CTL, ki predstavljajo 60 do 90 % celotnega strupa; pet sekundarnih družin toksinov (DIS, LAO, CRISP, KUN in žilni endotelijski rastni dejavniki F) predstavlja 6 do 15 % strupa; šest manj pomembnih družin toksinov, živčni rastni dejavnik, 5' nukleotidaze, fosfodiesteraze, hialuronidaze, fosfolipaze B in cistatin iz rodu *Bitis*, so določili v manj kot polovici proteomov s skupnim povprečnim deležem 13 %; več redkih

družin toksinov pa je prisotnih v povprečnem 1 % deležu: glutaminil ciklotransferaza, aspartana proteaza, različne proteaze in triprstni proteini. Izmed peptidov prevladujejo inhibitorji MPKS, natriuretični peptidi in peptidi, ki potencirajo bradikinin.

Rod *Vipera* šteje 21 različnih vrst in številne podvrste. Taksonomska raznolikost se odraža tudi v sestavi njihovega strupa. Med vsemi kačami iz družine Viperidae imajo tiste iz rodu *Vipera* v svojem strupu največ CRISP (5–30 %) in najmanj DIS (le nekaj 1 %). Izjema je strup stepskega gada (*V. renardi*) iz Rusije, pri katerem toksini iz družine DIS predstavljajo četrtino strupa (Kovalchuk in sod. 2016). V strupih *V. ursini* (Hrvaška), *V. b. berus* (Slovaška), *V. nikolskii* (Rusija) ter *V. a. montadoni* in *V. transcaucasiana* (Turčija) pa DIS niso odkrili (Bocian in sod. 2016; Kovalchuk in sod. 2016; Hempel in sod. 2018; Lang Balija in sod. 2020). Medtem ko imajo nekateri strupi kač iz rodu *Vipera* visoko vsebnost MPKS (npr. 40–50 % pri *V. ursini* in *V. anatolica*), so drugi bogati s $sPLA_2$ (npr. 45–52 % pri *V. a. montandoni* in *V. transcaucasiana*) ali SPKS (npr. 20–30 % pri *V. b. berus*, *V. nikolskii* in *V. orlovi* iz Rusije) (Göçmen in sod. 2015; Kovalchuk in sod. 2016; Latinović in sod. 2016; Hempel in sod. 2020). Zanimivo je opažanje, da sta količini MPKS in $sPLA_2$ v strupih obratno sorazmerni.

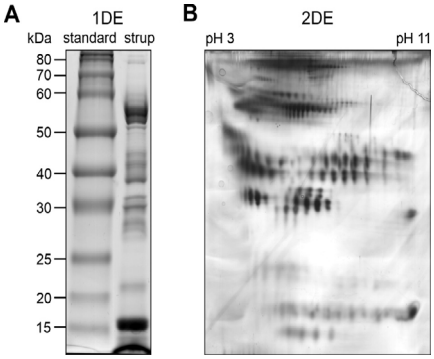
V nadaljevanju bom bolj podrobno opisala venomiko treh kač, modrasa (Sl. 2), gada in travniškega gada, ki je bila tema naših raziskav (Latinović in sod. 2016; Leonardi in sod. 2019; Lang Balija in sod. 2020) V vseh primerih smo uporabili proteomiko »od spodaj navzgor«. Naše rezultate bom primerjala z rezultati drugih raziskovalcev.

Slika 2: Modras (*Vipera a. ammodytes*). Njegov prepoznavni znak je rožiček na nosu, od koder izvira tudi njegovo angleško ime nose-horned viper ali long-nosed viper. Na hrbtu svetlo rjave, rdečkasto rjave ali sive barve ima cikcak vzorec temnejše barve (foto: Neven Vrbanić).

Figure 2: The nose-horned viper (*Vipera ammodytes ammodytes*). Its distinct feature is a single "horn" on the snout, hence its English name nose-horned or long-nosed viper. It has a dark zigzag pattern on its pale brown, reddish brown, or grey back (photo: Neven Vrbanić).

Venomika modrasovega strupa

Proteom in peptidom modrasovega strupa hrvaškega izvora smo analizirali s kombinacijo metod 2DE/LC-MS/MS in gelska filtracija/RP-HPLC/LC-MS/MS. Ključnega pomena za identifikacijo proteinov je bila transkriptomska analiza strupne žleze modrasa, s pomočjo katere smo zgradili cDNA knjižnico strupne žleze, ki je vsebovala prepise za prekurzorje 45 različnih proteinov in peptidov. Proteine iz surovega strupa z molekulsko maso večjo od 10 kDa smo ločili z 2DE in jih nato identificirali z MS. Za boljšo ločbo proteinov smo optimizirali vsak korak v 2DE metodi. V prvi dimenziji smo proteine ločili na poliakrilamidnih trakovih z imobiliziranim pH gradientom (IPG) v območju pH 3–11. Uspešnost analize je odvisna predvsem od priprave vzorca, to je njegove topnosti v pogojih izoelektričnega fokusiranja. Zato smo sestavo pufra za popolno raztapljanje vzorca in rehidracijo IPG trakov optimizirali z modificirano Taguchijevo metodo, ki omogoča enostavno in hitro optimizacijo večkomponentnih sistemov (Ahmad in Sharma, 2009). Pufer je vseboval standardne komponente, denaturante ureo in tioureo, ki smo jim za boljšo topnost proteinov dodali detergenta (CHAPS in ASB-14) in amfolite v optimalnih koncentracijah (Sl. 3). Proteine v IPG trakovih, fokusirane pri pH svojih izoelektričnih točk, smo reducirali in alkilirali pred analizo v drugi dimenziji. Slednja je potekala v 10 % (m/v) poliakrilamidnem gelu v puferskem sistemu Tris/tavrin (Sl. 3) (Tastet in sod. 2003). Ta puferski sistem ima v proteomiki prednost pred klasičnim NaDS-PAGE puferskim sistemom Tris/glicin, ker za ločbo proteinov v širokem masnem območju (5-250 kDa) potrebujemo manj zamrežene gele (le 9-11,5 % (m/v) namesto klasičnih 7,5-15 % (m/v)). Razgradnja proteinov, predvsem tistih z višjo maso, in ekstrakcija peptidov za MS analizo je bolj uspešna v manj zamreženih gelih. Pod temi pogoji smo strup modrasa z 2DE ločili na 208 proteinskih lis, ki smo jih vizualizirali z občutljivim reverznim barvanjem v prisotnosti imidazola in Zn^{2+} (Castellanos-Serra in sod. 2001). Gel se obarva motno belo, medtem ko kompleksi protein-NaDS-imidazol ostanejo prozorni. Prednost te metode pred barvanjem s koloidnim srebrom ali barvilom Coomassie modro je, da je zelo hitra in ne zahteva fiksacije proteinov v gelu, pri čemer lahko nastanejo netopni agregati. Proteinske lise smo izrezali iz gela, jih razgradili s tripsinom in analizirali s tandemsko MS.



Slika 3: Optimizirana 1DE (A) in 2DE (B) analiza modrasovega strupa. Strup smo analizirali z 1DE v 10 % (m/v) gelu v puferskem sistemu Tris/tavrin. Pri 2DE smo prvo dimenzijo (izoelektrično fokusiranje) izvedli na 7 cm IPG trakovih v optimiziranem rehidracijskem pufru, ki je vseboval detergenta 2,5 % (m/v) CHAPS in 0,25 % (m/v) ASB-14 ter 1 % (v/v) amfolitov. Pogoji v drugi dimeziji so bili enaki kot pri 1DE. Gele smo pobarvali s koloidnim srebrom.

Figure 3: Optimized 1DE (A) and 2DE (B) analysis of *V. a. ammodytes* venom. (A) - the venom was analysed in a 10% (w/v) gel in a Tris/Taurine buffer system. For 2DE, the first dimension (isoelectric focusing) was performed on 7 cm IPG strips in an optimised rehydration buffer containing detrgents 2.5% (w/v) CHAPS and 0.25% (w/v) ASB-14, and 1% (v/v) ampholytes. The conditions in the second dimension were the same as for 1DE. The gels were stained with colloidal silver.

Za identifikacijo proteinov smo masne spektre primerjali s podatkovno bazo neredundantnih proteinskih zaporedij NCBI, ki smo jo dopolnili z zaporedji prepisov iz naše cDNA knjižnice. V 176 proteinskih lisah smo identificirali 57 proteinov iz 16 različnih proteinskih/toksinskih družin, med katerimi so najštevilčnejši SPKS, MPKS, CTL in $sPLA_2$ (Tab. 1). Pomemben dosežek naše proteomske študije je bilo odkritje novega P-IIIe podrazreda MPKS, ki je nastal tekom evolucije s podvajanjem predniškega gena in izgubo celotne proteinazne domene (Požek in sod. 2022). MPKS predstavljajo izjemen primer evolucije večgenskih proteinskih družin, katerih zgodovino so zaznamovale številne epizode izgube domen (katalitičnih in nekatalitičnih), posttranslacijske modifikacije in pospešena evolucija s pozitivno selekcijo, kar je povzročilo pogoste spremembe strukturnega ogrodja (Casewell in sod. 2011).

Polipeptide in peptide iz modrasovega strupa z molekulsko maso pod 10 kDa smo najprej ločili z gelsko filtracijo celotnega strupa, ki ji je sledila RP-HPLC analiza nizkomolekulskih frakcij. V njih smo z MS in Edmanovim sekvenciranjem določili proteine DIS, KUN in žilni endotelijski rastni dejavnik F, izmed peptidov pa natriuretične peptide, peptide, ki potencirajo bradikinin in inhibitorje MPKS.

V podobni proteomski študiji so strup bolgarskega modrasa ločili z 2DE na le 139 proteinskih lis (Georgieva in sod. 2008). Proteine so uspešno identificirali le v eni tretjini lis, skupno 38 proteinov iz 9 proteinskih družin, kar je lahko posledica manjše sekvenčne identitete proteinov modrasovega strupa s proteini v takrat dostopnih podatkovnih bankah. Primerjava dveh omenjenih proteomskih študij poudarja pomen optimizacije protokola za predhodno ločevanje proteinov iz strupa in uporabo vrstno specifičnega referenčnega transkriptoma za njihovo MS identifikacijo.

V nedavno objavljeni kvalitativni analizi strupa modrasa iz Srbije so uporabili proteomski pristop »od spodaj navzgor«, ki temelji na MS/MS analizi tripsinskega hidrolizata celotnega strupa (Gopcevic in sod. 2021). Identificirali so 99 proteinov iz 9 proteinskih družin in s tem dobro pokrili visokomolekulski del proteoma. Ta pristop omogoča zelo hitro analizo celotnih kompleksnih nefrakcioniranih proteomov, vendar zahteva visokoločljive nanoLC-MS/MS sisteme. Zato je bil do sedaj uporabljen le v osmih proteomskih študijah strupov iz poddružine *Viperinae*, od tega pet iz rodu *Vipera* (Damm in sod. 2021). Omejitev uporabljenega pristopa je slabša identifikacija nizkomolekulskih proteinov in peptidov.

Po podatkih WHO (2017) je modras uvrščen na seznam vrst strupenih kač največjega medicinskega pomena v Evropi. Modrasov strup na majhne živali (mali glodavci, ptiči, kuščarji), ki so njegov naravni plen, deluje predvsem nevrotoksično zaradi delovanja nevrotoksičnih $sPLA_2$, amoditoksinov (Križaj, 2011). Ob zastrupitvi človeka pa so najbolj izraženi lokalna in sistemska hemoragija, lokalna poškodba tkiva in motnje strjevanja krvi, v manjši meri nevrotoksičnost, poročila o smrtnih izidih pa so zelo redka (Luksić in sod. 2006; Karabuva in sod. 2016a). Skladno s to zelo zapleteno klinično sliko je proteomska analiza strupa modrasa potrdila njegovo izredno kompleksno sestavo. Posamezne komponente smo povezali s patofiziološkim delovanjem strupa tako, da smo z različnimi metodami tekočinske kromatografije postopoma načrtno ločevali sestavine strupa in testirali njihov vpliv na srčno-žilni sistem (Sajevic in sod. 2014; Karabuva in sod. 2016b; Karabuva in sod. 2017). Na ta način smo pokazali, da so toksini iz štirih najštevilčnejših in najbolj raznolikih družin, SPKS, $sPLA_2$, CTL in MPKS, ki predstavljajo 80 % vseh proteinov strupa, odgovorni za glavne toksične učinke strupa, vključno s krvavitvami, koagulopatijo, zaviranjem agregacije trombocitov, kardiološkimi in nevrološkimi motnjami. Protein iz novega podrazreda MPKS, sestavljen le iz nekatalitskih domen, zavira agregacijo trombocitov.

Tabela 1: Sestava in relativni deleži identificiranih družin proteinov/toksinov v strupu modrasa, navadnega gada in malega gada.

Table 1: Composition and relative abundances of the identified protein/toxin families in the venom of *Vipera a. ammodytes*, *Vipera b. berus* in *Vipera ursinii* ssp.

Proteinska/toksinska družina	**Masni delež v strupu (%)**		
	modras (*V. a. ammodytes*)	**navadni gad (*V. b. berus*)**	**mali gad (*V. ursinii* ssp.)**
Encimi			
serinske proteaze (SPKS)	25	31	6,3
fosfolipaze (PLA_2)	21,5	10	11,5
metaloproteinaze (MPKS)	14,4	19	55,2
oksidaze *L*-aminokislin (LAO)	2,9	1,6	ni zaznan
aspartatne proteaze	< 1	< 1	ni zaznan
glutaminil ciklotransferaza	< 1	ni zaznan	ni zaznan
5' nukleotidaza	< 1	ni zaznan	ni zaznan
Brez encimske aktivnosti			
lektini tipa C (CTL)	19,3	1,6	1,8
s cisteini bogati sekretorni proteini (CRISP)	7,7	8,2	12,2
disintegrini (DIS)	2	< 1	ni zaznan
žilni endotelijski rastni faktor	< 1	ni zaznan	ni zaznan
živčni rastni dejavnik	< 1	ni zaznan	< 1

Venomika gadovega strupa

Navadni gad (*V. b. berus*, Sl. 4) je druga nevarna strupenjača, ki jo lahko srečamo v naravi v Sloveniji in je zaradi razširjenosti in zato večje pogostosti srečevanja z ljudmi medicinsko najbolj pomembna kača v Evropi. V naši venomski študiji smo analizirali strup ruskega izvora (moskovska regija) in ga primerjali s strupom modrasa iz prej opisane raziskave (Latinović in sod. 2016). Kvalitativni primerjalni analizi obeh strupov z RP-HPLC in 2DE sta razkrili manjšo kompleksnost gadovega strupa. Zaradi tega in manjše količine razpoložljivega strupa smo za strukturno in kvantitativno analizo gadovega strupa izbrali klasični proteomski pristop Calveteja in sod. (2007) RP-HPLC/1DE/MS. Surovi strup smo najprej ločili v 14 frakcij z RP-HPLC, ki smo jih naprej analizirali z 1DE pod ne-reducirajočimi pogoji. Na gelu smo zaznali 30 diskretnih proteinskih lis z masami v razponu od 15 do 150 kDa. V njih smo s tandemsko MS določili 31 različnih proteinov, predstavnike 7 glavnih proteinskih družin iz strupov gadov, SPKS, MPKS, $sPLA_2$, CRISP, LAO, CTL in DIS (Tab. 1). Prvič smo na proteinskem nivoju v nekem kačjem strupu identificirali tudi aspartatno proteazo. Komponente z nizko molekulsko maso v frakcijah RP-HPLC smo analizirali neposredno z uporabo razgradnje po Edmanu ali ESI-QTOF-MS/MS in identificirali inhibitorje MPKS, natriuretične peptide in KUN.

Bistvene razlike v sestavi strupov navadnega gada in modrasa, ki lahko na molekularni ravni razložijo razlike v kliničnih slikah po zastrupitvah s temi strupi, so naslednje: i) delež SPKS in MPKS sta večja v strupu gada, deleži $sPLA_2$, LAO, CTL in DIS pa manjši, ii) nevrotoksičnih $sPLA_2$, amoditoksinov, v gadovem strupu ni, iii) prav tako ne proteinov CTL. Slednje smo v strupu gada identificirali le kot kovalentno vezane podenote MPKS in ne pa tudi kot proste proteine v strupu (Tab. 1). Njihov delež v strupu modrasa pa je 10 % in lahko povzročijo življenjsko nevarno trombocitopenijo,

t.j. znižanje števila trombocitov v krvi. V skladu z odsotnostjo amoditoksinov v gadovem strupu so poročila o nevrotoksičnih učinkih po zastrupitvi z njegovim strupom zelo redka. Nasprotno pa so se nevrotoksični znaki (npr. pareza ali paraliza kranialnih živcev) pojavili v približno 6 % pacientov zastrupljenih z modrasovim strupom. Ti zahtevajo nujno medicinsko pomoč, saj lahko napredujejo od ptoze (povešenost zgornjih vek) do močne mišične oslabelosti, ki lahko traja celo več ur (Luksić in sod. 2006). Najbolj učinkovita terapija po zastrupitvah s kačjimi strupi je imunoterapija s protistrupi. Naša raziskava imunološke navzkrižne reaktivnosti obeh strupov je pokazala, da protistrupi proti modrasovem strupu lahko nudijo popolno zaščito v primeru zdravljenja po strupenem ugrizu gada. Po drugi strani pa ne moremo pričakovati, da antiserum proti strupu gada nudi zadostno zaščito v primeru zastrupitve po ugrizu modrasa, še zlasti ne v primeru resne zastrupitve z močno izraženimi nevrološkimi učinki in trombocitopenijo.

Tako kot modrasov je tudi strup navadnega gada bil predmet še dveh proteomskih raziskav, ki so lepo pokazale, kako je sestava strupov iz iste vrste kač odvisna od geografskega porekla. Al-Shekhadat in sod. (2019) so uporabili enak standardni venomski pristop kot smo ga uporabili mi za analizo prav tako strupa ruskega izvora, toda iz regij Tver in Novosibirsk. Tudi ta strup vsebuje kompleksen nabor toksinov, saj so določili 80 različnih proteinov in peptidov iz 13 družin. Za razliko od gadovega strupa iz moskovske regije je imel gadov strup iz Sibirije dvakrat več $sPLA_2$, dvainpolkrat več LAO, a dvakrat manj SPKS in CRISP. Delež ostalih komponent, MPKS, DIS, CTL (le kot podenote MPKS), KUN in natriuretičnih peptidov je bil primerljiv. Od peptidov so poleg natriuretičnih peptidov za razliko od nas določili tudi peptide, ki potencirajo bradikinin in sicer v 9,5 % deležu. Ker so za analizo uporabili večjo količino strupa, so lahko identificirali tudi komponente, ki so v strupih navadno prisotne v zelo majhnih količinah (pod 1 %), kot so hialuronidaza, 5' nukleotidaza, glutaminil ciklotransferaza, fosfodiesteraza in živčni rastni dejavnik. Z izjemo hialuronidaze smo jih lahko določili tudi v strupu modrasa.

Bocian in sod. (2016) so s proteomskim pristopom »od spodaj navzgor« in kombinacijo metod 2DE/MALDI TOF/TOF MS analizirali strup navadnega gada iz Slovaške. 2DE analiza v širokem območju pH 3–10 je pokazala, da se večina proteinskih lis nahaja v ožjem območju pH 5–8. Zato so za boljšo ločbo različnih izooblik proteinov strup analizirali tudi v tem ožjem območju, izrezali vse lise iz obeh gelov in proteine identificirali s tandemsko MS. Slovaški strup se je od ruskega razlikoval na kvalitativni in kvantitativni ravni. V njem so določili manjše število različnih proteinov, 25 iz 6 toksinskih družin, izmed peptidov pa le peptide, ki potencirajo bradikinin. Poleg tega, da niso našli proteinov iz družine DIS, je vsebnost proteinov iz družine CTL le 6 %. Presenetljivo pa so več kot polovico strupa sestavljale $sPLA_2$, med njimi tudi nevrotoksične, sledili so jim SPKS, LAO in CRISP ter MPKS, ki jim je pa pripadel le nekaj odstotni delež. Do sedaj so poročali o primerih nevrotoksičnega učinka strupa navadnega gada po ugrizih v Romuniji in na Madžarskem, različni surovi strupi modrasa po poreklu iz Madžarske pa so povzročili paralizo izoliranih živčno-mišičnih preparatov iz piščanca (Malina in sod. 2017). Opaženi znaki paralize pri človeku in popolna ohromelost pri miših, ki so jim vbrizgali madžarski strup so tudi značilni za delovanje $sPLA_2$ nevrotoksinov. Čeprav so te strupe analizirali le z 1DE, pa so iz intenzitet lis z maso 13-15 kDa, kar ustreza masi $sPLA_2$, lahko sklepali, da so to dominantne komponente. Pričakujemo lahko torej, da je sestava strupov navadnega gada iz nekaterih regij Madžarske in Romunije bolj podobna sestavi slovaškega kot pa ruskega strupa.

Slika 4: Navadni gad (*Vipera berus berus*). Telo mu krasi neprekinjena temna cikcakasta proga vzdolž hrbta, ki se začne za ovalno glavo. Navadno je sivkaste ali rjavkaste barve, čeprav je lahko tudi popolnoma črn (foto: Neven Vrbanić).

Figure 4: Common or European adder (*Vipera berus berus*). Its body is decorated with a continuous dark zigzag stripe along the back, starting behind the oval head. It is usually greyish or brownish in colour, although it could be also completely black (photo: Neven Vrbanić).

Venomika strupa malega gada

Mali gad (*V. ursinii*) je ogrožena vrsta evropske kače. Zaradi nevarnosti izumrtja je Svet Evrope leta 2005 sprejel načrt za njegovo zaščito. V Sloveniji te kače ni, na Hrvaškem pa najdemo njeno podvrsto *V. ursinii* ssp. (Sl. 5). Poseljuje visokogorske suhe travnate predele na jugu in jugovzhodu države. Je medicinsko manj pomembna kot druge kače iz vrste *Vipera* zaradi redkih srečanj s človekom in zelo majhne količine strupa, ki ga lahko injicira s svojimi le nekaj milimetrov dolgimi strupniki. V svojem naravnem okolju se večinoma prehranjuje z žuželkami.

Strup hrvaškega malega gada je dosti manj kompleksen od prej opisanih strupov modrasa in gada (Lang Balija in sod. 2020). Surovi strup kač iz naravnega okolja, smo ločili z 1DE na le sedem in z 2DE na le 50 proteinskih lis. V izrezanih proteinskih lisah smo s tandemsko MS identificirali 25 proteinov, uvrščenih v sedem družin, MPKS, SPKS, $sPLA_2$, CRISP, CTL in KUN in živčni rastni dejavnik (Tab. 1). Dobro polovico strupa sestavljajo visokomolekulske MPKS, ki so večinoma homologi modrasovih hemoragičnih MPKS. Skladno s tem sta oba strupa, strup malega gada in strup modrasa, v testu v podganah pokazala primerljivo visoko hemoragično aktivnost. $sPLA_2$ iz strupa malega gada so encimi brez nevrotoksične aktivnosti, zato je strup za podgano precej manj toksičen kot strup modrasa. Po drugi strani je bil strup modrasa bistveno manj toksičen za črička kot strup malega gada. To je lepa demonstracija naravne prilagoditve, saj so črički naravna hrana malega gada, ne pa tudi modrasa.

Za namene ohranjanja in raziskav nekatere primerke malega gada gojijo tudi v ujetništvu, kjer imajo drugačen režim prehranjevanja kot v naravi;

hranijo jih namreč z mišmi namesto z žuželkami. Sprememba sestave strupa zaradi spremembe prehrane je bila že opisana pri drugih kačah (Barlow in sod. 2009; Gibbs in sod. 2013; Amazonas in sod. 2019). Da bi ugotovili, kako različna prehrana v primeru malega gada vpliva na sestavo strupa, smo strupe, zbrane od kač v ujetništvu, in strupe, pridobljene od divjih živali, primerjalno analizirali z metodo 2DE. Opažene spremembe v sestavi strupa kač v ujetništvu v primerjavi s tistimi, ki živijo v naravi, so predvsem večja količina $sPLA_2$ ter manjša količina MPKS in CRISP. Ta ugotovitev podpira hipotezo, da pridobitev različnih izooblik $sPLA_2$ v strupu s pospešeno evolucijo (Ogawa in sod. 1996) predstavlja močno selektivno prednost, npr. za hitro prilagajanje razpoložljivemu plenu s spremembo v izražanju genov (Aird in sod. 2015).

Slika 5: Mali ali Ursinijev gad (*Vipera ursinii* ssp.). Ime je dobil po italijanskem naravoslovcu Antoniu Orsiniju (1788-1870). Ima majhno srčasto oblikovano glavo, telo je pa kratko in čokato. Je sive ali oker barve s črno obrobljeno temno rjavo cikcakasto progo na hrbtu (foto: Neven Vrbanić).

Figure 5: Meadow or Ursini's viper (*Vipera ursinii* ssp.). It is named after the Italian naturalist Antonio Orsini (1788–1870). It has a small, heart-shaped head and a short, stocky body, which is grey or ochre in colour and has a black-edged, dark brown zigzag stripe on the back (photo: Neven Vrbanić).

Antivenomika

Po podatkih WHO (2019) strupene kače vsak dan ugriznejo skoraj 7400 ljudi na vseh kontinentih, od posledic ugriza pa jih umre 220 do 380. To pomeni približno 2,7 milijona primerov zastrupitev in 81.000 do 138.000 smrtnih primerov na leto. Problematika najbolj prizadeva države v razvoju z velikim številom ruralnega prebivalstva. Zato je WHO pripravila celovito strategijo za zmanjšanje umrljivosti in invalidnosti zaradi zastrupitev po kačjih ugrizih za 50 % do leta 2030. Pri tem je proizvodnja in dostopnost učinkovitih protistrupov eden glavnih ukrepov za obvladovanje kačjih ugrizov. Protistrupi so zaenkrat edino res učinkovito specifično zdravilo proti sistemskim

učinkom zastrupitev s kačjimi strupi (Gutiérrez in sod. 2017).

Sam izraz antivenomika opisuje proteomski proces identifikacije tistih polipeptidov v strupu, ki imajo epitope, ki jih protistrup slabo ali pa sploh ne prepozna (Calvete in sod. 2009). Vzrok za slabši zaščitni učinek protistrupa, pridobljenega z imunizacijo živali s celotnim strupom, je lahko tvorba protiteles z nizko afiniteto ali pa sploh izostanek tvorbe protiteles proti določenim toksičnim komponentam v strupu. Antivenomika dopolnjuje *in vitro* in *in vivo* teste nevtralizacije aktivnosti strupa ter tradicionalne imunološke metode, kot so analize ELISA in prenos po Westernu, za predklinično oceno nevtralizacijskega spektra protistrupov. Predstavlja alternativni pristop testiranju na živalih za določanje učinkovitosti zaščite protistrupov pred letalno nevarnostjo, ki jo povzroča strup. Prva generacija antivenomike, ki je temeljila na imunoprecipitaciji kompleksov antigen-protitelo v raztopini in ji je sledila kromatografska kvantifikacija prostega antigena v supenatantu, je bila primerna le za protistrupe, ki so vsebovali celotne IgG (Núñez in sod. 2009; Lingam in sod. 2020). Ta pristop je bil pozneje preoblikovan tako, da je bil primeren tudi za protistrupe, ki jih sestavljajo $F(ab')_2$ fragmenti. Ključna posodobitev v anivenomiki druge generacije je priprava imunoafinitetne kolone z vezavo molekul protistrupa na kromatografski nosilec (Villalta in sod. 2012; Lomonte in Calvete, 2017; Patra in sod. 2017; Pla in sod. 2017a). S kombinacijo imunoafinitetne kromatografije in proteomske analize sestavin strupa v nevezanih in vezanih frakcijah takšen pristop zagotavlja kvalitativne in tudi kvantitativne informacije o obeh skupinah proteinov strupa, tistih, ki jih protistrup dobro prepozna, in tistih, ki jih ne (imajo slabšo imunoreaktivnost). Ob predpostavki, da je stopnja imunoprecipitacije toksičnih sestavin v strupu s protistrupom *in vitro* enaka stopnji nevtralizacije toksičnih učinkov teh sestavin *in vivo*, rezultati antivenomike zagotavljajo podatke o tem, s katerimi sestavinami strupa je treba dodatno obogatiti imunizacijsko mešanico ali katerim sestavinam strupa je treba z določenimi postopki izboljšati imunogenost, da dobimo visoko učinkovit antiserum.

Antivenomika tretje generacije nadgrajuje predhodni pristop z določitvijo največje kapacitete vezave različnih toksinov iz strupa s protistrupom in kvantifikacijo celotnega deleža protiteles v protistrupu, ki imajo imunoafiniteto do toksinov iz strupa – terapevtska protitelesa (Pla in sod. 2017b). Dejansko so pri skoraj vseh obstoječih protistrupih najpogostejše molekule protitelesa proti antigenom, ki niso strup (60-90 %) (Sanny, 2011). Ta pristop se uporablja tudi v molekularnih študijah navzkrižne reaktivnosti protistrupov in heterolognih strupov (Gutiérrez in sod. 2009). Protitelesa bodo verjetno navzkrižno nevtralizirala toksine znotraj iste družine toksinov v različnih kačjih strupih.

Zaključki

Venomika je orodje, ki nam omogoča boljše razumevanje kačjih strupov z evolucijskega, biološkega in kliničnega vidika. Izjemen pospešek raziskavam živalskih strupov je omogočil razvoj proteomskih, transkriptomskih in genomskih platform, ki jih podpirajo visoko zmogljive tehnologije sekvenciranja proteinov/peptidov, RNA in DNA. Tako se skokovito bogatijo baze aminokislinskih zaporedij, ki jih z analizo s pomočjo vse zmogljivejših bioinformatskih orodij prevajamo v vse bogatejše baze znanja. Proteomika strupov temelji predvsem na masni spektrometriji, s katero določamo i) kvalitativno in kvantitativno sestavo proteoma na nivoju proteinskih družin, ii) delna ali celotna zaporedja izoliranih proteinov/peptidov, in iii) njihove točne mase, kar omogoča identifikacijo različnih proteoform. Informacije o sestavi strupa so bistvene za določitev obsega medvrstnih in znotrajvrstnih razlik ter vpliva ekologije in prehrane na evolucijo strupa. Podprta z genomiko in transkriptomiko nam torej proteomika zagotavlja veliko informacij o procesih, ki uravnavajo izražanje genov, o alternativnem spajanju in drugih molekularnih mehanizmih, odgovornih za izražanje fenotipskih razlik. Primerjalni podatki o sestavi strupa so koristni tudi za nadaljnje raziskave na področju molekularne biologije, kot so regulacija, izguba in podvajanje genov. Izjemen potencial v prihodnosti leži v povezavi proteomike z naprednimi slikovnimi tehnologijami, tako imenovana prostorska venomika – kartiranje proteinskih toksinov in njihove aktivnosti neposredno v tkivu

strupne žleze – ki odstira pogled v morfološke in funkcionalne značilnosti strupnega sistema.

Natančno poznavanje sestave strupov je izjemno pomembno tudi za razumevanje patofiziologije zastrupitev in za razvoj ustreznih strategij zdravljenja zastrupitev s kačjimi strupi in pa za razvoj protistrupov. Zastrupitve s kačjimi strupi predstavljajo velik zdravstveni in ekonomski problem še posebej v večjem delu sveta v razvoju. To problematiko je prepoznala tudi WHO, ki je protistrupe uvrstila med ključna zdravila. Specifičnost in učinkovitost teh zdravil – večinoma antiserumov – sta neločljivo povezani s sestavo strupov, ki se uporabljajo za imunizacijo, variabilnost toksinov pa povzroča slabše prepoznavanje in nevtralizacijo toksinov iz različnih strupov. S pomočjo antivenomike lahko s proteomskimi orodji kvalitativno in kvantitativno ovrednotimo interakcijo (nevtralizacijo) strupov s protistrupi *in vitro*. S tem bistveno zmanjšamo potrebo po testiranju protistrupov v laboratorijskih živalih. Antivenomika nam torej pomaga nadzorovati kakovost in načrtovati najboljše mešanice strupov za imunizacijo živali za proizvodnjo učinkovitih protistrupov. S ciljem povečanja njihove učinkovitosti bi bil namesto imunizacije živali s celotnim strupom bolj primeren pristop s proizvodnjo protistrupa le proti najbolj nevarnim toksinom v strupu. Prihodnost torej leži v pripravi rekombinantnih toksinov – antigenov za imunizacijo. Ti v eni molekuli vsebujejo več ključnih epitopov, sicer prisotnih na različnih toksinih ali proteoformah toksinov (celo iz različnih rodov in vrst kač). Tako proizvedena protitelesa naj bi imela široko nevtralizacijsko moč proti podobnim toksinom v različnih kačjih strupih.

Summary

Snake venoms are complex mixtures of biologically active proteins and peptides that have evolved over the course of evolution to become one of the deadliest natural weapons. The pathological effect of the venom on the organism depends on its composition, which is specific to each venomous snake family and even to a single snake. Indeed, snake venom may vary depending on the age, sex, diet or geographical distribution of a snake. Proteomics of snake venoms, snake venomics, is primarily based on mass spectrometry, which allows us to determine i) the qualitative and quantitative composition of the proteome at the protein family level, ii) the partial or complete sequences of venom proteins/peptides, and iii) their exact masses, allowing the identification of the different proteoforms. The most commonly used approach to snake venom analysis is bottom-up proteomics. In this approach, the venom is first separated using liquid and/or gel-based chromatographic methods. The proteins are enzymatically degraded and the resulting peptides are analyzed using liquid chromatography-tandem mass spectrometry. Bioinformatics tools are then used to identify the proteins and find the best match between the experimental peptide tandem mass spectra and the theoretically generated spectra of proteins in sequence databases. The proteomes of snake venoms are increasingly being studied. Undoubtedly, snake venom research received an additional boost in 2017 when the World Health Organisation reclassified snakebite envenomation into the Category A of the Neglected Tropical Diseases, recognising that it is a major cause of suffering, disability and premature death in many developing countries. To date, venomics studies have identified numerous toxins classified into 63 enzymatic and non-enzymatic families of proteins and peptides. The medically important European vipers belong to the genus *Vipera*. Three of these snakes live in Slovenia, the nose-horned viper (*Vipera a. ammodytes*), the common adder (*Vipera b. berus*) and the asp viper (*Vipera aspis*, subspecies *francisciredi*). The nose-horned viper is the most venomous of the European venomous snakes, while the adder is the most widespread. We have studied their venoms using bottom-up proteomics and found characteristic differences in the composition and relative abundance of certain venom protein/toxin families. The most important difference, which is also reflected in the markedly different pharmacological effects of these two venoms, is the absence of the neurotoxic $sPLA_2$s and CTLs in the venom of *V. b. berus*. These two groups of toxins are the reason for the neurotoxicity and thrombocytopenia (a decrease in platelet count) in a victim intoxicated by the venom of *V. a. ammodytes*. We also conducted a venom study on the most endangered snake species in Europe, the meadow viper (*Vipera ursinii*). This medically

insignificant viper species produces only a small amount of venom and feeds mainly on insects. It was no surprise that its venom is much less complex than that of the nose-horned viper and the adder. We found that it consists of many fewer toxin families, which are also less diverse. By comparing the venoms of *V. ursinii* snakes from the wild with those in captivity fed on mice instead of insects, we were able to confirm that diet strongly influences the composition of the venom. Currently, antivenoms are the only effective treatment for the systemic effects of snakebite envenomation. In antivenomics, proteomics is used to identify the venom components that carry epitopes that are recognised by the antivenom weekly or not at all. The maximum binding capacity of different toxins by the antivenom and the proportion of antibodies in the antivenom that have immunoaffinity for the venom toxins to the total antibodies in the antivenom, *e.g.* the ratio of therapeutic antibodies in the antivenom, are determined by antivenomics. This is an effective alternative approach to animal testing to predict the protective efficacy of an antivenom in the case of snake envenomation.

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Optical properties of different structures of some herbaceous understorey plant species from temperate deciduous forests

Optične lastnosti različnih struktur pri nekaterih zelnatih rastlinskih vrstah v podrasti zmernega listopadnega gozda

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Abstract: This contribution discusses the optical properties of different structures of some herbaceous understorey plant species from temperate deciduous and mixed forests. These forests are marked by annual dynamics of radiation level that is related to the vegetation cycle of forest trees. During winter and early spring, the understorey is exposed to full solar radiation, while later in the growing season radiation is limited due to the closing of the tree storey. The plasticity of optical properties of photosynthetic structures of understorey plants is directly related to their structural and biochemical phenotypic plasticity that optimises harvesting and use of energy. The optimisation of energy harvesting is also achieved by specific adaptations of green leaves, such as variegation (*Pulmonaria officinalis*, *Cyclamen* sp.), anthocyanic lower epidermis (*Cyclamen* sp.), and by using structures other than green leaves for photosynthesis, such as bracts (*Hacquetia epipactis*) and sepals (*Helleborus* sp.). The optical properties of these structures are similar to those of green leaves. The understanding of optical responses of different structures contributes to the understanding of the forest understorey functioning.

Keywords: bracts, leaves, light conditions, optical properties, sepals, temperate deciduous forest, understorey plants

Izvleček: Prispevek povzema optične lastnosti različnih struktur nekaterih zelnatih rastlinskih vrst v podrasti listnatega in mešanega gozda zmernega pasu. Te gozdove zaznamuje letna dinamika ravni sevanja, ki je povezana z vegetacijskim ciklom gozdnega drevja. Pozimi in zgodaj spomladi je podrast izpostavljena polnemu sončnemu sevanju, kasneje pa je sevanje omejeno zaradi olistanja krošenj. Plastičnost optičnih lastnosti fotosinteznih struktur rastlin v podrasti je neposredno povezana z njihovo strukturno in biokemijsko plastičnostjo, ki optimizira privzem in rabo energije. Optimizacijo pridobivanja energije rastline dosežejo tudi s posebnimi prilagoditvami zelenih listov, kot sta pisanost (*Pulmonaria officinalis*, *Cyclamen* sp.) in antocianska spodnja povrhnjica (*Cyclamen* sp.) ter z uporabo drugih struktur poleg zelenih listov za fotosintezo, kot so podporni (*Hacquetia epipactis*) in čašni listi (*Helleborus* sp.). Optične lastnosti teh

struktur so podobne optičnim lastnostim zelenih listov. Razumevanje optičnih odzivov različnih struktur prispeva k razumevanju delovanja rastlin v podrasti.

Ključne besede: čašni listi, listi, optične lastnosti, podporni listi, podrast, svetlobne razmere, zmerni listopadni gozd

Introduction

Temperate deciduous and mixed forests are marked by annual dynamics of radiation regime that is related to the vegetation cycle of forest trees (Klančnik et al. 2015). The amount of radiation at the forest floor in a certain time of the year is a consequence of canopy structure, especially leaf area index (Larcher 2003). During winter and early spring, the understorey is exposed to full solar radiation, while later in the growing season radiation is limited due to the closing of the tree storey (Rothstein et al. 2001, Klančnik et al. 2015). In a fully foliated forest, light enters the system *via* gaps in the canopy forming sun flecks that provide up to 80% of solar energy for understorey species (Larcher 2003). However, these sun flecks are very variable regarding their quality, intensity, and duration (Chazdon et al. 1991, Lambers et al. 1998, Leakey 2004). The radiation conditions in the understorey layer during the vegetation period define the functional traits of photosynthetic organs, including their optical properties (Esteban et al. 2008, Grašič et al. 2020), which enable efficient light use (Reich et al. 2003, Yoshimura et al. 2010). Such traits are leaf tissue thickness and density, spotted and variegated leaves (Klančnik et al. 2016), and the production of additional pigments, such as anthocyanins (Smillie et al. 1999). Optical properties depend on plant tissue structure and may thus be species-specific (Marín et al. 2016). However, they can vary significantly during tissue ontogenetic development (Grašič et al. 2021 a,b) and due to species phenotypic plasticity, which is related to environmental changes in the habitat (Liew et al. 2008, Klančnik et al. 2016, Klančnik et al. 2014 a).

Herbaceous understorey species exhibit different life histories that are related to variable radiation environments during specific phenological phases. The first group of plants develops all organs before the full foliation of trees. These organs are based on storage accumulated in underground organs (Larcher 2003) and possess a variety of traits that support quick development in the period with abundant light (Kim et al. 2015). The second group develops leaves prior to tree foliation and reproductive organs after the closure of the canopy (Gilliam 2014). The third group consists of, for example, the genus *Vinca* (Darcy et al. 2002) and some *Helleborus* species (Bavcon et al. 2012). These species have evergreen leaves that enable photosynthesis throughout the whole year, especially during high-light conditions in late winter and early spring. Some species within these groups increase their carbon and energy budget through additional photosynthetic organs, such as sepals and bracts (Grašič et al. 2020, Aschan et al. 2003), and in some species, the colour of sepals turns green after pollination, as shown for *H. orientalis* cv. *Olympicus* (Shahri et al. 2011).

Photosynthetic organs optimise light harvesting by various adaptations of their biophysical structure (Ustin et al. 2001). Optical properties that comprise light reflectance, absorbance, and transmittance (Woolley 1971) vary among different ecological groups of plants (Klančnik et al. 2012, Klančnik et al. 2014 b, Klančnik and Gaberščik 2016). In addition, they can be tissue- or species-specific, or they can vary due to tissue ontogenetic development and species phenotypic plasticity in relation to environmental changes in the habitat (Liew et al. 2008, Klančnik et al. 2012). Therefore, they also vary among different understorey species and their organs in time and space. The reflectance spectra of photosynthetic organs are a very useful parameter since they reflect specific organ traits, such as biochemical structure (Carter et al. 2002, Gitelson et al. 2002, Castro et al. 2008, Kováč et al. 2013, Roelofsen et al. 2014, Klančnik and Gaberščik 2016), and content of nutrients and hydration (Baltzer et al. 2005, Lukeš et al. 2013, Roelofsen et al. 2014). They also provide information about energy

balance (Noda et al. 2013, Ullah et al. 2012), the potential presence of stress, and contribute to the understanding of photosynthetic performance (Coops et al. 2005). In some studies, it was shown that reflectance spectra act as a "plant signature", enabling the identification of different plant groups (Klančnik and Gaberščik 2016), or even species classification (Castro-Esau et al. 2006).

The present article presents the optical properties of different structures of some understorey plant species in temperate deciduous forests. The insight into these optical properties and the traits supporting them contributes to the understanding of the forest ecosystem and enables the maintenance of some of these plants in man-made environments, which usually differ significantly from those of the floor of deciduous forests.

Leaf traits and their optical properties

Phenotypic plasticity

Plant traits are a result of the evolutionary process, which favours a variety of adaptations that enable an optimal response to specific environmental conditions (Šraj Kržič et al. 2005, Rascio et al. 1999, Boeger et al. 2003). The persistence of these traits in plant species is related to the stability of conditions in the habitat. However, species phenotypic plasticity enables the acclimation of specific traits to current conditions (Larcher 2003). Therefore, phenotypic plasticity presents the potential of an organism to produce various phenotypes when exposed to different environmental conditions (Sommer 2020). The highest level of phenotypic plasticity is found in environments with pronounced environmental changes. Environmental conditions in the forest understorey may be very heterogeneous in time and space, as is the case in temperate deciduous and mixed forests (Valladares 2003). This highly heterogeneous environment differs in multiple factors. Besides variable light conditions, understorey plants are subjected to changes in temperature, soil moisture, and fertility. However, these factors usually co-vary in nonlinear ways (Valladares et al. 2007). The adaptation to light depends on a trade-off with plant responses to other factors (Larcher 2003). Different species in the forest understorey exhibit different levels of phenotypic plasticity, as shown for *Asarum arifolium* and *Hepatica nobilis*, the latter showing a higher level of plasticity (Warren et al. 2013). Plasticity may be expressed in different organs and at different levels of plant structure and function, and it also differs among different environments, as is the case in *Hacquetia epipactis* (Grašič et al. 2021 a). The plasticity of understorey plants in response to light is relatively low in shade-tolerant woody species in the tropics, where the environment is rather stable (Valladares et al. 2000). Warren et al. (2013) reported an ecological convergence in trait values along environmental gradients between ecologically similar, but phylogenetically different evergreen understorey herbs. The plastic response of plant structural traits due to different environmental conditions results in undisturbed functioning, including the processing of available energy. Thus, the plasticity of leaf optical properties is directly related to their structural and biochemical phenotypic plasticity (Klančnik et al. 2014 a, Grašič et al. 2021 a,b). Differences in species responses to variable light environments affect the success of understorey species in forest dynamics (Santos et al. 2021). In the case of fern *Phyllitis scolopendrium*, light along with other environmental factors significantly affected the frond biochemical structure, and consequently also their optical properties (Fig. 1). However, the photochemical efficiency of PS II remained the same (Grašič et al. 2020). Thus, photosynthetic acclimation to specific light conditions, which also includes pigment levels, is one of the most important plant abilities (Popović et al. 2006). Changes in biochemical leaf traits and their optical properties during the growing season were observed in *Cyclamen purpurascens*, where the contents of carotenoids and anthocyanins decreased between February and April, and affected optical properties (Klančnik et al. 2016). This is also a consequence of lower levels of radiation that reached their habitat due to canopy closing (Rothstein et al. 2001). Besides anthocyanins, changes in carotenoid contents are also important since they can present accessory pigments under light limitation (Demmig-Adams et al. 1996).

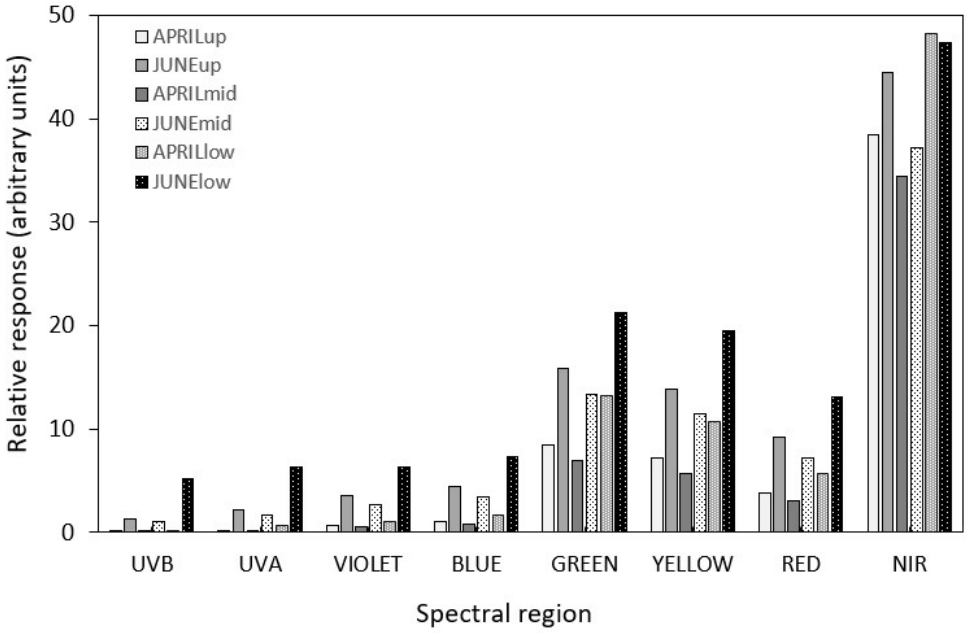

Figure 1: Mean reflectance of radiation in *Phyllitis scolopendrium* fronds in different spectral regions from locations with various light regimes at different times of the year. Locations along the light gradient: up - upper (high light level), mid – in the middle (middle light level), low - lower (low light level).

Slika 1: Povprečna odbojnost sevanja pri listih vrste *Phyllitis scolopendrium* v različnih območjih sevanja z lokacij z različnimi svetlobnimi režimi v različnih delih sezone. Lokacije vzdolž svetlobnega gradienta: up - zgoraj (visoka raven svetlobe), mid - sredina (srednja raven svetlobe), low - spodaj (nizka raven svetlobe).

The optical properties of understorey species are shaped by leaf structure and thickness, which are represented by specific leaf area (SLA). SLA of understorey species may vary from 0.66 to 0.01 cm^2/mg (Prado et al. 2015), which results in different light capture efficiency (Reich et al. 2003). Lower SLA increases light backscattering, which positively affects light absorbance, reduces sieving effects, and prolongs the path of photons within the tissue (Lee et al. 2000). It determines photosynthetic efficiency per leaf mass, which increases with increasing SLA (Evans et al. 2001). Correlations between SLA and light reflectance spectra were observed by Asner et al. (2008), who studied the optical properties of tropical forest canopy species.

Leaf colouration

Plant biochemical and morphological structure is determined by organ-specific interactions with the environment (Bongers et al. 2019), which also includes light conditions. Many studies have revealed an important role of pigments in shaping leaf optical properties (Slaton et al. 2001, Gitelson et al. 2002, Baltzer et al. 2005, Levizou et al. 2005, Castro et al. 2008, Klančnik, et al. 2014 a, Klančnik et al. 2016). The contents of chlorophylls, which are the main light-harvesting pigments, usually negatively affect the reflectance spectra (Klančnik et al. 2014 a). This effect may be altered in plants with different structures at the leaf surface, e.g., in many understorey species. For example, the presence of trichomes as the first target of light may significantly affect leaf optical properties (Baldini et al. 1997, Klančnik et al. 2012).

Anthocyanins play an important role in the adaptive strategy of plants to their radiation environment, including in forest understorey species. Anthocyanins mitigate or prevent plant stress, as they function as sunscreens, antioxidants, and chelating substances (Landi et al. 2015). Anthocyanins in leaves of understorey plants filter high-intensity radiation during sun flecks (Gould et al. 1995, Gould 2004). *In vivo* anthocyanins exhibit an absorption peak around 550 nm, and this peak magnitude is related to anthocyanin content (Gitelson et al. 2022). In general, anthocyanins accumulate in upper leaf layers (Chalker-Scott 1999, Lev-Yadun 2002, la Rocca et al. 2014). However, some understorey plant species accumulate them in their abaxial epidermis as well (Hughes et al. 2008, Lee et al. 1979, Lee et al. 2001), as is the case in the genus *Cyclamen* (Klančnik et al. 2016) (Fig. 2).

Figure 2: The red abaxial epidermis in *Cyclamen purpurascens* may vary in colour intensity and homogeneity.
Slika 2: Rdeča spodnja povrhnjica vrste *Cyclamen purpurascens* se lahko razlikuje po intenzivnosti in homogenosti barve.

Some researchers suggest that the reduction of light transmission through the leaf due to anthocyanins might negatively affect competitors, especially in spring before the development of the canopy. A study of leaves of tropical trees at the beginning of the last century showed that red abaxial epidermis contributes to enhanced leaf temperatures, however, this was not confirmed in later studies (Gould et al. 1995, Lee et al. 1979). Klančnik et al. (2016) showed significant differences in transmittance in the visible and NIR regions between the leaves with and without the red abaxial epidermis. The visible region is used for photosynthesis but also has a thermal effect, while the effect of NIR is mainly thermal (Ross 1981). In addition, lower transmittance in the green and yellow regions was measured for the red-coloured lower epidermis in comparison to the epidermis with fewer anthocyanins. The study of *Begonia heracleifolia* revealed that the red anthocyanic lower epidermis did not affect the reflectance of red light in the mesophyll (Hughes et al. 2008). The study of *Colocasia esculenta* leaves with different anthocyanin contents showed no differences in CO_2 uptake under shade conditions between the studied leaf types (Hughes et al. 2014). *Erytronium dens-canis* red patches are due to a single layer of cells in the upper parenchyma that accumulate anthocyanins and have lower photochemical efficiency in comparison to the green sections (Esteban et al. 2008).

Variegated leaves

Plant leaves are usually uniformly coloured. However, some understorey plants develop leaves in such a way that they have different colour patterns at their surface, optimising the use of both high and low light levels in the forest understorey (Tsukaya et al. 2004). These coloured patterns are very popular, therefore, such species can be used as ornamental plants (e.g., *Aglaonema*, *Begonia*, *Cyclamen*). *Pulmonaria officinalis*, a perennial forest herb that grows in biodiverse, mixed, and open forests, has variable light green spots at the green leaf surface (Fig. 3). Variegation patterns are mainly not related to pigments, but rather to the differences in the palisade mesophyll (Konoplyova et al. 2008). Light green spots in *P. officinalis* are caused by the presence of loosely arranged cells instead of a well-established layer of packed cells in the palisade parenchyma (Esteban et al. 2008). SLA in light green parts was higher in comparison to dark green parts (3.16 and 2.75 dm^2/g DM, respectively). Consequently, dark green parts had somewhat higher contents of all pigments, however, the differences were not significant. All these aspects affected plant optical properties, as shown in Figure 4. Light green parts reflected and transmitted more light in the green, yellow, and red regions, while shorter wavelengths and NIR showed a similar pattern in both light green and dark parts of the leaf. In addition, chlorophyll fluorescence imaging revealed a decrease in photochemical efficiency for light green spots in comparison to the green sections (Esteban et al. 2008). Under higher levels of UV radiation that are found in more open habitats, the light green spots become less transparent to visible light (Gaberščik et al. 2001).

Figure 3: *Pulmonaria officinalis* leaves with light green spots.
Slika 3: Listi vrste *Pulmonaria officinalis* s svetlozelenimi pikami.

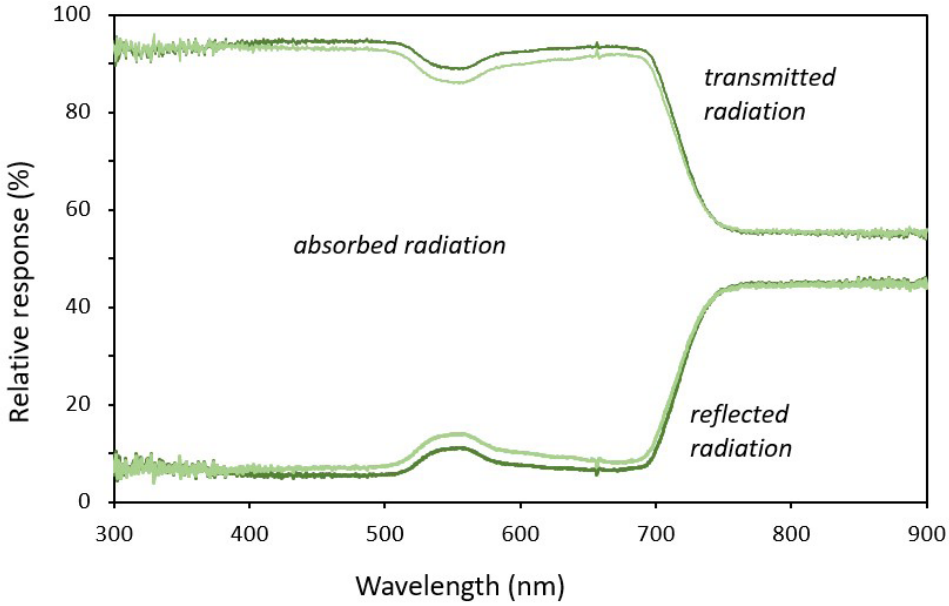

Figure 4: Mean radiation reflectance (area below the lower curves), light transmittance (area above the upper curves), and absorbance (area between the upper and lower curves) measured on the dark (dark green curves) and light green (light green curves) parts of the *Pulmonaria officinalis* leaves.

Slika 4: Povprečna odbojnost sevanja (površina pod spodnjimi krivuljami), prepustnost sevanja (površina nad zgornjimi krivuljami) in absorbanca (površina med zgornjo in spodnjo krivuljo), izmerjene na temnih (temnozelene krivulje) in svetlozelenih (svetlozelene krivulje) delih listov vrste *Pulmonaria officinalis*.

In *Cyclamen purpurascens* leaves, more evident differences in light reflectance of dark and light green parts were obtained in comparison to *P. officinalis* (Figs. 5 and 6). These differences were negligible in the UV region, but very pronounced in VIS, and then again less pronounced in NIR. The light green leaf parts also transmitted more radiation than the dark green leaf parts, wherein the most differences in transmission were seen for the green region (Klančnik et al. 2016). In spite of the differences in light management, the light green parts of the variegated leaves perform photosynthetic activities similar to those of the dark green leaf parts or of fully green leaves (Konoplyova et al. 2008, la Rocca et al. 2011, Sheue et al. 2012, la Rocca et al. 2014).

Figure 5: The light green pattern at the upper leaf surface of *Cyclamen purpurascens* may vary in intensity and shape.
Slika 5: Svetlozelen vzorec na zgornji površini listov vrste *Cyclamen purpurascens* se lahko razlikuje po jakosti in obliki.

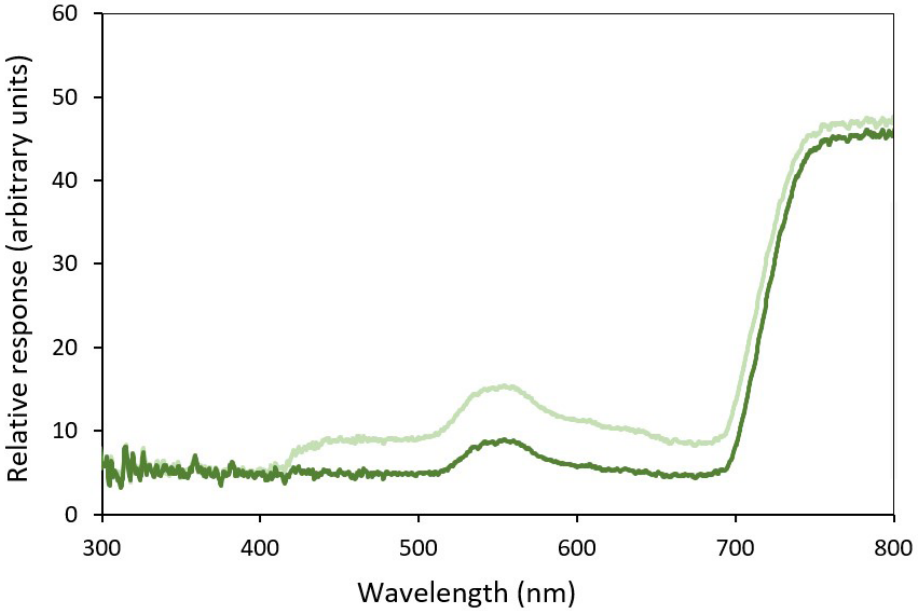

Figure 6: Reflectance of radiation measured on the dark green (dark green curve) and light green (light green curve) parts of the *Cyclamen purpurascens* leaves.
Slika 6: Odbojnost sevanja, izmerjena na temnozelenih (temnozelena krivulja) in svetlozelenih (svetlozelena krivulja) delih listov vrste *Cyclamen purpurascens*.

The higher reflectance in the light green leaf parts is mainly a consequence of the morphological differences. The mesophyll below the light green leaf parts shows a polygonal light reflection pattern, composed of white polygons formed around the epidermal cell edges (Zhang et al. 2009, Sheue et al. 2012, Klančnik et al. 2016). This pattern is associated with air spaces between the epidermal and mesophyll cells (Zhang et al. 2009), and thus the light green colouration is also a consequence of leaf mesophyll structure (Sheue et al. 2012). The palisade mesophyll cells of these leaf parts are larger and loosely arranged, therefore having a greater volume of intercellular air spaces (Konoplyova et al. 2008, Sheue et al. 2012, la Rocca et al. 2011), which increase light reflection and the scattering of light (Esteban et al. 2008). In *C. purpurascens* the differences in tissue density between the light and dark green leaf parts were most pronounced in April under high light conditions, when tissue density was significantly higher in the dark green leaf sections (Klančnik et al. 2016). This additionally supports the importance of variegation for light management. This increased light reflectance of the light green leaf parts may serve as photoprotection and may prevent damage caused by high light during sun flecks (Holmes et al. 2002, Esteban et al. 2008). However, the dark green leaf parts are protected against excessive radiation by carotenoids (Filella et al. 1999, Schulze et al. 2005), as their carotenoid contents were higher when the canopies were not yet closed (Klančnik et al. 2016). In the case of *Actinidia kolomikta* leaf colour was also related to leaf structure and leaf pigment contents (Wang et al. 2015), and the reflectance of white leaves was significantly higher than that of green leaves (Wang et al. 2020).

Structures other than leaves

Some species in the understorey of mixed and deciduous forests may use structures other than leaves, such as bracts and sepals, for harvesting energy in the early period with abundant light.

Sepals may function as petals, as they attract pollinators, protect flowers, and regulate flower temperature, however, they can also serve as photosynthetic organs (Grašič et al. 2021 b, Herrera 2005). This is also the case in the genus *Helleborus*, which comprises 22 species of herbaceous or evergreen perennials originating in Europe and Asia (Bavcon et al. 2012, Fassou et al. 2020, Grašič et al. 2021 b). In some species, the colour of flowers changes during flower development. In *H. orientalis* cv. *Olympicus*, creamy white sepals turned green at later developmental stages (Shahri et al. 2011) and sepals of pollinated flowers contained more chlorophyll in comparison to non-pollinated and senescent flowers (Schmitzer et al. 2013). In some *Helleborus* species with coloured sepals, the evolutionary selection in sepals was not directed to floral function, but rather to the development of sepals into photosynthetic organs (Salopek-Sondi 2002, Salopek-Sondi et al. 2000). This was confirmed by the presence of stomata in the sepals (Grašič et al. 2021 b), even though their density is relatively low in comparison to leaves (Aschan et al. 2005). In *H. odorus* with green-coloured sepals, photochemical efficiency is permanently high, whereas this is not the case in *H. niger* with initially white sepals (Grašič et al. 2021 b). However, the photochemical efficiency of *H. niger* sepals increases during flower development, as they turn green since their chlorophyll content increases (Grašič et al. 2021 b). Along with chlorophylls, sepal carotenoid, anthocyanin, and UV-B–absorbing substances contents were also gradually increasing (Grašič et al. 2021 b). A study of *H. niger* showed an increase in the contents of total anthocyanins, but not flavonols, which absorb in the UV region (Schmitzer et al. 2013). The reflectance and transmittance spectra of the green sepals in *H. odorus* and *H. niger* (Grašič et al. 2021 b) had similar shapes as those of green leaves (Klančnik et al. 2012). Sepal reflectance in VIS and NIR regions was in a negative relationship with chlorophylls and anthocyanins in all phases of flower development. In the case of transmittance, negative relationship between the visible regions (with the exception of green) and anthocyanins and chlorophyll *a* and *b* was obtained in the developing phase, while UV-B–absorbing substances were more important in the flowering phase (Grašič et al. 2021 b).

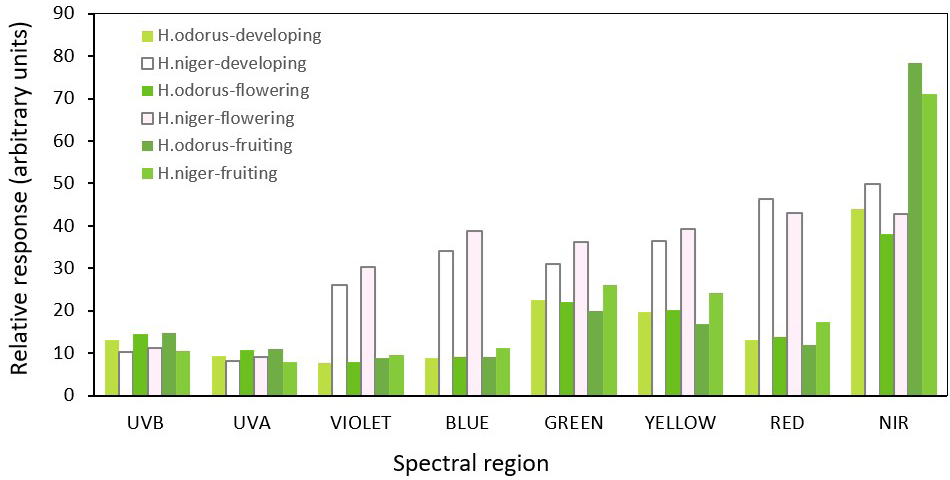

Figure 7: Mean reflectance of radiation in *Helleborus odorus* and *H. niger* sepals in the different spectral regions at different phases of flower development.

Slika 7: Povprečna odbojnost sevanja čašnih listov vrst *Helleborus odorus* in *H. niger* v različnih spektralnih območjih v različnih fazah razvoja cvetov.

In some genera, floral bracts serve as a protective structure and replace the lacking perianth by enclosing floral organs (von Balthazar et al. 1999). Their photosynthetic ability presumably increases the importance of bracts early in the season. Bracts are also extremely important for the attraction of pollinators in some species (Gagliardi et al. 2018) since in 25% of angiosperm flowers, the reflection of ultraviolet light represents important visual information for pollinators (Klomberg et al. 2019). An example of such plant species is *Hacquetia epipactis*, which develops leaves, flowers, and fruits before the canopy layer closes (Gilliam 2014). It has a narrow ecological range and it is sensitive to changes in light conditions and water availability (Ellenberg 1996). *H. epipactis* has umbels that are supported by green bracts (von Balthazar et al. 1999). The shape of the spectral curves of bract reflectance reveals spectra typical of green leaves with peaks in the green and NIR regions, and with low reflectance in the shorter wavelengths (Klančnik et al. 2012). During umbel development, the traits of these bracts change along with changes of the basal leaves, wherein the most evident difference in the reflectance spectra was observed in the UV range (Fig. 8), which increased with age for bracts, while it decreased with age for basal leaves (Grašič et al. 2021 a). Some similarity was observed for bracts of immature and flowering umbels, which may be of relevance for pollinators (Arnold et al. 2010).

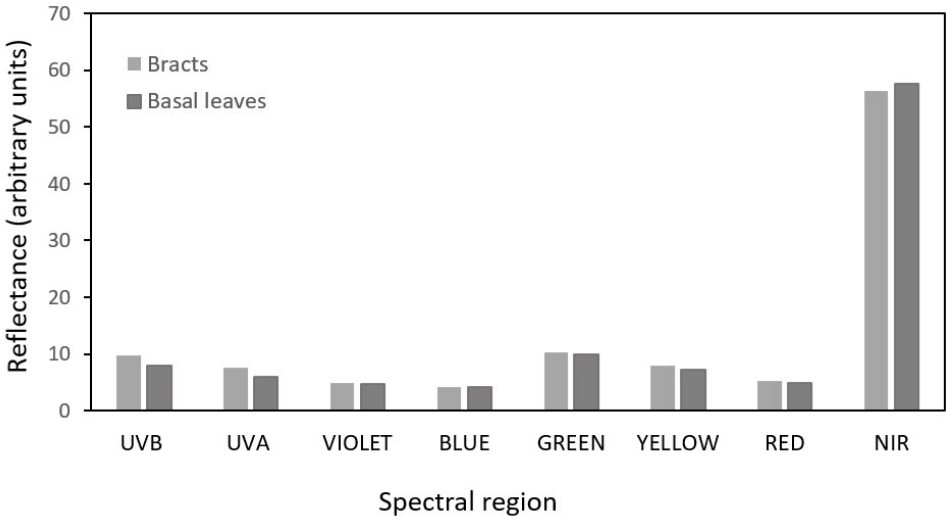

Figure 8: Mean reflectance of radiation in *Hacquetia epipactis* basal leaves and bracts in the different spectral regions.
Slika 8: Povprečna odbojnost sevanja bazalnih in podpornih listov *Hacquetia epipactis* v različnih območjih spektra.

Summary

Temperate deciduous and mixed forests are marked by annual dynamics of radiation level that is related to the vegetation cycle of forest trees. The amount of radiation on the forest floor at a certain time of the year is a consequence of the canopy structure. During winter and early spring, the understorey is exposed to full solar radiation, while later in the growing season radiation is limited due to the closing of the tree storey. In a fully foliated forest, light enters the system *via* gaps in the canopy forming sun flecks that are very variable regarding their quality, intensity, and duration. The radiation conditions in the understorey layer during the vegetation period define the functional traits of photosynthetic organs, including their optical properties, which support efficient light use. The present article presents the optical properties of different structures in some understorey plants species in temperate deciduous forests. The understanding of the functioning of these optical responses and the traits supporting them contributes to the understanding of the forest ecosystem and enables the maintenance of some of these plants in man-made environments, which usually differ significantly from those of the floor of deciduous forests. Plant traits are a result of the evolutionary process, which favours a variety of adaptations that enable an optimal response to specific environmental conditions. The adaptation to light depends on a trade-off in plant responses to other factors. However, plant plasticity that enables the development of specific traits may also enhance light harvesting. Different species in the forest understorey exhibit different levels of phenotypic plasticity. Plasticity may be expressed in different organs and at different levels of plant structure and function, and it also differs among different environments. The plasticity of leaf optical properties is directly related to their structural and biochemical phenotypic plasticity. Plant biochemical and morphological structure, including photosynthetic pigments and anthocyanins, plays an important role in the adaptive strategy of plants to the radiation environment in the forest understorey. Plant leaves are usually uniformly coloured. However, some understorey plants develop leaves in such a way that they have different colour patterns at their surface, optimising the use of both high and low light levels in the forest understorey. The higher light reflectance in

the light green leaf parts is mainly a consequence of the morphological differences and to a lesser extent of pigment contents. Some species may use structures other than leaves, such as bracts and sepals, for efficient energy harvesting.

Povzetek

Za zmerne listnate in mešane gozdove je značilna letna dinamika ravni sevanja, ki je povezana z vegetacijskim ciklom gozdnega drevja. Količina sevanja v gozdnih tleh skozi čas je posledica strukture krošnje. Pozimi in zgodaj spomladi je podrast izpostavljena polnemu sončnemu sevanju, kasneje v rastni dobi pa je sevanje omejeno zaradi zaprtja drevesnih krošenj. Sevanje v podrasti med vegetacijskim obdobjem vpliva na funkcionalne poteze fotosinteznih organov, med drugim tudi na njihove optične lastnosti, ki podpirajo učinkovito rabo svetlobe. Pričujoči članek predstavlja optične lastnosti različnih struktur nekaterih vrst rastlin podrasti v zmernem listnatem gozdu. Poznavanje optičnih odzivov in funkcionalnih potez, ki jih podpirajo, prispeva k razumevanju gozdnega ekosistema in omogoča ohranjanje določenih tovrstnih rastlin v umetnih okoljih, ki se običajno bistveno razlikujejo od razmer v gozdu. Funkcionalne poteze rastlin so rezultat procesa evolucije, ki daje prednost različnim prilagoditvam, ki omogočajo optimalen odziv na specifične okoljske razmere. Prilagajanje na svetlobo je kompromis med odzivom rastlin na vse dejavnike, medtem ko fenotipska plastičnost rastlin, ki omogoča razvoj specifičnih lastnosti, lahko izboljša tudi prestrezanje svetlobe. Različne vrste podrasti kažejo različno stopnjo fenotipske plastičnosti. Plastičnost se lahko izraža v različnih organih in na različnih ravneh zgradbe in delovanja rastline, razlikuje pa se tudi med različnimi okolji. Plastičnost optičnih lastnosti listov je neposredno povezana z njihovo strukturno in biokemijsko fenotipsko plastičnostjo. Biokemijska in morfološka zgradba rastlin, vključno s fotosinteznimi pigmenti in antocianini, ima pomembno vlogo v strategiji prilagajanja rastlin na sevalno okolje v gozdni podrasti. Listi rastlin so običajno enakomerno obarvani, vendar imajo nekatere rastline liste z različnimi barvnimi vzorci na površini, kar optimizira rabo tako visoke kot tudi nizke ravni svetlobe v podrasti. Večji odboj svetlobe v svetlozelenih delih listov je predvsem posledica morfoloških razlik in v manjši meri vsebnosti pigmentov. Nekatere vrste lahko za povečanje učinkovitosti prestrezanja svetlobe poleg listov uporabljajo tudi druge strukture, kot so podporni in čašni listi.

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Aerobic bacteria in holy water from Catholic churches in Slovenia

Aerobne bakterije v blagoslovljeni vodi iz katoliških cerkva v Sloveniji

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Abstract: Holy water plays an important role in various religions. It is used for baptisms, to bless people, places and objects. In Catholic churches, it is usually offered in the holy water fonts at the entrance of the church. But it is also considered a source of potential pathogens, such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and enterobacteria. To estimate the potential risk, we studied the composition and antimicrobial resistance of bacteria in holy water from fonts and reservoirs of ten selected Catholic churches in Ljubljana, Slovenia. Bacterial contamination of holy water from fonts was moderate (10^2 - 10^5 CFU ml^{-1}), but one to two orders of magnitude higher than in reservoirs, probably due to frequent immersion of fingers in the water. Some genera/species occurred only in fonts (*Acinetobacter beijerinckii*, *A. haemolyticus*, *Brevundimonas aurantiaca*, *B. mediterranea*, *Delftia*, *Kocuria*, *Sphingobacterium*, *Staphylococcus warneri*), while few fecal indicator bacteria were isolated. Isolated bacteria have relatively low pathogenic potential, some of them are skin commensals. Bacterial strains isolated in this study were susceptible to antibiotics. While according to our results, the potential of holy water for spreading bacterial infections is modest, to further limit the risks, water should be changed regularly, the fonts cleaned thoroughly, and the water should not be brought in contact with the eyes, ingested or aerosolized and inhaled.

Keywords: antibiotic resistance, bacteria, fonts, holy water, NaCl, Roman Catholic churches, pathogens

Izvleček: Blagoslovljena voda ima pomembno vlogo v različnih religijah. Uporablja se pri svetem krstu, za blagoslov ljudi, krajev in predmetov. V katoliških cerkvah se običajno nahaja v kropilnikih ob vhodu v cerkev. Lahko pa predstavlja tudi vir oportunističnih patogenov, kot so *Pseudomonas aeruginosa*, *Acinetobacter baumanii* in enterobakterije. Da bi bolje razumeli to tveganje, smo proučili sestavo in protimikrobno odpornost bakterij v blagoslovljeni vodi kropilnikov in rezervarjev desetih izbranih katoliških cerkva v Ljubljani (Slovenija). Bakterijska kontaminacija blagoslovljene vode iz krstilnikov je bila zmerna (10^2 - 10^5 CFU ml^{-1}), a za red velikosti do dva višja kot v rezervarjih, verjetno zaradi pogostega pomakanja prstov vernikov v vodo kropilnika ob vstopu in izstopu iz cerkve. Nekateri bakterijski rodovi/vrste so se pojavili le v kropilnikih (*Acinetobacter beijerinckii*, *A. haemolyticus*, *Brevundimonas aurantiaca*, *B. mediterranea*, *Delftia, Kocuria*, *Sphingobacterium*, *Staphylococcus warneri*), izoliranih

pa je bilo le nekaj fekalnih indikatorskih bakterij. Izolirane bakterije imajo razmeroma nizek patogeni potencial, nekatere med njimi so kožni komenzali. Bakterijski sevi v tej študiji so bili občutljivi proti antibiotikom. Čeprav je potencial blagoslovljene vode za širjenje bakterijskih okužb glede na naše rezultate majhen, lahko tveganja dodatno zmanjšamo z redno menjavo vode, s temeljitim čiščenjem kropilnikov in s preprečevanjem vnosa vode v oči, njenega zaužitja ter aerosolizacije in vdihavanja.

Ključne besede: bakterije, blagoslovljena voda, kropilniki, NaCl, odpornost proti antibiotikom, patogeni, rimskokatoliške cerkve

Introduction

Water is considered a sign of cleanliness and purification and has been used in many ancient and modern religious traditions (Oestigaard 2017). Holy water is water that has been blessed by a priest or comes from a well or spring considered sacred. In the Roman Catholic tradition, holy water is used as a sacrament for baptisms, to bless people, places, and objects, or to protect against evil and danger (Kirschner et al. 2012). It is commonly used to wash away sins (Flemming 2011). The Roman Catholic Church recommends adding an unregulated amount of blessed salt (sodium chloride) to the water during the blessing, which also results in varying salt concentrations of the holy water (Kirschner et al. 2012). Holy water is usually made only once a year at Easter, when tap water is blessed and then stored in metal reservoirs (tanks) located in the church. Holy water is offered in the holy water fonts at the entrance of the church (or sometimes at a separate location, the baptistery). Smaller vessels, called stoups, are usually placed on the walls of church entrances for people to bless themselves with as they enter the church. They may be larger or smaller and made of stone, marble, glass, metal, or porcelain. When the faithful enter and leave the church, they dip the fingers of their right hand into the holy water and make the sign of the cross on their forehead, lips and chest. Holy water can also be used to bless food, objects, places, or people by sprinkling them (Jurado et al. 2002, Kirschner et al. 2012).

However, it is well known that water is both a reservoir and source of pathogens that can lead to transmission of infectious diseases (Denham et al. 2013). Holy water has also been identified as a potential source of microbial and viral infections, including COVID-19 (Gajurel and Deresinski 2021). As early as the late 19th century, bacteriologist L. Vincenzi found large numbers of microbes - staphylococci, streptococci, coli bacilli, Klebs-Loeffler bacillus (*Corynebacterium diphtheriae*), and other bacteria - in samples of holy water from a church in Sassari, Italy (Leffmann 1898). In a study from 1998, coliforms, staphylococci, yeasts, and molds were cultured from holy water from County Clare, Ireland (Payne, 2001). An examination of holy water fonts from churches in Seville, Spain, revealed heavy contamination with bacterial pathogens. The number of coliforms in the fonts exceeded 10^3 per 100 ml of water. The presence of potential pathogens was demonstrated by the identification of *Acinetobacter*, *Aeromonas*, *Haemophilus*, *Neisseria*, *Salmonella*, and *Staphylococcus* species. The most common genera in holy water were *Pseudomonas* and *Bacillus*, followed by *Staphylococcus*, *Sphingobacterium,* and *Delftia*. However, species diversity varied greatly from one church to another. According to the authors, the presence of the genera *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Pseudomonas,* and several others in holy water was associated with hand contamination and human skin transmission (Jurado et al. 2002). Contamination of holy water fonts has also been reported in Vienna, Austria. All holy water samples from churches and hospital chapels had extremely high concentrations of heterotrophic plate counts, up to 10^7 colony-forming units (CFU) ml^{-1}; while fecal indicators such as enterococci and *E. coli*, as well as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, were found only in the most frequently visited churches (Kirschner et al. 2012). A similar study was conducted in the Villingen-Schwenningen area, Germany. Colony

counts revealed an average aerobic microbial load of 5.85±3.98 × 10^3 CFU ml^{-1}. Urban churches had significantly higher bacterial contamination levels than rural churches, likely due to a higher number of visitors. The majority of the bacteria identified were typical human skin commensals, mainly belonging to the genus *Staphylococcus*. Fifty percent of the identified species were classified as potential pathogens: *Staphylococcus* (*S. aureus*, *S. epidermidis*, *S. homini*, *S. pettenkoferi*, *S. pasteuri*), *Bacillus cereus*, *Actinomyces oris*, *Acinetobacter johnsonii*, and *Enterobacter hormaechei* (König et al. 2017).

Water that comes from Christian shrines and churches around the world is often used by hospital patients. Holy water is often administered by sprinkling, but can also be ingested, dripped into the eyes, or used to bathe affected body parts. There have been some reports of hospital patients experiencing serious complications after contact with holy water during hospital treatment. A case of hospital-acquired infection by *Acinetobacter baumanii* in a patient with burns after contact with holy water (sprinkling) has been described (Rees and Allen 1996). Based on the analysis of the holy water used (from Lourdes, Walsingham, and River Jordan), the majority of organisms isolated from the holy water samples were Gram-negative bacilli, including opportunistic pathogens such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter* spp., and *Stenotrophomonas maltophilia* (Rees and Allen 1996). A case has also been reported in which an adult male contracted *Pseudomonas aeruginosa* pneumonia while recovering from several injuries. His aunt was observed sprinkling holy water on the patient, and this water was confirmed as a source of the pathogen (Greaves and Porter 1992). Similarly, an 11-year-old boy with recurrent epilepsy requiring mechanical ventilation was found to have recurrent multidrug-resistant *Acinetobacter baumannii* pneumonia. The patient's mother had regularly sprinkled him with holy water over several months. Microbiological examination of this holy water detected the multidrug-resistant *Acinetobacter baumanii* strain previously isolated from the patient (Michel et al. 2013).

Because holy water may pose a risk for infection with pathogenic microorganisms through inoculation by hands, in this study we investigated the quantity and diversity of the cultivable bacterial community from holy water fonts and reservoirs located in some of the most visited Roman Catholic churches of Ljubljana, Slovenia. The bacterial isolates were identified and their resistance to selected antibiotics was analyzed to assess the potential health risk.

Materials and methods

Sampling and water analyses

The sampling of holy water was carried out in ten churches in different parts of Ljubljana and the city area. In order to preserve anonymity, the names of the churches are not listed, but are labelled here with two-letter codes. Sampling took place three times at three-week intervals (the first sampling on March 11 and 12, 2019, the second on April 1 and 2, 2019, and the third on April 23 and 24, 2019). The third (final) sampling took place after Easter holiday, when churches are the most crowded. Samples were collected in sterile containers in the morning hours, before, during, or after morning Mass from the same fonts at the entrance and from the holy water reservoirs. We also collected a sample from tap water in church DC. Within two hours of collection, samples were inoculated onto culture media and incubated. The remaining water samples were stored at 4 °C until chemical analysis. During the final sampling, the temperature and pH of the holy water in the wells and reservoirs were measured. The water activity of the water samples from the first sampling was measured using the AquaLab 3TE instrument (Meter, Germany) according to manufacturer's instructions. Sodium concentration of selected water samples from the first sampling was measured using a Varian AA240 atomic absorption spectrophotometer.

Bacterial isolation and identification

Within two hours of collection, 10 µl and 100 µl of the collected water samples were inoculated onto blood agar (BA, Fluka) and onto UriSelect

4 agar (URI, Bio-Rad) and incubated aerobically at 37 °C. After three days of incubation, colonies were counted, and all morphologically different colonies were selected and isolated in pure culture on Brain Heart Infusion (BHI) agar plates (Biolife). DNA was extracted from the isolated pure cultures using PrepMan Ultra reagent (Applied Biosystems) according to the manufacturer's instructions. The 16S rRNA gene was amplified using primers 27f- CM (5'- AGAGTTTGATCMTGGCTCAG -3') (Frank et al. 2008) and 1492R (5'- GGTTACCTTGTTACGACTT -3') (Turner et al. 1999). The 16S rDNA gene amplicons were sequenced by Microsynth AG (Switzerland) using Sanger sequencing. The resulting sequences were analyzed using MUSCLE software (Edgar, 2004) implemented in the MEGA7 package (Kumar et al., 2016) and compared against the GenBank database (16S ribosomal RNA (Bacteria and Archaea) database) using BLASTN software (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi). All isolated strains from this study were deposited in the Ex Culture Collection of the Infrastructural Centre Mycosmo (MRIC UL) at the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia.

Antibiotic susceptibility testing

For antimicrobial susceptibility testing, the isolated bacterial strains were cultured on LB agar plates (Biolife) with 8 different commonly used antibiotics (Sigma) and incubated at 37 °C. The results were observed after 3 days. The following antibiotics and concentrations were used: Ampicillin (AMP) 100 mg/l; Chloramphenicol (CHL) 25 mg/l; Cefotaxime (CTX) 2 mg/l; Colistin (COL) 3.5 mg/l; Enrofloxacin (ENR) 0.5 mg/l; Erythromycin (ERY) 15 mg/l; Imipenem (IPM) 4 mg/l; Kanamycin (KAN) 50 mg/l); Tetracycline (TET) 10 mg/l. Wild-type *Escherichia coli* strains EXB L-4239 A5 and EXB L-4240 A6 isolated from poultry with known resistance profiles were used as positive controls (recovered from Ex Culture Collection of the Infrastructural Centre Mycosmo (MRIC UL), University of Ljubljana).

Community analyses using machine learning

To analyze the obtained data and discover the connections between them, machine learning methods were used. All data analyses were performed using the R statistical programming language and environment and Microsoft Excel 2016. The hierarchical clustering method was used to determine the similarity between samples, using the function "hclust()" from the package stats v3.6.1 (Müllner 2013). The R package randomForest was used to find how well the presence of individual bacterial species predicts the various characteristics of the sample (e.g. material of vessels) using a random forests approach. We calculated 10001 trees for each condition studied and determined from the calculated models the bacterial species whose presence best predicted each characteristic of the sample (Tang et al. 2014).

Results

The holy water is located in the churches in holy water fonts and containers, which can be made of different materials. The fonts in the studied churches were made of stone, porcelain, glass or metal, while the reservoirs in all the studied churches were made of metal - stainless steel (Fig. 1).

Figure 1: Examples of holy water fonts made of different materials in the studied churches (top left metal, top right stone, bottom left glass, and bottom right porcelain).
Slika 1: Primeri kropilnikov za blagoslovljeno vodo iz različnih materialov v proučevanih cerkvah (zgoraj levo kovinski, zgoraj desno kamnit, spodaj levo steklen in spodaj desno porcelanast).

Physico-chemical parameters of holy water samples

The values of selected physicochemical parameters such as temperature, pH, water activity (a_w), and sodium mass concentration in the holy water were measured (Tab. 1). These parameters were monitored with the aim of determining their influence on the growth of the cultivable aerobic bacterial community in fonts and reservoirs.

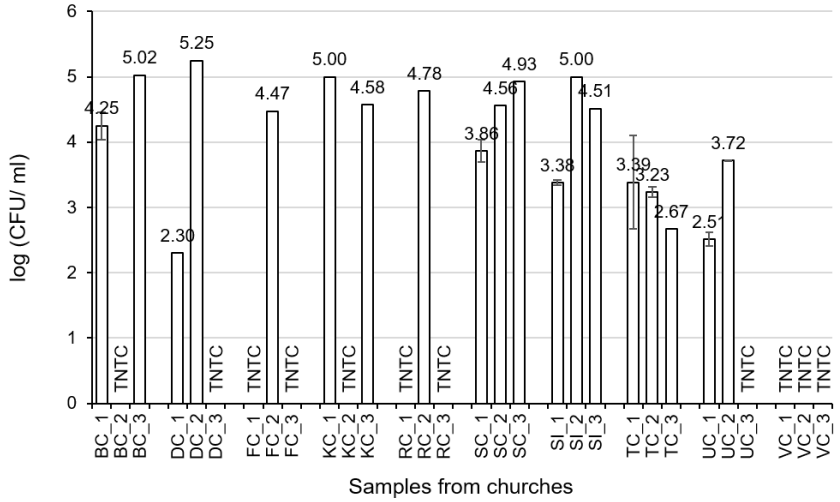
Table 1: Results of measurements of temperature, pH, water activity (a_w) and sodium (Na^+) mass concentration in holy water from various churches in Ljubljana and its surroundings.
Tabela 1: Rezultati merjenja temperature, pH, vodne aktivnosti (a_w) in masne koncentracije natrija (Na^+) v blagoslovljeni vodi, vzorčeni v različnih cerkvah v Ljubljani in okolici.

Church	Sampling site	Material of container	Water T [°C]	Water pH	Water acitvity a_W	Na^+ [mg/l]
FC	Font	Glass	18	7.30	0.998	11.53
	Reservoir	Metal	21	7.84	0.997	8.56
SC	Font	Porcelain	18	7.76	0.998	-
	Reservoir	Metal	19	7.83	0.998	-
UC	Font	Glass	18	7.49	0.999	-
	Reservoir	Metal	17	7.79	0.999	5.72
VC	Font	Glass	19	7.49	0.998	-
	Reservoir	Metal	18	7.93	0.998	-
TC	Font	Stone	15	7.99	0.979	14958.00
	Reservoir	-	-	-	-	-
RC	Font	Metal	20	7.45	0.998	-
	Reservoir	Metal	17	7.81	0.998	-
BC	Font	Glass	17	7.67	0.998	-
	Reservoir	Metal	-	7.74	0.998	-
SI	Font	Stone	20	7.70	0.997	-
	Reservoir	Metal	18	7.79	0.997	4.72
KC	Font	Stone	18	7.38	0.997	-
	Reservoir	Metal	17	7.80	-	-
DC	Font	Glass	16	7.68	0.995	2044.80
	Reservoir	Metal	16	7.83	0.991	4109.00
Tap water (W)	-	-	17	7.26	0.999	6.17

The temperature of the holy water was measured during the last, third sampling on April 23 and 24, 2019, it ranged from 15 °C to 21 °C. The pH of holy water samples from all three samplings was measured, and the average pH ranged from 7.30 to 7.99. In all sampled churches, the average pH of the holy water in the reservoir was slightly higher than in the font. The pH of the tap water (W) was 7.26. The water activity (a_w) of the samples from the first sampling was also determined and ranged from 0.979 to 0.999, with the lowest a_w value in the font of the church TC (0.979) and in the reservoir (0.991) and font (0.995) of the church DC. We also measured the sodium mass concentration of the selected samples. Holy water from the font of the church TC had the highest measured mass concentration of sodium (14.96 g/l), followed by holy water from the reservoir (4.11 g/l) and from the font (2.04 g/l) of the church DC. Sodium concentration in most samples of holy water from churches was 0.005-0.012 g/l, which is in the range of Na^+ concentration in tap water (Tab. 1).

Load of cultivable aerobic bacteria in holy water samples

The number of cultivable aerobic bacteria in holy water samples was determined by the plate count method on blood agar after incubation at 37 °C. The colony counts of the samples from fonts (Fig. 2) spanned four orders of magnitude (10^2 – 10^5 colony-forming units (CFU) ml^{-1}).



For one third of the samples no value could be determined because of confluent overgrowth (too numerous to count – TNTC). The colony counts of samples from reservoirs spanned three orders of magnitude (10^1 – 10^3 CFU ml^{-1}; Fig. 3). Comparing CFU numbers between samples from the font and the reservoir for a single church, the CFU count in the font was higher than in the reservoir in almost all cases. In some cases, it was not possible to obtain samples of holy water from reservoirs (BC, KC, RC, and especially TC; Fig. 3). There were also considerable differences in the number of CFUs in each church between samplings.

Figure 2: Aerobic colony counts (CFU ml^{-1}) in holy water samples from fonts in ten churches in different parts of Ljubljana and the city area. Values represent mean counts. TNTC – confluent overgrowth.

Slika 2: Štetje aerobnih kolonijskih enot (CFU ml^{-1}) v vzorcih blagoslovljene vode iz kropilnikov desetih cerkva v različnih predelih Ljubljane in njeni okolici. Vrednosti predstavljajo povprečja vzorčenj. TNTC – konfluentna rast.

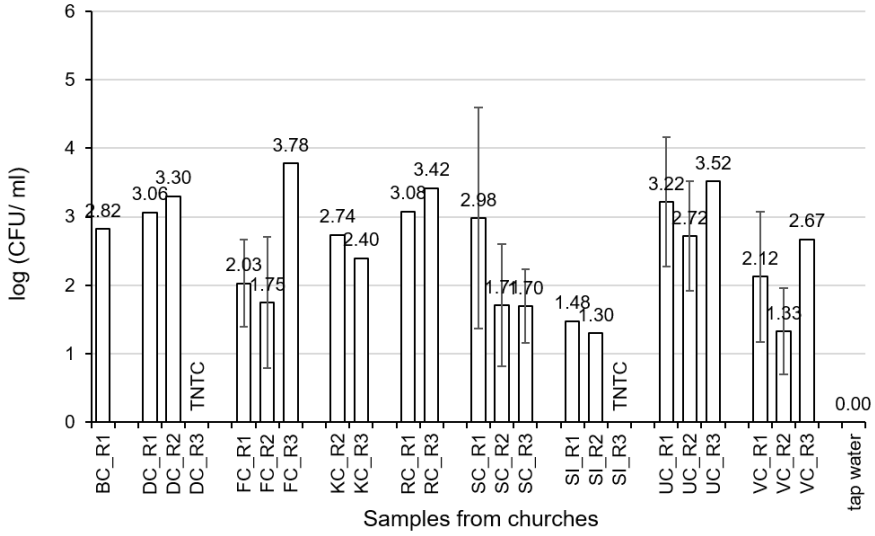


Figure 3: Aerobic colony counts (CFU ml^{-1}) in tap water and holy water samples from reservoirs in nine churches in different parts of Ljubljana and the city area. Values represent mean counts. TNTC – confluent growth.
Slika 3: Štetje aerobnih kolonijskih enot (CFU ml^{-1}) v pitni vodi in v vzorcih blagoslovljene vode iz rezervarjev devetih cerkva v različnih predelih Ljubljane in njeni okolici. Vrednosti predstavljajo povprečja vzorčenj. TNTC – konfluentna rast.

Bacterial identification

From 56 samples, one of which was tap water, 585 bacterial strains were isolated and identified. The results of the identification of the bacterial strains are given in the supplementary material (S1). Identification was performed by searching for homologous sequences of the 16S rRNA gene for our isolates in the GenBank database. Most of the identified strains were classified into the classes *Gammaproteobacteria* and *Actinomycetes*. The cultivable aerobic bacterial community differs from church to church, as not all taxa are present in all churches (Fig. 4). In addition, different bacterial taxa are present in the font and reservoir of each church. The highest number of species (21 and 22) was found in the holy water of the church RC from the font and reservoir (Fig. 4). Otherwise, the studied churches differed in the number of species isolated from the fonts compared to the reservoirs. In the four studied churches, the number of different species was similar in fonts and reservoirs (DC, RC, UC, VC), in FC the species diversity in the holy water from the reservoir was higher (22) than in the font (14), and in four churches (BC, KC, SC and SI) the number of species in the water samples from the fonts was higher.

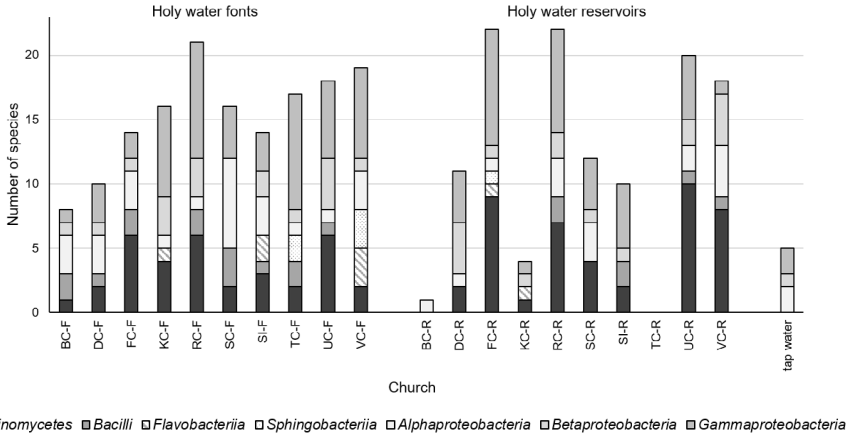


Figure 4: The number of identified species of cultivable aerobic bacteria isolated from the holy water fonts and reservoirs of the studied churches, grouped by classes.

Slika 4: Prikaz števila identificiranih vrst kultivabilnih aerobnih bakterij, izoliranih iz blagoslovljene vode kropilnikov in rezervarjev vzorčenih cerkva, združenih v razrede.

Bacterial isolates were assigned to 52 genera. Genera whose species appeared in at least two samples were compared for their occurrence in holy water fonts and reservoirs (Fig. 5). Some genera occurred only in fonts (*Delftia, Kocuria, Sphingobacterium, Staphylococcus*), and only species of two genera (*Acinetobacter, Pseudomonas*) were found in all three sample groups. These two genera were also the most represented.

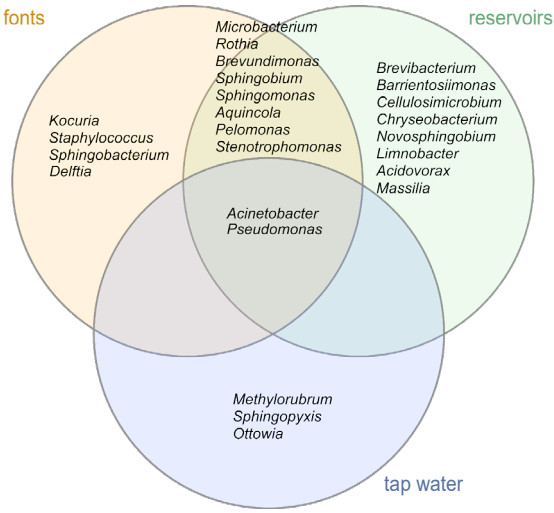


Figure 5: Venn diagram (Heberle et al. 2015) of the occurrence of the identified bacterial genera in samples of holy water from fonts and reservoirs and from tap water.

Slika 5: Vennov diagram (Heberle s sod. 2015) prisotnosti identificiranih bakterijskih rodov v vzorcih blagoslovljene vode kropilnikov in rezervarjev ter pitne vode.

Bacterial community analysis using machine learning

Machine learning methods were used to analyze the data obtained (bacterial species at different sampling sites) and to investigate the presence of bacterial species as a function of sampling site. We were interested in whether the presence of any of the species could be associated with the type of sample (font, reservoir). Certain bacterial species (*Acinetobacter beijerinckii*, *Acinetobacter haemolyticus*, *Brevundimonas aurantiaca*, *Brevundimonas mediterranea*, *Staphylococcus warneri*) were present only in holy water fonts (Fig. 6). Similarly, some species were more abundant in fonts than in reservoirs (*Acinetobacter johnsonii*, *Aquincola tertiaricarbonis*, *Rothia amarae*, *Sphingobium hydrophobicum*). The opposite phenomenon was observed for *Microbacterium maritypicum*, which was more abundant in holy water reservoirs (in seven of nine sampled reservoirs) than in fonts (in three of ten sampled fonts).

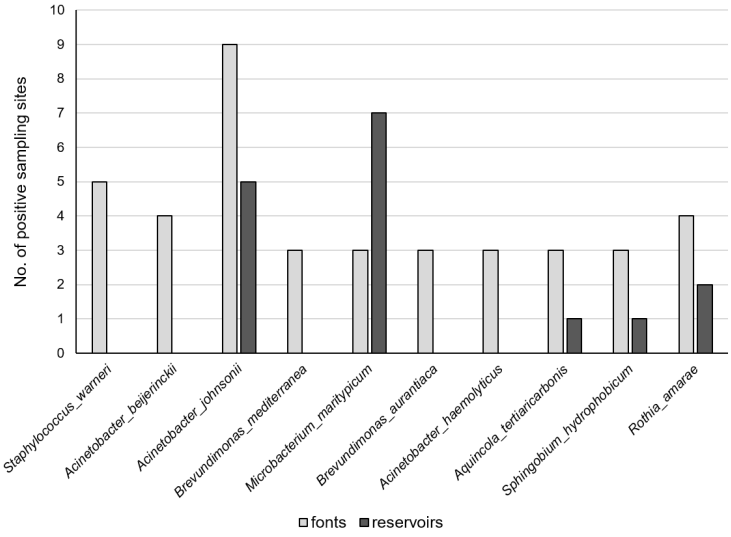


Figure 6: Occurrence of selected bacterial species depending on the container with holy water.
Slika 6: Pojavnost izbranih bakterijskih vrst v odvisnosti od posode z blagoslovljeno vodo.

The hierarchical clustering method was used to investigate the similarity between sampling sites (fonts and reservoirs) based on the matrix of the presence of different bacterial species. The more similar the bacterial communities of the sampling sites, the closer they are in the clustering tree (Fig. 7). Some reservoirs (churches DC, KC and BC) cluster together with tap water. In most cases, there is no grouping by church (font and reservoir of each church).

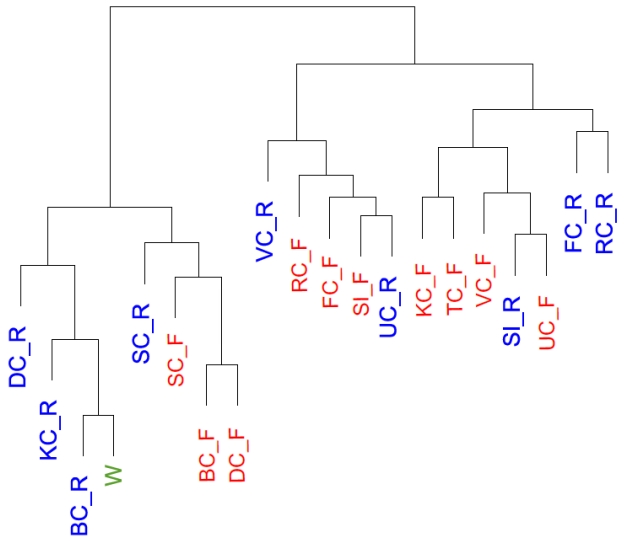


Figure 7: Display of hierarchical clustering of sample sites into groups based on the bacterial species occurrence matrix. Color marks: blue - reservoirs, red - fonts, green - tap water (W).
Slika 7: Prikaz hierarhičnega združevanja vzorčnih mest v skupine na osnovi matrice pojavljanja bakterijskih vrst. Barvne oznake: modra - rezervarji, rdeča – kropilniki, zelena - vodovodna voda (W).

A connection between the container material and the occurrence of bacterial species was also tested (not shown). *Acinetobacter beijerinckii*, *Brevundimonas mediterranea*, *Brevundimonas aurantiaca*, *Kocuria uropygioeca*, and *Sphingobacterium multivorum* were isolated only from nonmetallic fonts. However, no association was found between a single bacterial species and a metal vessel, nor with other materials (glass, stone, porcelain).

Resistance of bacterial isolates to selected antibiotics

The effects of nine antibiotics on 83 selected bacterial strains isolated from the holy water fonts and reservoirs was investigated, namely ampicillin (AMP, 100 mg/l), tetracycline (TET, 12.5 mg/l), imipenem (IPM, 4 mg/l), erythromycin (ERY, 15 mg/l), chloramphenicol (CHL, 25 mg/l), kanamycin (KAN, 50 mg/l), cefotaxime (CTX, 2 mg/l), enrofloxacin (ENR, 0.5 mg /l) and colistin (COL, 3.5 mg/l). Results are shown in the supplemental material (S2). Most strains were sensitive to tetracycline (12.5 mg/l) and chloramphenicol (25 mg/l) (Fig. 8), and more than half of the strains were resistant to colistin (3.5 mg/l) and cefotaxime (2 mg/l).

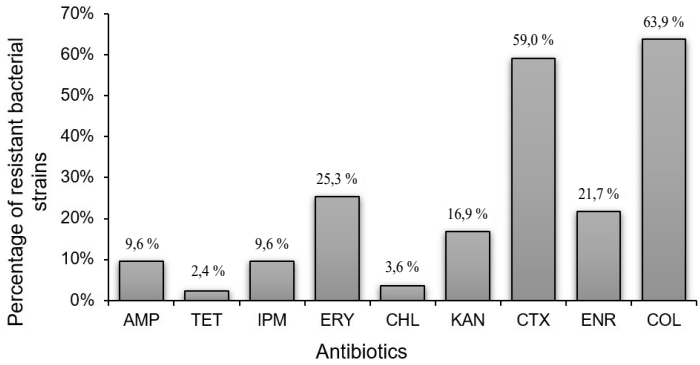


Figure 8: Summary of results for antimicrobial susceptibility for the 83 isolates tested, presented are percentages of isolates that are resistant. Ampicillin (AMP) 100 mg/l; chloramphenicol (CHL) 25 mg/l; cefotaxime (CTX) 2 mg/l; colistin (COL) 3.5 mg/l; enrofloxacin (ENR) 0.5 mg/l; erythromycin (ERY) 15 mg/l; imipenem (IPM) 4 mg/l; kanamycin (KAN) 50 mg/l); tetracycline (TET) 10 mg/l.

Slika 8: Povzetek rezultatov občutljivosti proti antibiotikom za 83 testiranih izolatov, predstavljen je odstotek izolatov, ki so odporni. Ampicilin (AMP) 100 mg/l; kloramfenikol (CHL) 25 mg/l; cefotaksim (CTX) 2 mg/l; kolistin (COL) 3.5 mg/l; enrofloksacin (ENR) 0.5 mg/l; eritromicin (ERY) 15 mg/l; imipenem (IPM) 4 mg/l; kanamicin (KAN) 50 mg/l); tetraciklin (TET) 10 mg/l.

Resistance profiles for individual species of the same genus are similar. Among bacterial species potentially pathogenic to humans and vertebrates, *Pseudomonas aeruginosa* (resistant to 7 antibiotics tested at selected concentrations) and *Stenotrophomonas maltophilia* (resistant to 4 antibiotics tested) were the most resistant to studied antibiotics.

Discussion

In Slovenia, 57.8% of the population declared themselves Roman Catholic, according to the last census in 2002 (Črnič et al. 2013). As in other Christian religions, for Catholics, holy water is water that has been blessed by a priest and is used for baptism and to bless people, churches, homes, and objects of devotion (Jurado et al. 2002, Kirschner et al. 2012). Some studies have presented holy water as a potential source of infection with pathogenic organisms (Rees and Allen 1996, Greaves and Porter 1992, Michel et al. 2013, Gajurel and Deresinski 2021). Due to COVID -19 concern, many churches emptied their holy water fonts and, in some churches, a non-contact holy water dispenser was installed (Pullella 2020; Drogo 2022). In this study, we investigated the microbiological quality of holy water in the fonts and reservoirs of ten selected Roman Catholic churches in Ljubljana and its surroundings, focusing on bacteria. In order to assess the potential health risk of the bacterial strains isolated from the holy water, they were identified and their resistance to selected antibiotics was analyzed.

The aerobic bacterial load of the holy water was determined by the plate count method. In most of the churches studied, the number of CFU ml^{-1} was higher in the holy water from fonts (10^2 - 10^5 CFU ml^{-1}; Fig. 2) than in the reservoirs (10^1 - 10^3 CFU ml^{-1}; Fig. 3), which is to be expected since the church visitors have direct daily contact with the holy water from the fonts. The bacterial load varied among the different churches and also among the sampling of a single church. This relatively moderate bacterial contamination of holy water from fonts is consistent with previous studies from churches in Vienna, Austria (Kirschner et al. 2012) and in the Villingen-Schwenningen area, Germany (König et al. 2017), where similar cultivation conditions (rich medium, incubation at 37 °C) were used. In Vienna, all holy water samples from the investigated churches and hospital chapels had high concentrations of heterotrophic microbial counts at 37 °C, up to 3×10^7 CFU ml^{-1}, higher than in our study, while in Villingen-Schwenningen

(Germany) the colony count showed an average aerobic microbial load of 5.85±3.98 × 10^3 CFU ml^{-1}. In a study of holy water fonts from churches in Seville, Spain, total aerobic bacteria and coliforms in certain churches were 'too numerous to count' (Jurado et al. 2002). Bacterial load is certainly influenced by the number of visitors and the frequency of cleaning and replacement of water from fonts (König et al. 2017, Jurado et al. 2002). This type of data would help us understand the differences between the bacterial load of the sampled churches in Ljubljana, but these data were not available. Nevertheless, our results confirm that the frequent immersion of fingers in holy water fonts is probably the main reason for the higher bacterial load in the fonts compared to the reservoirs. In this way, microbes – and also nutrients for their growth – are transferred from the skin to the holy water (Kirschner et al. 2012).

The Roman Catholic Church recommends adding blessed salt (NaCl) to the water during the blessing (Kirschner et al. 2012). Salt was found only in holy water samples from two churches and its concentration was low, ranging from 0.2 to 1.5% NaCl (m/v) (Tab.1). In most churches, the sodium concentration was of the same order of magnitude as the concentration in tap water. In a study from Spain, the viability of selected bacterial species was measured as a function of different NaCl concentrations. At NaCl concentrations of 20% or more, both pathogenic and nonpathogenic bacteria lysed (Jurado et al. 2002). This process, called plasmolysis, occurs due to water efflux leading to a decrease in cytoplasmic volume. This can lead to retraction of cell walls, detachment and wrinkling of the plasmalema, and disruption of protein assemblies that extend across the cell wall (Wood 2011). In a study by Hrenovic and Ivankovic (2009) testing the survival of *E. coli* and *Acinetobacter junii* at different NaCl concentrations, complete death of *E. coli* was achieved after 72 hours at concentrations of 20% NaCl. Thus, to prevent contamination of the holy water, larger amounts of NaCl would have to be added than was detected in our samples because only then would the growth of most microorganisms be completely inhibited. However, high NaCl concentrations can damage the fonts, so the use of NaCl should be carefully balanced with protection of cultural heritage (Jurado et al. 2002). An alternative is to change the holy water daily and to clean the holy water fonts regularly.

From 56 samples of holy water, 585 bacterial isolates were obtained and identified based on the homologous sequences of the 16S rRNA gene in the GenBank database. The aerobic cultivable community of the holy water consisted mainly of bacterial species from the classes *Gammaproteobacteria* and *Actinomycetes* (Fig. 4). The bacterial community differed between churches as well as between the font and the reservoir of each church. Some genera were present only in the holy water from fonts (*Delftia*, *Kocuria*, *Sphingobacterium*, *Staphylococcus*), and only species of two genera (*Acinetobacter*, *Pseudomonas*) were found in the holy water from fonts, reservoirs, and tap water (Fig. 5). It is likely that some of the identified bacteria in holy water fonts originated from the skin of church visitors. In the microbiota of human hands studied by amplicon sequencing, species from the phyla *Pseudomonadota*, *Actinomycetota*, and *Bacillota* were found, accounting for 94% of all identified bacterial species, with the most abundant genera *Cutibacterium* (31.6% of all sequences; *Actinomycetota*), *Streptococcus* (17.2%; *Bacillota*), *Staphylococcus* (8.3%; *Bacillota*), *Corynebacterium* (4.3%; *Actinomycetota*), and *Lactobacillus* (3.1%; *Bacillota*) (Egert and Simmering 2016, Byrd et al. 2018, Carmona-Cruz et al. 2022). In agreement with previous studies (Rees and Allen 1996, Jurado et al. 2002, Kirschner et al. 2012, König et al. 2017), we also detected bacteria of probable fecal origin, i.e., enterococci and enterobacteria, albeit in very low abundance. The representative of fecal indicator bacteria, *Enterococcus* sp., was found in only one sample of holy water from the reservoir, while *Citrobacter freundii* and *Enterobacter cloacae* were detected in two samples from fonts.

Using machine learning methods, we aimed to investigate the occurrence of bacterial species as a function of sampling location (font or reservoir). Some bacterial species occurred only in holy water from fonts (*Acinetobacter beijerinckii*, *Acinetobacter haemolyticus*, *Brevundimonas aurantiaca*, *Brevundimonas mediterranea*, *Staphylococcus warneri*), while others, such as *Microbacterium maritypicum*, were more abundant

in holy water reservoirs (Fig. 6). These bacteria have relatively low pathogenic potential, and some of them are considered to be skin commensals. The genus *Acinetobacter* (*Pseudomonadota*) is generally found in aqueous environments, with the majority of species being non-pathogenic. The most common species causing infections is *A. baumannii*, followed by species also found on human skin, such as *A. calcoaceticus* and *A. lwoffii*, which has also been isolated from our holy water samples. These are largely opportunistic pathogens that cause infections, especially in immunocompromised patients. Other species, including *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. nosocomialis*, *A. pittii*, *A. schindleri*, and *A. ursingii*, have occasionally been reported as pathogens (Wong et al. 2017). *Brevundimonas* spp. (*Pseudomonadota*) are a genus of non-fermenting Gram-negative bacteria and, particularly *Brevundimonas diminuta* and *Brevundimonas vesicularis*, are considered to be of minor clinical importance (Ryan and Pembroke 2018). *Microbacterium* species (*Actinomycetota*), non-spore-forming, Gram-positive rods, have also rarely been associated with human disease. However, increasing amount of literature shows that *Microbacterium* species are opportunistic human pathogens, causing, for example, infective endocarditis associated with *Microbacterium maritypicum* bacteremia (Yeung et al. 2020). But *Staphylococcus warneri*, a coagulase-negative staphylococcus (CNS) commonly found in the microbiota of human and animal epithelia and mucosa, is considered an opportunistic pathogen that causes serious infections in humans and animals (Liu et al. 2020).

The hierarchical clustering was used to determine the similarity between sampling sites (fonts and reservoirs) based on the matrix of occurrence of different bacterial species. Some of the reservoirs were clustered together with the tap water, indicating that an important part of bacterial community in these reservoirs originated from the tap water (Fig. 7). However, this is not true for all sampled churches. Perhaps unexpectedly, we also did not observe clustering of samples from the reservoirs and fonts from the same church. Since the fonts in the sampled churches were made of stone, porcelain, glass, and metal, we tested whether there was a relationship between the font material and the presence of bacterial species. Although certain bacterial species were isolated only from non-metallic fonts, unfortunately no correlation was found between the material of the vessel (metal, stone, glass, porcelain) and the presence of certain bacterial species. The choice of the material of the vessel containing the holy water affects the growth of microorganisms through the smoothness of its surface. Rougher surfaces provide a better substrate for microbial attachment and biofilm formation. Microorganisms adhere more quickly to hydrophobic and nonpolar materials (plastic) than to hydrophilic materials (glass or metal) (Donlan 2002). To reduce contamination of the holy water, it would be better to use fonts made of glass or metal than fonts made of stone, for example.

Testing of the selected bacterial isolates for antibiotic resistance to nine antibiotics showed that the majority of the strains tested were sensitive to tetracycline (12.5 mg/l) and chloramphenicol (25 mg/l) and resistant to cefotaxime (2 mg/l) and colistin (3.5 mg/l) (Fig. 8). The use of colistin has increased recently mainly because of the emergence of multidrug-resistant Gram-negative bacteria. It is used as a last resort antibiotic against most *Enterobacterales* species and non-fermenting Gram-negative bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Conversely, colistin is not active against Gram-positive bacteria, Gram-negative cocci, and anaerobic bacteria (Torres et al. 2021). However, such a high level of resistance (63,9% of bacterial isolates) could be alarming. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimal inhibitory concentration (MIC) breakpoints for colistin (EUCAST 2022) for interpretation are for *Acinetobacter* spp. and *Enterobacterales* ≤ 2 mg l^{-1} susceptible (S), > 2 mg l^{-1} resistant (R), while for *Pseudomonas* spp. ≤ 4 mg l^{-1} susceptible, > 4 mg l^{-1} resistant. However, the Clinical and Laboratory Standards Institute (CLSI) recommends higher susceptibility breakpoints for *P. aeruginosa* ($S \leq 2$ mg l^{-1}, $R \geq 8$ mg l^{-1}) and for *Enterobacterales* and *Acinetobacter* spp. ($S \leq 2$ mg l^{-1}, $R \geq 4$ mg l^{-1}) (CLSI 2020). According to CLSI recommendations, the colistin concentration in the culture medium was too low to correctly assess susceptibility to colistin. The same may be

true for cefotaxime, where the EUCAST recommendations are for *Enterobacterales* and other non-species related ($S \leq 1$ mg l^{-1}, $R \geq 2$ mg l^{-1}) (EUCAST 2022), whereas the CLSI breakpoint recommendations are for aerobic bacteria ($S \leq 1$ mg l^{-1}, $R \geq 4$ mg l^{-1}) (Humphries et al. 2019). The observed high resistance in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* strains, both of which are most commonly associated with respiratory infections in humans, is somewhat expected and consistent with the literature, as these multidrug-resistant species are intrinsically resistant to a variety of antibiotics (Brooke 2012, Luczkiewicz et al. 2015).

According to our results, the risk of bacterial infection from holy water is modest, especially if only applied to unbroken skin. Many of the isolated bacteria from holy water may actually have been introduced by churchgoers. This is especially true for those bacteria that are considered part of skin microbiota and are not normally associated with aqueous environments. However, the unpredictability of contamination source of holy water means that the water may sporadically contain more problematic species than the ones identified in this study, or strains of the species found here, but with higher resistance to antibiotics. Therefore, sprinkling or other actions that can lead to inhalation or ingestion of the holy water or its introduction into eyes is not recommended, particularly for immunocompromised individuals, the elderly, neonates, and patients with severe burns, trauma, postoperative wounds, or intravenous access.

Conclusions

Holy water may pose a risk of infection with pathogenic microorganisms. Adding salt (NaCl) to holy water is an accepted practice in Catholic churches, but the concentrations required to limit most microbial growth (20% w/v or more) are often incompatible with the protection of fonts as part of the cultural heritage. Since the complete removal of water from fonts would likely be poorly accepted for religious reasons, the recommended practice to limit the transmission of potentially pathogenic microorganisms is regular and rigorous cleaning of the fonts, use of vessels with easy-to-clean surfaces and regular replacement, e.g., after several days during non-holiday period, and daily after attending Mass in churches during church holidays. The public, especially individuals with increased susceptibility for infection, can protect themselves by avoiding the contact of the holy water with eyes, nose, mouth, ears and broken skin, and by practising good hand hygiene.

Povzetek

V Sloveniji se je po zadnjem popisu prebivalstva iz leta 2002 za katoličane opredelilo 57,8 % prebivalcev (Črnič in sod. 2013). Tako kot v drugih krščanskih religijah je za katoličane blagoslovljena voda tista, ki jo je blagoslovil duhovnik in se uporablja pri svetem krstu ter za blagoslov ljudi, živali, cerkva, domov in predmetov (Jurado in sod. 2002, Kirschner in sod. 2012). Nekatere študije so blagoslovljeno vodo prepoznale kot potencialni vir okužbe s patogenimi organizmi (Rees in Allen 1996, Greaves in Porter 1992, Michel in sod. 2013, Gajurel in Deresinski 2021). Zaradi zaskrbljenosti zaradi COVID-19 so številne cerkve izpraznile kropilnike za blagoslovljeno vodo, v nekaterih cerkvah so celo namestili brezkontaktne razdelilnike blagoslovljene vode (Pullella 2020, Drogo 2022). V tej raziskavi smo preučevali mikrobiološko kakovost blagoslovljene vode, s poudarkom na bakterijah, v kropilnikih in rezervarjih desetih izbranih rimskokatoliških cerkva v Ljubljani in njeni okolici. Da bi ocenili potencialno tveganje za zdravje, smo bakterijske seve, izolirane iz blagoslovljene vode, identificirali in preučili njihovo odpornost proti izbranim antibiotikom.

Obremenitev blagoslovljene vode z aerobnimi bakterijami smo določili z metodo štetja na ploščah. V večini proučevanih cerkva je bilo število kolonijskih enot (CFU) na ml vzorca večje v blagoslovljeni vodi iz kropilnikov (10^2 - 10^5 CFU ml^{-1}; sl. 2) kot v rezervoarjih (10^1 - 10^3 CFU ml^{-1}; sl. 3), kar je pričakovano, saj imajo obiskovalci cerkve neposreden vsakodnevni stik s blagoslovljeno vodo iz kropilnikov. Obremenitev z bakterijami se je razlikovala med različnimi cerkvami in tudi med različnimi vzorčenji ene same cerkve. Ugotovljena relativno zmerna bakterijska kontaminacija blagoslovljene vode iz kropilnikov je

skladna s prejšnjimi študijami iz cerkva na Dunaju v Avstriji (Kirschner in sod. 2012) in na območju Villingen-Schwenningen v Nemčiji (König in sod. 2017), kjer so uporabili podobne pogoje gojenja (bogato gojišče, inkubacija pri 37 °C). Na Dunaju so imeli vsi vzorci blagoslovljene vode iz preiskovanih cerkva in bolnišničnih kapelic visoko število heterotrofnih mikrobov pri 37 °C, do 3×10^7 CFU ml^{-1}, več kot v naši študiji, medtem ko je bila na območju Villingen-Schwenningena (Nemčija) povprečna mikrobna obremenitev blagoslovljene vode z aerobi $5{,}85 \pm 3{,}98 \times 10^3$ CFU ml^{-1}. V študiji blagoslovljene vode iz kropilnikov cerkva v Sevilli v Španiji je bilo število aerobnih ter koliformnih bakterij v nekaterih cerkvah previsoko, da bi jih lahko prešteli (»too numerous too count«, TNTC) (Jurado in sod. 2002). Na obremenitev z bakterijami zagotovo vpliva število obiskovalcev ter pogostost čiščenja in menjave vode v kropilnikih (König in sod. 2017, Jurado in sod. 2002). Tovrstni podatki bi nam pomagali razumeti razlike med bakterijsko obremenitvijo blagoslovljene vode iz kropilnikov preučevanih cerkva v Ljubljani, vendar ti podatki niso bili na voljo. Kljub temu naši rezultati potrjujejo, da je pogosto pomakanje prstov vernikov v vodo kropilnika ob vstopu in izstopu iz cerkve verjetno glavni razlog za večjo bakterijsko obremenitev v kropilnikih v primerjavi z rezervoarji. Na ta način se mikrobi – in tudi hranila za njihovo rast – prenesejo s kože v blagoslovljeno vodo (Kirschner in sod. 2012).

Rimskokatoliška cerkev priporoča dodajanje blagoslovljene soli (NaCl) vodi ob blagoslovu (Kirschner in sod. 2012). Sol smo določili le v vzorcih blagoslovljene vode iz dveh cerkva, njena koncentracija pa je bila nizka in se je gibala od 0,2 do 1,5 % NaCl (m/v) (Tab.1). V večini cerkva je bila koncentracija natrija enakega reda velikosti kot v vodovodni vodi. V študiji iz Španije so merili sposobnost preživetja izbranih bakterijskih vrst kot funkcijo različnih koncentracij NaCl. Pri koncentraciji NaCl 20 % ali več naj bi tako patogene kot nepatogene bakterije lizirale (Jurado in sod. 2002). Do tega procesa, imenovanega plazmoliza, pride zaradi izhajanja vode, ki povzroči zmanjšanje volumna citoplazme. To lahko privede do umika celične stene, odcepitve in gubanja plazmaleme ter prekinitev proteinskih kompleksov, ki segajo čez celično steno (Wood 2011). V študiji Hrenovic in Ivankovic (2009), kjer so testirali preživetje *E. coli* in *Acinetobacter junii* pri različnih koncentracijah NaCl, je bilo popolno uničenje *E. coli* pri koncentraciji 20 % NaCl doseženo po 72 urah. Da bi preprečili kontaminacijo blagoslovljene vode, bi tako morali dodati večje količine NaCl, kot smo jih zaznali v naših vzorcih, saj bi le tako popolnoma zavrli rast večine mikroorganizmov. Visoke koncentracije NaCl pa lahko poškodujejo kropilnike, zato moramo uporabo NaCl skrbno uravnotežiti z varovanjem kulturne dediščine (Jurado in sod. 2002). Druga alternativa je, da blagoslovljeno vodo dnevno menjamo in redno čistimo kropilnike.

Iz 56 vzorcev blagoslovljene vode smo osamili 585 bakterijskih izolatov, ter jih identificirali na podlagi homolognih zaporedij gena za 16S rRNA v podatkovni zbirki GenBank. Združbo aerobnih gojljivih bakterij iz blagoslovljene vode so sestavljale predvsem bakterijske vrste iz razredov *Gammaproteobacteria* in *Actinomycetes* (slika 4). Sestava bakterijske združbe se je razlikovala med cerkvami, pa tudi med kropilnikom in rezervoarjem posamezne cerkve. Nekateri rodovi so bili prisotni samo v blagoslovljeni vodi iz kropilnikov (*Delftia*, *Kocuria*, *Sphingobacterium*, *Staphylococcus*), v blagoslovljeni vodi iz kropilnikov, rezervoarjev in vodovodne vode pa smo identificirali vrste le dveh rodov (*Acinetobacter*, *Pseudomonas*) (sl. 5). Verjetno so nekatere od identificiranih bakterij iz kropilnikov izvirale s kože obiskovalcev cerkve. V mikrobioti kože človeških rok, ki so jo preučevali s sekvenciranjem pomnožkov, so bile najdene vrste iz bakterijskih debel *Pseudomonadota*, *Actinomycetota* in *Bacillota*, ki so predstavljale 94 % vseh identificiranih bakterijskih vrst, z najbolj razširjenimi rodovi *Cutibacterium* (31,6 % vseh zaporedij; *Actinomycetota*), *Streptococcus* (17,2 %; *Bacillota*), *Staphylococcus* (8,3 %; *Bacillota*), *Corynebacterium* (4,3 %; *Actinomycetota*) in *Lactobacillus* (3,1 %; *Bacillota*) (Egert in Simmering 2016, Byrd in sod. 2018, Carmona-Cruz in sod. 2022). V skladu s predhodnimi študijami (Rees in Allen 1996, Jurado in sod. 2002, Kirschner in sod. 2012, König in sod. 2017) smo odkrili tudi bakterije fekalnega izvora, to so enterokoki in enterobakterije, čeprav v zelo nizkem številu. Tako je bil predstavnik fekalnih indikatorskih bakterij *Enterococcus* sp. najden le

v enem vzorcu blagoslovljene vode iz rezervoarja, *Citrobacter freundii* in *Enterobacter cloacae* pa v dveh vzorcih vode iz kropilnikov.

Z uporabo metod strojnega učenja smo želeli raziskati pojavljanje bakterijskih vrst kot funkcijo lokacije vzorčenja (kropilnik ali rezervoar). Nekatere bakterijske vrste so se pojavljale le v blagoslovljeni vodi iz kropilnikov (*Acinetobacter beijerinckii*, *Acinetobacter haemolyticus*, *Brevundimonas aurantiaca*, *Brevundimonas mediterranea*, *Staphylococcus warneri*), medtem ko so bile druge, kot je *Microbacterium maritypicum*, bolj pogoste v rezervoarjih blagoslovljene vode (sl. 6). Večina teh bakterij ima razmeroma nizek patogeni potencial in nekatere od njih veljajo za kožne komensale. Rod *Acinetobacter* (*Pseudomonadota*) se običajno nahaja v vodnem okolju, pri čemer je večina vrst nepatogenih. Najpogostejša vrsta, ki povzroča okužbe, je *A. baumannii*, sledijo pa ji vrste, ki jih najdemo tudi na človeški koži, kot sta *A. calcoaceticus* in *A. lwoffii*, ki je bil prav tako izoliran iz naših vzorcev blagoslovljene vode. To so večinoma oportunistični patogeni, ki povzročajo okužbe, zlasti pri bolnikih z oslabljenim imunskim sistemom. Druge vrste, vključno z *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. nosocomialis*, *A. pittii*, *A. schindleri* in *A. ursingii*, naj bi le redko povzročale okužbe (Wong in sod. 2017). *Brevundimonas* spp. (*Pseudomonadota*) so rod nefermentirajočih gramnegativnih bakterij in so, zlasti *Brevundimonas diminuta* in *Brevundimonas vesicularis,* le malo klinično pomembne (Ryan in Pembroke 2018). Vrste rodu *Microbacterium* (*Actinomycetota*), ki so grampozitivne paličice in ne tvorijo spor, so prav tako redko povezovali z okužbami pri ljudeh. Vse več raziskav pa kaže, da so vrste rodu *Microbacterium* oportunistični človeški patogeni, ki lahko na primer povzročijo infektivni endokarditis, povezan z bakteriemijo, povzročeno z *Microbacterium maritypicum* (Yeung in sod. 2020). Po drugi strani pa *Staphylococcus warneri*, koagulazno negativni stafilokok (CNS), ki ga pogosto najdemo v mikrobioti človeških in živalskih epitelijev in sluznic, velja za oportunističnega patogena, ki povzroča resne okužbe pri ljudeh in živalih (Liu in sod. 2020).

Hierarhično združevanje smo uporabili za ugotavljanje podobnosti med mesti vzorčenja (kropilniki in rezervoarji) na podlagi matrike pojavljanja različnih bakterijskih vrst. Nekateri rezervoarji so se združevali skupaj z vodovodno vodo, kar kaže, da pomemben del bakterijske združbe v teh rezervoarjih izvira iz vodovodne vode (sl. 7). Vendar to ne velja za vse vzorčene cerkve. Morda nepričakovano tudi nismo opazili združevanja vzorcev iz rezervoarjev in kropilnikov posamezne cerkve. Ker so bili kropilniki v preučevanih cerkvah izdelani iz kamna, porcelana, stekla in kovine, smo ugotavljali, ali obstaja povezava med materialom kropilnika in prisotnostjo posameznih bakterijskih vrst. Čeprav so bile določene bakterijske vrste izolirane samo iz nekovinskih kropilnikov, žal nismo ugotovili povezave med materialom posode (kovina, kamen, steklo, porcelan) in prisotnostjo določenih bakterijskih vrst. Material posode z blagoslovljeno vodo vpliva na rast mikroorganizmov z gladkostjo površine. Bolj grobe površine zagotavljajo boljšo podlago za pritrditev mikrobov in tvorbo biofilma. Mikroorganizmi se hitreje prilepijo na hidrofobne in nepolarne materiale (plastika) kot na hidrofilne materiale (steklo ali kovina) (Donlan 2002). Da bi zmanjšali onesnaženje blagoslovljene vode, bi bilo bolje uporabiti kropilnike na primer iz stekla ali kovine kot iz kamna.

Protimikrobno testiranje izbranih bakterijskih izolatov proti devetim antibiotikom je pokazalo, da je večina testiranih sevov občutljivih proti tetraciklinu (12,5 mg/l) in kloramfenikolu (25 mg/l) ter odpornih proti cefotaksimu (2 mg/l) in kolistinu (3,5 mg/l) (sl. 8). Uporaba kolistina se je v zadnjem času povečala predvsem zaradi pojava gramnegativnih bakterij, odpornih proti več antibiotikom. Uporablja se kot antibiotik zadnje obrambne linije proti večini vrst reda *Enterobacterales* in nefermentirajočih gramnegativnih bakterij, kot sta *Acinetobacter baumannii* in *Pseudomonas aeruginosa*. Nasprotno pa kolistin ne deluje proti grampozitivnim bakterijam, gramnegativnim kokom in anaerobnim bakterijam (Torres in sod. 2021). Zato bi lahko bila tako visoka stopnja odpornosti (63,9 % bakterijskih izolatov) zaskrbljujoča. Smernice mejnih vrednosti minimalne inhibitorne koncentracije (MIC) za kolistin (EUCAST 2022) po EUCAST (European Committee on Antimicrobial Susceptibility Testing) so za *Acinetobacter* spp. in *Enterobacterales* $\leq$ 2 mg l^{-1} občutljiv (S), > 2 mg l^{-1} odporen (R),

medtem ko za *Pseudomonas* spp. velja ≤ 4 mg l^{-1} občutljiv, > 4 mg l^{-1} odporen. Vendar Inštitut za klinične in laboratorijske standarde (CLSI) priporoča višje mejne vrednosti občutljivosti za *P. aeruginosa* (S ≤ 2 mg l^{-1}, R ≥ 8 mg l^{-1}) ter za *Enterobacterales* in *Acinetobacter* spp. (S ≤ 2 mg l^{-1}, R ≥ 4 mg l^{-1}) (CLSI 2020). Glede na smernice CLSI je bila koncentracija kolistina v gojišču prenizka za pravilno oceno občutljivosti proti kolistinu. Enako bi lahko veljalo za cefotaksim, kjer so smernice EUCAST za *Enterobacterales* in druge nesorodne vrste (S ≤ 1 mg l^{-1}, R ≥ 2 mg l^{-1}) (EUCAST 2022), medtem ko so smernice CLSI za mejne vrednosti za aerobne bakterije (S ≤ 1 mg l^{-1}, R ≥ 4 mg l^{-1}) (Humphries in sod. 2019). Opažena visoka odpornost sevov vrst *Pseudomonas aeruginosa* in *Stenotrophomonas maltophilia*, ki sta najpogosteje povezani z okužbami dihal pri ljudeh, je pričakovana in skladna z objavami, saj sta ti vrsti intrinzično odporni proti več antibiotikom (Brooke 2012, Luczkiewicz in sod. 2015).

Glede na naše rezultate je tveganje za bakterijsko okužbo z blagoslovljeno vodo majhno, še posebej, če se jo nanaša samo na nepoškodovano kožo. Precej izoliranih bakterij iz blagoslovljene vode so najbrž vanjo vnesli obiskovalci cerkve. To še posebej velja za tiste bakterije, ki so prepoznane kot del mikrobiote kože in običajno niso povezane z vodnim okoljem. Vendar pa nepredvidljivost vira kontaminacije blagoslovljene vode pomeni, da lahko voda občasno vsebuje več problematičnih vrst od tistih, opredeljenih v tej študiji, ali sevov vrst, ki jih najdemo tukaj, vendar z večjo odpornostjo proti antibiotikom. Zato načini uporabe blagoslovljene vode, ki bi lahko vodili do vdihavanja, zaužitja vode ali njenega vnosa v oči ali druga tkiva, niso priporočljivi, zlasti za imunsko oslabljene posameznike, starejše, novorojenčke in bolnike s hudimi opeklinami, poškodbami, pooperativnimi ranami ali z vzpostavljeno periferno vensko potjo.

Blagoslovljena voda tako lahko predstavlja nevarnost okužbe s patogenimi mikroorganizmi. Ker bi bila popolna odstranitev vode iz kropilnikov verjetno slabo sprejeta zaradi verskih razlogov, je priporočena praksa za omejitev prenosa potencialno patogenih mikroorganizmov redno in temeljito čiščenje kropilnikov, uporaba posod s površinami, ki jih je enostavno čistiti, in redna oziroma vsakodnevna zamenjava vode, zlasti po povečanem obisku cerkva (npr. ob cerkvenih praznikih). Javnost, zlasti posamezniki s povečano dovzetnostjo za okužbe, se lahko zaščitijo tako, da se izogibajo stiku blagoslovljene vode z očmi, nosom, usti, ušesi in poškodovano kožo ter z vzdrževanjem dobre higiene rok.

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Supplementary material

Table S1: List of identified bacterial isolates from holy water of fonts and reservoirs.

Table S2: List of the isolates selected for the antimicrobial susceptibility testing with the corresponding antimicrobial profile.

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Supplementary information

Table S1: List of identified bacterial isolates from holy water of fonts and reservoirs.
Risk group determined according to TRBA 466 Classification of Prokaryotes (Bacteria and Archaea) into Risk Groups (Federal Institute for Occupational Safety and Health. https://www.baua.de/EN/Service/Legislative-texts-and-technical-rules/Rules/TRBA/TRBA-466.html).

Tabela S1: Seznam identificiranih bakterijskih izolatov iz blagoslovljene vode kropilnikov in rezervoarjev.

Mycosmo culture collection no. (exb)	Genbank	Identity	Risk group	Phylum, class, order, family	Sample	Medium and growth temperature
L-5025	*Kocuria salsicia*	99.72	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_1	blood agar, 37 °C
L-5026	*Brevundimonas aurantiaca*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_1	blood agar, 37 °C
L-5027	*Brevundimonas vesicularis/ Brevundimonas nasdae*	99.9	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_1	blood agar, 37 °C
L-5028	*Staphylococcus lugdunensis*	100	2	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	FC_1	blood agar, 37 °C
L-5029	*Kocuria arsenatis/ Kocuria rhizophila*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_1	blood agar, 37 °C
L-5030	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_1	blood agar, 37 °C
L-5031	*Brevundimonas vesicularis/ Brevundimonas nasdae*	99.9	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_1	UriSelect4 agar, 37 °C
L-5032	*Brevundimonas aurantiaca*	99.91	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_1	UriSelect4 agar, 37 °C
L-5033	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	blood agar, 37 °C
L-5034	*Ralstonia pickettii*	99.7	2	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	FC_R1	blood agar, 37 °C
L-5035	*Pseudomonas alcaliphila/ Pseudomonas oleovorans*	99.89	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	blood agar, 37 °C

L-5036	*Pseudomonas chloritidismutans/ Pseudomonas knackmussii*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5037	*Ralstonia pickettii*	99.82	2	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5038	*Microbacterium invictum*	98.42	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R1	blood agar, 37 °C
L-5039	*Pseudomonas chloritidismutans/ Pseudomonas knackmussii*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	blood agar, 37 °C
L-5040	*Brevibacterium sanguinis*	100	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R1	blood agar, 37 °C
L-5041	*Ralstonia pickettii*	99.79	2	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	FC_R1	blood agar, 37 °C
L-5042	*Microbacterium maritypicum*	99.82	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R1	blood agar, 37 °C
L-5043	*Microbacterium maritypicum*	99.8	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5044	*Pseudomonas chloritidismutans/ Pseudomonas knackmussii*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5045	*Pseudomonas chengduensis*	99.82	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5046	*Pseudomonas chengduensis*	99.89	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5047	*Microbacterium invictum*	98.35	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5048	*Pseudomonas chengduensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R1	UriSelect4 agar, 37 °C
L-5049	*Rothia kristinae*	99.56	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	FC_2	blood agar, 37 °C
L-5050	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_2	blood agar, 37 °C
L-5051	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_2	UriSelect4 agar, 37 °C

L-5052	*Sphingomonas hankookensis*	99.41	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_2	UriSelect4 agar, 37 °C
L-5053	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	FC_2	UriSelect4 agar, 37 °C
L-5054	*Actinomyces haliotis*	99.97	1	*Actinomycetota, Actinomycetes, Micrococcales, Actinomycetaceae*	FC_2	UriSelect4 agar, 37 °C
L-5055	*Rothia amarae*	97.88	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_2	UriSelect4 agar, 37 °C
L-5056	*Rothia terrae*	98.96	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_2	blood agar, 37 °C
L-5057	*Staphylococcus haemolyticus*	99.82	2	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	FC_2	blood agar, 37 °C
L-5058	*Rothia kristinae*	99.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_2	blood agar, 37 °C
L-5059	*Brevundimonas aurantiaca*	99.9	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_2	blood agar, 37 °C
L-5060	*Brevundimonas aurantiaca*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_2	UriSelect4 agar, 37 °C
L-5061	*Staphylococcus haemolyticus*	99.91	2	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	FC_2	UriSelect4 agar, 37 °C
L-5062	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_2	UriSelect4 agar, 37 °C
L-5063	*Rothia kristinae*	99.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_2	UriSelect4 agar, 37 °C
L-5064	*Rothia kristinae*	99.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	FC_2	UriSelect4 agar, 37 °C
L-5067	*Microbacterium testaceum*	99.05	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R2	blood agar, 37 °C
L-5068	*Brevibacterium sanguinis*	99.72	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R2	blood agar, 37 °C
L-5069	*Brevibacterium sanguinis*	99.72	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5070	*Brevundimonas vesicularis/ Brevundimonas nasdae*	99.79	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	FC_R2	blood agar, 37 °C
L-5071	*Chryseobacterium shandongense*	99.91	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5072	*Brevibacterium casei*	99.22	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R2	UriSelect4 agar, 37 °C

L-5073	*Sphingobacterium daejeonense*	99.63	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5074	*Pseudomonas rhodesiae*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5075	*Pseudomonas chloritidismutans/ Pseudomonas knackmussii*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5076	*Stenotrophomonas maltophilia*	99.35	2	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	FC_R2	UriSelect4 agar, 37 °C
L-5077	*Microbacterium saccharophilum*	99.14	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5078	*Pseudomonas chengduensis*	99.8	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5079	*Pseudomonas chloritidismutans/ Pseudomonas knackmussii*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R2	UriSelect4 agar, 37 °C
L-5080	*Acinetobacter haemolyticus*	99.2	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_1	blood agar, 37 °C
L-5081	*Brevundimonas mediterranea*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_1	blood agar, 37 °C
L-5082	*Acinetobacter johnsonii*	99.61	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_1	blood agar, 37 °C
L-5083	*Brevundimonas vesicularis/ Brevundimonas nasdae*	99.61	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_1	blood agar, 37 °C
L-5084	*Sphingomonas hankookensis*	99.42	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_1	blood agar, 37 °C
L-5085	*Sphingomonas hankookensis*	99.42	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_1	blood agar, 37 °C
L-5086	*Sphingomonas hankookensis*	99.42	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_1	blood agar, 37 °C

L-5087	*Sphingomonas hankookensis*	99.42	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_1	blood agar, 37 °C
L-5089	*Sphingomonas hankookensis*	98.87	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_1	UriSelect4 agar, 37 °C
L-5090	*Staphylococcus vitulinus*	99.91	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	SC_1	UriSelect4 agar, 37 °C
L-5091	*Acinetobacter haemolyticus*	99.14	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_1	UriSelect4 agar, 37 °C
L-5092	*Sphingomonas hankookensis*	99.51	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_1	UriSelect4 agar, 37 °C
L-5093	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_1	UriSelect4 agar, 37 °C
L-5094	*Acinetobacter johnsonii*	99.57	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_1	UriSelect4 agar, 37 °C
L-5095	*Staphylococcus vitulinus*	99.9	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	SC_1	UriSelect4 agar, 37 °C
L-5097	*Pseudomonas peli*	99.63	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SC_R1	blood agar, 37 °C
L-5098	*Bacillus drentensis/ Bacillus infantis*	99.7	1	*Bacillota, Bacilli, Bacillales, Bacillaceae*	UC_1	blood agar, 37 °C
L-5099	*Microbacterium testaceum*	99.12	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_1	blood agar, 37 °C
L-5100	*Aquincola tertiaricarbonis*	98.25	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_1	blood agar, 37 °C
L-5101	*Aquincola tertiaricarbonis*	98.4	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_1	blood agar, 37 °C
L-5102	*Microbacterium testaceum*	99.15	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_1	UriSelect4 agar, 37 °C
L-5103	*Aquincola tertiaricarbonis*	98.18	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_1	UriSelect4 agar, 37 °C

L-5104	*Aquincola tertiaricarbonis*	98.24	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_R1	blood agar, 37 °C
L-5105	*Microbacterium lacus*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R1	blood agar, 37 °C
L-5107	*Aquincola tertiaricarbonis*	98.29	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_R1	UriSelect4 agar, 37 °C
L-5108	*Enterococcus ureilyticus*	99.9	1	*Bacillota, Bacilli, Lactobacillales, Enterococcaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5109	*Cellulosimicrobium funkei*	99.72	1	*Actinomycetota, Actinomycetes, Micrococcales, Promicromonosporaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5110	*Microbacterium maritypicum*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5111	*Microbacterium saccharophilum*	98.28	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5112	*Aquincola tertiaricarbonis*	98.38	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_R1	UriSelect4 agar, 37 °C
L-5113	*Microbacterium maritypicum*	99.8	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5114	*Kocuria carniphila*	99.72	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5115	*Acinetobacter johnsonii*	99.78	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5116	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5117	*Pseudomonas koreensis*	99.65	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5120	*Janibacter indicus*	99.09	1	*Actinomycetota, Actinomycetes, Micrococcales, Intrasporangiaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5121	*Rothia amarae*	98.04	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	UC_R1	UriSelect4 agar, 37 °C
L-5122	*Acinetobacter beijerinckii*	98.93	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	blood agar, 37 °C
L-5123	*Acinetobacter johnsonii*	99.9	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	blood agar, 37 °C

L-5124	*Acinetobacter johnsonii*	98.76	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	blood agar, 37 °C
L-5125	*Acinetobacter johnsonii*	99	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	blood agar, 37 °C
L-5126	*Acinetobacter johnsonii*	99.9	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	UriSelect4 agar, 37 °C
L-5127	*Chryseobacterium hispalense*	99.79	1	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	VC_1	UriSelect4 agar, 37 °C
L-5128	*Microbacterium maritypicum*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_1	UriSelect4 agar, 37 °C
L-5129	*Kocuria carniphila*	99.71	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	VC_1	UriSelect4 agar, 37 °C
L-5130	*Pseudomonas koreensis*	99.62	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	VC_1	UriSelect4 agar, 37 °C
L-5131	*Acinetobacter johnsonii*	99.89	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	UriSelect4 agar, 37 °C
L-5132	*Pseudomonas rhodesiae*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	VC_1	UriSelect4 agar, 37 °C
L-5133	*Acinetobacter johnsonii*	99.89	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_1	UriSelect4 agar, 37 °C
L-5134	*Barrientosiimonas humi*	99.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Dermacoccaceae*	VC_R1	blood agar, 37 °C
L-5136	*Novosphingobium aquaticum/ Novosphingobium subterraneum/ Novosphingobium lentum*	97.82	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae*	VC_R1	blood agar, 37 °C
L-5137	*Ponticoccus gilvus*	100	1	*Pseudomonadota, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae*	VC_R1	blood agar, 37 °C
L-5139	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R1	blood agar, 37 °C
L-5140	*Bacillus aerius*	99.68	1	*Bacillota, Bacilli, Bacillales, Bacillaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5141	*Microbacterium chocolatum*	98.6	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5142	*Microbacterium chocolatum*	98.6	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R1	UriSelect4 agar, 37 °C

L-5143	*Acinetobacter johnsonii*	98.82	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5144	*Cellulosimicrobium funkei*	99.53	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5145	*Microbacterium maritypicum*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5146	*Brevundimonas vesicularis/ Brevundimonas nasdae*	99.9	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	VC_R1	UriSelect4 agar, 37 °C
L-5147	*Rothia amarae*	99.89	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5149	*Naumannella halotolerans*	100	1	*Actinomycetota, Actinomycetes, Propionibacteriales, Propionibacteriaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5150	*Sphingomonas panaciterrae*	99.8	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	VC_R1	UriSelect4 agar, 37 °C
L-5151	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_1	blood agar, 37 °C
L-5153	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_1	UriSelect4 agar, 37 °C
L-5154	*Pseudomonas rhodesiae*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_1	UriSelect4 agar, 37 °C
L-5155	*Staphylococcus epidermidis*	99.89	2	*Firmicutes, Bacilli, Bacillales, Staphylococcaceae*	TC_1	UriSelect4 agar, 37 °C
L-5156	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_1	UriSelect4 agar, 37 °C
L-5157	*Acinetobacter johnsonii*	99.6	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_1	blood agar, 37 °C
L-5158	*Acinetobacter johnsonii*	98.98	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_1	blood agar, 37 °C
L-5159	*Microbacterium testaceum*	99.04	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_1	blood agar, 37 °C
L-5160	*Brevundimonas aurantiaca*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	KC_1	blood agar, 37 °C

L-5161	*Acidovorax facilis*	99.44	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_1	blood agar, 37 °C
L-5162	*Chryseobacterium shandongense*	99.36	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	KC_1	blood agar, 37 °C
L-5163	*Acinetobacter beijerinckii*	99.41	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_1	blood agar, 37 °C
L-5164	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_1	UriSelect4 agar, 37 °C
L-5165	*Acinetobacter johnsonii*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_1	UriSelect4 agar, 37 °C
L-5166	*Acinetobacter johnsonii*	99.32	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_1	UriSelect4 agar, 37 °C
L-5167	*Microbacterium testaceum*	98.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_1	UriSelect4 agar, 37 °C
L-5168	*Brevundimonas aurantiaca*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	KC_1	UriSelect4 agar, 37 °C
L-5169	*Acidovorax facilis*	99.42	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_1	UriSelect4 agar, 37 °C
L-5170	*Kocuria uropygioeca/ Kocuria uropygialis*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	KC_1	UriSelect4 agar, 37 °C
L-5171	*Acinetobacter beijerinckii*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	BC_1	blood agar, 37 °C
L-5173	*Acinetobacter beijerinckii*	99.61	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	BC_1	UriSelect4 agar, 37 °C
L-5174	*Sphingobium hydrophobicum*	99.5	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	BC_1	UriSelect4 agar, 37 °C
L-5177	*Sphingomonas paucimobilis*	100	2	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	BC_R1	UriSelect4 agar, 37 °C
L-5178	*Microbacterium testaceum*	99.13	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_1	blood agar, 37 °C
L-5179	*Brevundimonas mediterranea*	99.72	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	DC_1	blood agar, 37 °C

L-5180	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_1	blood agar, 37 °C
L-5181	*Kocuria uropygioeca*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	DC_1	blood agar, 37 °C
L-5182	*Brevundimonas vesicularis/ Brevundimonas nasdae*	97.7	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	DC_1	blood agar, 37 °C
L-5183	*Acinetobacter johnsonii*	99.73	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	DC_1	UriSelect4 agar, 37 °C
L-5184	*Microbacterium testaceum*	98.24	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_1	UriSelect4 agar, 37 °C
L-5185	*Microbacterium testaceum*	99.15	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_1	UriSelect4 agar, 37 °C
L-5186	*Microbacterium testaceum*	99.1	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_1	UriSelect4 agar, 37 °C
L-5187	*Stenotrophomonas rhizophila*	99.61	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	DC_R1	blood agar, 37 °C
L-5188	*Sphingobium hydrophobicum*	99.9	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_R1	blood agar, 37 °C
L-5189	*Stenotrophomonas chelatiphaga*	99.55	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	DC_R1	blood agar, 37 °C
L-5190	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R1	UriSelect4 agar, 37 °C
L-5191	*Sphingobium hydrophobicum*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_R1	UriSelect4 agar, 37 °C
L-5192	*Microbacterium testaceum*	99.08	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_R1	UriSelect4 agar, 37 °C
L-5193	*Stenotrophomonas rhizophila*	99.61	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	DC_R1	UriSelect4 agar, 37 °C
L-5194	*Stenotrophomonas rhizophila*	99.67	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	DC_R1	UriSelect4 agar, 37 °C
L-5195	*Microbacterium testaceum*	99.17	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_R1	UriSelect4 agar, 37 °C

L-5196	*Acidovorax facilis*	99.34	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R1	UriSelect4 agar, 37 °C
L-5197	*Sphingobium hydrophobicum*	99.81	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_1	blood agar, 37 °C
L-5198	*Sphingobium hydrophobicum*	99.9	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_1	blood agar, 37 °C
L-5200	*Sphingobium hydrophobicum*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_1	UriSelect4 agar, 37 °C
L-5201	*Chryseobacterium sediminis*	98.17	1	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	SI_1	blood agar, 37 °C
L-5202	*Rothia aeria*	99.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	SI_1	blood agar, 37 °C
L-5203	*Staphylococcus warneri*	99.91	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	SI_1	UriSelect4 agar, 37 °C
L-5204	*Rothia amarae*	97.99	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	SI_1	UriSelect4 agar, 37 °C
L-5205	*Microbacterium paraoxydans*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_1	UriSelect4 agar, 37 °C
L-5207	*Staphylococcus haemolyticus*	99.91	2	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	SI_1	UriSelect4 agar, 37 °C
L-5208	*Pseudomonas koreensis*	99.63	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R1	blood agar, 37 °C
L-5209	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R1	blood agar, 37 °C
L-5210	*Aerococcus urinaeequi*	99.91	1	*Bacillota, Bacilli, Bacillales, Aerococcaceae*	SI_R1	blood agar, 37 °C
L-5211	*Sphingomonas olei/ Sphingomonas panaciterrae*	100	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_R1	blood agar, 37 °C
L-5212	*Pseudomonas koreensis*	99.54	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R1	UriSelect4 agar, 37 °C
L-5213	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R1	UriSelect4 agar, 37 °C
L-5214	*Brachybacterium paraconglomeratum*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Dermabacteraceae*	SI_R1	UriSelect4 agar, 37 °C

L-5215	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R1	UriSelect4 agar, 37 °C
L-5216	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R1	UriSelect4 agar, 37 °C
L-5217	*Acinetobacter johnsonii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_1	blood agar, 37 °C
L-5218	*Pseudomonas koreensis*	99.9	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_1	blood agar, 37 °C
L-5220	*Acinetobacter johnsonii*	99.13	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_1	blood agar, 37 °C
L-5221	*Rothia amarae*	98.04	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	RC_1	blood agar, 37 °C
L-5222	*Acinetobacter haemolyticus*	99.86	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_1	blood agar, 37 °C
L-5223	*Microbacterium lacus*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_1	blood agar, 37 °C
L-5224	*Pseudomonas koreensis*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_1	UriSelect4 agar, 37 °C
L-5225	*Acinetobacter johnsonii*	99.42	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_1	UriSelect4 agar, 37 °C
L-5226	*Brevundimonas diminuta*	99.53	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	RC_1	UriSelect4 agar, 37 °C
L-5227	*Delftia lacustris*	100	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_1	UriSelect4 agar, 37 °C
L-5228	*Acinetobacter johnsonii*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_1	UriSelect4 agar, 37 °C
L-5229	*Stenotrophomonas bentonitica*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	RC_1	UriSelect4 agar, 37 °C
L-5230	*Pseudomonas koreensis*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_1	UriSelect4 agar, 37 °C
L-5231	*Pseudomonas helmanticensis*	99.82	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R1	blood agar, 37 °C

L-5232	*Acinetobacter johnsonii*	99.73	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R1	blood agar, 37 °C
L-5234	*Microbacterium paraoxydans*	99.82	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R1	blood agar, 37 °C
L-5235	*Pseudomonas turukhanskensis*	99.62	-	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R1	blood agar, 37 °C
L-5236	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R1	blood agar, 37 °C
L-5238	*Sphingomonas panaciterrae*	99.9	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	RC_R1	blood agar, 37 °C
L-5239	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R1	blood agar, 37 °C
L-5240	*Pseudomonas turukhanskensis*	99.62	-	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R1	blood agar, 37 °C
L-5241	*Tsukamurella pulmonis*	100	2	*Actinomycetota, Actinomycetes, Mycobacteriales, Tsukamurellaceae*	RC_R1	blood agar, 37 °C
L-5242	*Pseudomonas putida*	99.2	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R1	blood agar, 37 °C
L-5243	*Pseudomonas chloritidismutans*	99.73	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R1	blood agar, 37 °C
L-5244	*Acinetobacter johnsonii*	99.72	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5245	*Acinetobacter johnsonii*	99.72	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5246	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5247	*Acinetobacter johnsonii*	99.81	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5248	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	RC_R1	UriSelect4 agar, 37 °C

L-5249	*Pseudomonas koreensis*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5250	*Stenotrophomonas maltophilia*	99.54	2	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	RC_R1	UriSelect4 agar, 37 °C
L-5251	*Sphingomonas olei*	99.81	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5252	*Acinetobacter johnsonii*	99.6	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5253	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	RC_R1	UriSelect4 agar, 37 °C
L-5254	*Brachybacterium conglomeratum*	99.8	1	*Actinomycetota, Actinomycetes, Actinomycetales, Dermabacteraceae*	RC_R1	UriSelect4 agar, 37 °C
L-5255	*Microbacterium paraoxydans*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R1	UriSelect4 agar, 37 °C
L-5256	*Acinetobacter haemolyticus*	99.11	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_2	blood agar, 37 °C
L-5257	*Acinetobacter johnsonii*	99.6	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_2	blood agar, 37 °C
L-5258	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_2	blood agar, 37 °C
L-5259	*Stenotrophomonas maltophilia/ Pseudomonas hibiscicola*	98.96	2	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	SC_2	UriSelect4 agar, 37 °C
L-5260	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_2	blood agar, 37 °C
L-5261	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_2	blood agar, 37 °C
L-5262	*Brevundimonas aurantiaca*	99.91	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_2	blood agar, 37 °C
L-5263	*Curtobacterium oceanosedimentum*	99.63	-	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SC_2	UriSelect4 agar, 37 °C
L-5264	*Asticcacaulis excentricus*	99.62	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_2	UriSelect4 agar, 37 °C

L-5265	*Acinetobacter haemolyticus*	99.26	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_2	UriSelect4 agar, 37 °C
L-5266	*Brevundimonas aurantiaca*	99.9	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_2	UriSelect4 agar, 37 °C
L-5267	*Sphingomonas hankookensis*	99.13	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	SC_2	UriSelect4 agar, 37 °C
L-5268	*Pantoea dispersa*	99.17	1	*Pseudomonadota, Gammaproteobacteria, Enterobacteriales, Erwiniaceae*	SC_R2	blood agar, 37 °C
L-5269	*Stenotrophomonas pavanii/ Stenotrophomonas maltophilia/ Pseudomonas geniculata*	99.79	2	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	SC_R2	blood agar, 37 °C
L-5270	*Curtobacterium oceanosedimentum*	99.73	-	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SC_R2	blood agar, 37 °C
L-5271	*Curtobacterium oceanosedimentum*	99.81	-	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SC_R2	blood agar, 37 °C
L-5272	*Cellulomonas pakistanensis*	99.89	1	*Actinomycetota, Actinomycetes, Actinomycetales, Cellulomonadaceae*	SC_R2	blood agar, 37 °C
L-5273	*Pantoea dispersa*	99.19	1	*Pseudomonadota, Gammaproteobacteria, Enterobacteriales, Erwiniaceae*	SC_R2	UriSelect4 agar, 37 °C
L-5274	*Pantoea dispersa*	99.02	1	*Pseudomonadota, Gammaproteobacteria, Enterobacteriales, Erwiniaceae*	SC_R2	UriSelect4 agar, 37 °C
L-5275	*Pseudomonas oryzihabitans*	99.54	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SC_R2	UriSelect4 agar, 37 °C
L-5276	*Curtobacterium citreum*	99.43	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SC_R2	UriSelect4 agar, 37 °C
L-5278	*Microbacterium foliorum*	99.44	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SC_R2	UriSelect4 agar, 37 °C
L-5279	*Acinetobacter haemolyticus*	99.17	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	blood agar, 37 °C
L-5280	*Delftia acidovorans*	99.91	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	FC_3	blood agar, 37 °C
L-5281	*Delftia acidovorans*	99.91	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	FC_3	blood agar, 37 °C

L-5282	*Acinetobacter haemolyticus*	99.23	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	blood agar, 37 °C
L-5283	*Acinetobacter johnsonii*	99.54	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	blood agar, 37 °C
L-5284	*Acinetobacter haemolyticus*	99.25	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	UriSelect4 agar, 37 °C
L-5285	*Acinetobacter johnsonii*	99.72	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	UriSelect4 agar, 37 °C
L-5286	*Acinetobacter johnsonii*	99.82	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	UriSelect4 agar, 37 °C
L-5288	*Microbacterium testaceum*	98.99	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	blood agar, 37 °C
L-5289	*Microbacterium zeae/ Microbacterium proteolyticum*	98.48	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	blood agar, 37 °C
L-5290	*Microbacterium zeae/ Microbacterium proteolyticum*	98.42	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	blood agar, 37 °C
L-5291	*Brevibacterium sanguinis*	99.9	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R3	blood agar, 37 °C
L-5292	*Pseudomonas aeruginosa*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	blood agar, 37 °C
L-5293	*Pseudomonas chloritidismutans*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	blood agar, 37 °C
L-5294	*Microbacterium zeae/ Microbacterium proteolyticum*	98.42	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	blood agar, 37 °C
L-5295	*Sphingomonas koreensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5296	*Acinetobacter johnsonii*	99.9	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5297	*Brevibacterium sanguinis*	99.72	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5298	*Microbacterium zeae/ Microbacterium proteolyticum*	98.61	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	UriSelect4 agar, 37 °C

L-5299	*Aquincola tertiaricarbonis*	98.23	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	blood agar, 37 °C
L-5300	*Aquincola tertiaricarbonis*	98.23	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	blood agar, 37 °C
L-5301	*Microbacterium lacus*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_2	blood agar, 37 °C
L-5302	*Aquincola tertiaricarbonis*	98.27	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	UriSelect4 agar, 37 °C
L-5303	*Aquincola tertiaricarbonis*	98.22	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	UriSelect4 agar, 37 °C
L-5304	*Aquincola tertiaricarbonis*	98.23	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	blood agar, 37 °C
L-5305	*Acinetobacter johnsonii*	99.81	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_2	blood agar, 37 °C
L-5306	*Limnobacter thiooxidans*	99.53	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	UC_2	blood agar, 37 °C
L-5307	*Aquincola tertiaricarbonis*	98.19	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	blood agar, 37 °C
L-5308	*Aquincola tertiaricarbonis*	98.25	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_2	UriSelect4 agar, 37 °C
L-5313	*Aquincola tertiaricarbonis*	98.21	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_R2	blood agar, 37 °C
L-5314	*Microbacterium paraoxydans*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R2	blood agar, 37 °C
L-5317	*Aquincola tertiaricarbonis*	98.21	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_R2	blood agar, 37 °C

L-5318	*Aquincola tertiaricarbonis*	98.1	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	UC_R2	UriSelect4 agar, 37 °C
L-5319	*Kocuria arsenatis/ Kocuria rhizophila*	99.71	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcaceae*	UC_R2	UriSelect4 agar, 37 °C
L-5320	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R2	UriSelect4 agar, 37 °C
L-5321	*Chryseobacterium echinoideorum*	99.54	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	VC_2	blood agar, 37 °C
L-5322	*Sphingobacterium faecium*	99.11	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	VC_2	blood agar, 37 °C
L-5323	*Acinetobacter johnsonii*	99.71	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_2	blood agar, 37 °C
L-5324	*Sphingomonas panni*	100	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	VC_2	blood agar, 37 °C
L-5325	*Chryseobacterium hominis*	99.63	2	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	VC_2	blood agar, 37 °C
L-5327	*Chryseobacterium echinoideorum*	99.53	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	VC_2	UriSelect4 agar, 37 °C
L-5328	*Chryseobacterium hominis*	98.32	2	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	VC_2	UriSelect4 agar, 37 °C
L-5329	*Sphingobacterium faecium*	99.62	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	VC_2	UriSelect4 agar, 37 °C
L-5330	*Brevundimonas bullata*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	VC_2	UriSelect4 agar, 37 °C
L-5331	*Pelomonas aquatica*	98.99	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	VC_2	UriSelect4 agar, 37 °C
L-5332	*Acinetobacter johnsonii*	99.81	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_2	UriSelect4 agar, 37 °C
L-5333	*Microbacterium aurum*	99.8	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R2	blood agar, 37 °C
L-5334	*Limnobacter thiooxidans*	99.68	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	VC_R2	blood agar, 37 °C
L-5335	*Pelomonas puraquae*	99.34	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	VC_R2	blood agar, 37 °C

L-5336	*Pelomonas aquatica*	99.18	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	VC_R2	blood agar, 37 °C
L-5337	*Novosphingobium lentum*	99.86	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae*	VC_R2	blood agar, 37 °C
L-5338	*Pelomonas aquatica*	99.15	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	VC_R2	blood agar, 37 °C
L-5339	*Pelomonas aquatica*	99.01	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	VC_R2	UriSelect4 agar, 37 °C
L-5340	*Pelomonas aquatica*	99.16	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	TC_2	blood agar, 37 °C
L-5341	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	blood agar, 37 °C
L-5342	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	blood agar, 37 °C
L-5343	*Pseudomonas xanthomarina*	98.97	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	blood agar, 37 °C
L-5344	*Pseudomonas chloritidismutans*	99.9	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	UriSelect4 agar, 37 °C
L-5345	*Pseudomonas xanthomarina*	98.97	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	UriSelect4 agar, 37 °C
L-5346	*Pseudomonas rhodesiae*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	UriSelect4 agar, 37 °C
L-5347	*Staphylococcus hominis*	99.9	2	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	TC_2	UriSelect4 agar, 37 °C
L-5348	*Pseudomonas chloritidismutans*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	UriSelect4 agar, 37 °C

L-5349	*Pseudomonas zhaodongensis*	99.48	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	blood agar, 37 °C
L-5350	*Pseudomonas knackmussii*	99.9	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	blood agar, 37 °C
L-5351	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	blood agar, 37 °C
L-5352	*Pelomonas aquatica*	99.16	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	TC_2	blood agar, 37 °C
L-5353	*Pelomonas aquatica*	96.94	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	TC_2	UriSelect4 agar, 37 °C
L-5354	*Pseudomonas xanthomarina*	98.97	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_2	UriSelect4 agar, 37 °C
L-5355	*Microbacterium testaceum*	98.98	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_2	blood agar, 37 °C
L-5356	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_2	blood agar, 37 °C
L-5357	*Pelomonas aquatica*	99.07	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_2	blood agar, 37 °C
L-5358	*Microbacterium testaceum*	99.08	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_2	blood agar, 37 °C
L-5359	*Pelomonas aquatica*	99.15	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_2	UriSelect4 agar, 37 °C
L-5360	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_2	UriSelect4 agar, 37 °C
L-5361	*Sphingobium hydrophobicum*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_2	UriSelect4 agar, 37 °C
L-5362	*Microbacterium testaceum*	99.11	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_2	UriSelect4 agar, 37 °C
L-5363	*Staphylococcus warneri*	100	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	DC_2	blood agar, 37 °C

L-5364	*Citrobacter freundii*	99.24	2	*Pseudomonadota, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae*	DC_2	blood agar, 37 °C
L-5365	*Pelomonas aquatica*	99.15	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_2	blood agar, 37 °C
L-5366	*Pelomonas aquatica*	99.08	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_2	UriSelect4 agar, 37 °C
L-5367	*Microbacterium testaceum*	99.08	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_2	UriSelect4 agar, 37 °C
L-5368	*Acinetobacter johnsonii*	99.81	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	DC_2	UriSelect4 agar, 37 °C
L-5369	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R2	blood agar, 37 °C
L-5370	*Sphingobium hydrophobicum*	100	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_R2	blood agar, 37 °C
L-5371	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R2	UriSelect4 agar, 37 °C
L-5372	*Sphingobium hydrophobicum*	99.9	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_R2	UriSelect4 agar, 37 °C
L-5373	*Sphingobium hydrophobicum*	99.81	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_R2	UriSelect4 agar, 37 °C
L-5374	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_R2	blood agar, 37 °C
L-5375	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R2	blood agar, 37 °C
L-5376	*Microbacterium maritypicum*	99.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_R2	blood agar, 37 °C
L-5377	*Pelomonas aquatica*	99.23	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R2	blood agar, 37 °C
L-5378	*Acidovorax facilis*	99.44	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R2	blood agar, 37 °C

L-5379	*Pelomonas aquatica*	99.08	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R2	blood agar, 37 °C
L-5380	*Stenotrophomonas rhizophila*	99.62	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	DC_R2	blood agar, 37 °C
L-5381	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R2	blood agar, 37 °C
L-5382	*Pelomonas aquatica*	99.04	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R2	UriSelect4 agar, 37 °C
L-5383	*Pelomonas aquatica*	99.15	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R2	UriSelect4 agar, 37 °C
L-5384	*Stenotrophomonas rhizophila*	99.63	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	DC_R2	UriSelect4 agar, 37 °C
L-5386	*Pelomonas puraquae*	99.26	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R2	UriSelect4 agar, 37 °C
L-5387	*Pseudomonas rhodesiae*	99.82	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_2	blood agar, 37 °C
L-5388	*Pseudomonas koreensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_2	blood agar, 37 °C
L-5390	*Pseudomonas oryzihabitans*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_2	blood agar, 37 °C
L-5391	*Acinetobacter johnsonii*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_2	blood agar, 37 °C
L-5392	*Pelomonas puraquae*	98.46	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_2	UriSelect4 agar, 37 °C
L-5393	*Pelomonas aquatica*	99.17	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_2	UriSelect4 agar, 37 °C
L-5394	*Pseudomonas koreensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_2	UriSelect4 agar, 37 °C

L-5395	*Pelomonas aquatica*	97.36	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_2	UriSelect4 agar, 37 °C
L-5396	*Pelomonas puraquae*	99.21	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_2	blood agar, 37 °C
L-5397	*Acinetobacter lwoffii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_2	UriSelect4 agar, 37 °C
L-5398	*Chryseobacterium shandongense*	99.36	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	KC_R2	UriSelect4 agar, 37 °C
L-5399	*Chryseobacterium shandongense*	99.36	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	KC_R2	UriSelect4 agar, 37 °C
L-5400	*Chryseobacterium shandongense*	99.38	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	KC_R2	blood agar, 37 °C
L-5401	*Acinetobacter lwoffii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_R2	blood agar, 37 °C
L-5402	*Pelomonas aquatica*	98.89	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	KC_R2	blood agar, 37 °C
L-5403	*Microbacterium maritypicum*	99.71	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_R2	UriSelect4 agar, 37 °C
L-5404	*Microbacterium hominis*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_2	blood agar, 37 °C
L-5405	*Acinetobacter johnsonii*	99.73	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_2	blood agar, 37 °C
L-5408	*Pelomonas aquatica*	99.01	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_2	blood agar, 37 °C
L-5409	*Pelomonas aquatica*	98.77	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_2	UriSelect4 agar, 37 °C
L-5411	*Pseudomonas koreensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_2	UriSelect4 agar, 37 °C
L-5412	*Staphylococcus lentus*	100	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	RC_2	UriSelect4 agar, 37 °C
L-5413	*Pelomonas aquatica*	99.17	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_2	UriSelect4 agar, 37 °C
L-5414	*Staphylococcus warneri*	99.91	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	RC_2	UriSelect4 agar, 37 °C
L-5416	*Pelomonas puraquae*	99.23	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_2	UriSelect4 agar, 37 °C

L-5417	*Acinetobacter johnsonii*	99.9	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_2	UriSelect4 agar, 37 °C
L-5418	*Sphingomonas panni*	99.81	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_2	blood agar, 37 °C
L-5419	*Pseudomonas koreensis*	99.62	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_2	blood agar, 37 °C
L-5421	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SI_2	UriSelect4 agar, 37 °C
L-5422	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SI_2	UriSelect4 agar, 37 °C
L-5423	*Microbacterium maritypicum*	99.73	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	blood agar, 37 °C
L-5424	*Pseudomonas koreensis*	99.8	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R2	UriSelect4 agar, 37 °C
L-5425	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	UriSelect4 agar, 37 °C
L-5426	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	blood agar, 37 °C
L-5427	*Pseudomonas baetica*	99.35	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R2	blood agar, 37 °C
L-5428	*Microbacterium maritypicum*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	blood agar, 37 °C
L-5429	*Pseudomonas baetica*	99.42	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R2	blood agar, 37 °C
L-5430	*Microbacterium maritypicum*	99.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	blood agar, 37 °C
L-5431	*Pseudomonas koreensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R2	UriSelect4 agar, 37 °C
L-5432	*Microbacterium maritypicum*	99.79	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	UriSelect4 agar, 37 °C
L-5433	*Microbacterium maritypicum*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R2	UriSelect4 agar, 37 °C

L-5434	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	BC_2	blood agar, 37 °C
L-5435	*Brevundimonas mediterranea*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	BC_2	blood agar, 37 °C
L-5436	*Staphylococcus warneri*	100	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	BC_2	UriSelect4 agar, 37 °C
L-5437	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	BC_2	UriSelect4 agar, 37 °C
L-5438	*Streptococcus mitis*	88.36	2	*Bacillota, Bacilli, Lactobacillales, Streptococcaceae*	BC_2	blood agar, 37 °C
L-5439	*Rothia mucilaginosa*	99.27	2	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	BC_2	UriSelect4 agar, 37 °C
L-5440	*Acinetobacter parvus*	99.63	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	Tap water	blood agar, 37 °C
L-5441	*Methylorubrum populi/ Methylorubrum thiocyanatum*	99.81	1	*Pseudomonadota, Alphaproteobacteria, Hyphomicrobiales, Methylobacteriaceae*	Tap water	blood agar, 37 °C
L-5442	*Sphingopyxis alaskensis*	100	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	Tap water	blood agar, 37 °C
L-5443	*Ottowia shaoguanensis*	96.96	-	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	Tap water	blood agar, 37 °C
L-5444	*Pseudomonas peli*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	Tap water	blood agar, 37 °C
L-5445	*Sphingopyxis alaskensis*	100	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	Tap water	blood agar, 37 °C
L-5446	*Methylorubrum populi/ Methylorubrum thiocyanatum*	99.79	1	*Pseudomonadota, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae*	Tap water	UriSelect4 agar, 37 °C
L-5464	*Massilia varians*	99.72	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	VC_3	blood agar, 37 °C
L-5465	*Acinetobacter johnsonii*	99.35	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_3	blood agar, 37 °C

L-5466	*Acinetobacter johnsonii*	99.29	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_3	blood agar, 37 °C
L-5467	*Pseudomonas oryzihabitans*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	VC_3	blood agar, 37 °C
L-5469	*Sphingobacterium multivorum*	99.9	2	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	VC_3	UriSelect4 agar, 37 °C
L-5470	*Acinetobacter johnsonii*	99.53	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_3	UriSelect4 agar, 37 °C
L-5471	*Sphingobacterium multivorum*	99.91	2	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	VC_3	UriSelect4 agar, 37 °C
L-5472	*Massilia varians*	99.73	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	VC_3	UriSelect4 agar, 37 °C
L-5473	*Acinetobacter johnsonii*	98.26	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_3	UriSelect4 agar, 37 °C
L-5474	*Pseudomonas putida*	99.91	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	VC_3	UriSelect4 agar, 37 °C
L-5475	*Acinetobacter johnsonii*	99.46	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_3	UriSelect4 agar, 37 °C
L-5476	*Sphingomonas olei*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	VC_3	blood agar, 37 °C
L-5477	*Sphingobacterium hotanense*	99.9	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	VC_3	UriSelect4 agar, 37 °C
L-5478	*Pseudomonas plecoglossicida*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	VC_3	UriSelect4 agar, 37 °C
L-5479	*Limnobacter thiooxidans*	99.72	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	VC_R3	blood agar, 37 °C
L-5480	*Limnobacter thiooxidans*	99.61	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	VC_R3	blood agar, 37 °C
L-5481	*Barrientosiimonas humi*	99.9	1	*Actinomycetota, Actinomycetes, Actinomycetales, Dermacoccaceae*	VC_R3	blood agar, 37 °C

L-5482	*Cellulosimicrobium funkei*	99.51	1	*Actinomycetota, Actinomycetes, Micrococcales, Promicromonosporaceae*	VC_R3	blood agar, 37 °C
L-5483	*Microbacterium paraoxydans*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	VC_R3	UriSelect4 agar, 37 °C
L-5484	*Cellulosimicrobium funkei*	99.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Promicromonosporaceae*	VC_R3	UriSelect4 agar, 37 °C
L-5485	*Epidermidibacterium keratini*	100	-	*Actinomycetota, Actinomycetes, Geodermatophilales, Antricoccaceae*	VC_R3	UriSelect4 agar, 37 °C
L-5486	*Pseudomonas chloritidismutans*	99.9	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_3	UriSelect4 agar, 37 °C
L-5487	*Sphingobacterium cellulitidis*	100	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	TC_3	UriSelect4 agar, 37 °C
L-5488	*Sphingobacterium multivorum*	100	2	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	TC_3	UriSelect4 agar, 37 °C
L-5489	*Pseudoxanthomonas japonensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	TC_3	UriSelect4 agar, 37 °C
L-5490	*Acinetobacter johnsonii*	99.73	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	TC_3	UriSelect4 agar, 37 °C
L-5491	*Pseudomonas chloritidismutans*	99.82	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_3	UriSelect4 agar, 37 °C
L-5492	*Brevundimonas olei*	100	-	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	TC_3	UriSelect4 agar, 37 °C
L-5493	*Pseudomonas chloritidismutans*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_3	blood agar, 37 °C
L-5494	*Enterobacter cloacae*	100	2	*Pseudomonadota, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae*	TC_3	blood agar, 37 °C
L-5495	*Tsukamurella tyrosinosolvens*	100	2	*Actinomycetota, Actinomycetes, Mycobacteriales, Tsukamurellaceae*	TC_3	blood agar, 37 °C
L-5496	*Pseudomonas chloritidismutans*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_3	blood agar, 37 °C

L-5497	*Brachybacterium paraconglomeratum*	99.89	1	*Actinomycetota, Actinomycetes, Micrococcales, Dermabacteraceae*	TC_3	blood agar, 37 °C
L-5498	*Sphingobacterium cellulitidis*	100	1	*Bacteroidota, Sphingobacteriia, Sphingobacteriales, Sphingobacteriaceae*	TC_3	blood agar, 37 °C
L-5500	*Pseudomonas chloritidismutans*	99.81	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	TC_3	UriSelect4 agar, 37 °C
L-5501	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_3	UriSelect4 agar, 37 °C
L-5502	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_3	UriSelect4 agar, 37 °C
L-5503	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_3	blood agar, 37 °C
L-5504	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_3	blood agar, 37 °C
L-5505	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R3	blood agar, 37 °C
L-5506	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R3	blood agar, 37 °C
L-5507	*Acidovorax temperans*	99.64	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	DC_R3	blood agar, 37 °C
L-5508	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_R3	blood agar, 37 °C
L-5509	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R3	UriSelect4 agar, 37 °C
L-5510	*Microbacterium maritypicum*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	DC_R3	UriSelect4 agar, 37 °C
L-5511	*Pseudomonas baetica*	99.28	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	DC_R3	UriSelect4 agar, 37 °C

L-5513	*Brevundimonas aurantiaca*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	KC_3	blood agar, 37 °C
L-5514	*Microbacterium testaceum*	99.14	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_3	blood agar, 37 °C
L-5515	*Microbacterium hatanonis*	99.44	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_3	blood agar, 37 °C
L-5516	*Microbacterium chocolatum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_3	blood agar, 37 °C
L-5517	*Microbacterium chocolatum*	99.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_3	blood agar, 37 °C
L-5518	*Microbacterium testaceum*	99.17	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_3	UriSelect4 agar, 37 °C
L-5519	*Acinetobacter lwoffii*	99.71	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_3	UriSelect4 agar, 37 °C
L-5520	*Brevundimonas bullata*	100	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	KC_3	UriSelect4 agar, 37 °C
L-5521	*Pseudomonas chloritidismutans*	99.8	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_3	blood agar, 37 °C
L-5522	*Acinetobacter lwoffii*	99.9	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_3	blood agar, 37 °C
L-5523	*Acinetobacter johnsonii*	99.54	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_3	blood agar, 37 °C
L-5525	*Pseudomonas rhodesiae*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_3	blood agar, 37 °C
L-5526	*Acinetobacter johnsonii*	99.48	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	KC_3	UriSelect4 agar, 37 °C
L-5527	*Pseudomonas koreensis*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	KC_3	UriSelect4 agar, 37 °C
L-5528	*Chryseobacterium shandongense*	99.36	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	KC_R3	blood agar, 37 °C
L-5530	*Micrococcus aloeverae*	99.79	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	KC_R3	blood agar, 37 °C
L-5531	*Staphylococcus epidermidis*	99.82	2	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	KC_R3	blood agar, 37 °C
L-5532	*Chryseobacterium shandongense*	99.36	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	KC_R3	UriSelect4 agar, 37 °C

L-5533	*Micrococcus yunnanensis*	99.68	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	KC_R3	UriSelect4 agar, 37 °C
L-5534	*Acinetobacter haemolyticus*	99.04	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_3	blood agar, 37 °C
L-5535	*Sphingomonas panni*	100	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_3	blood agar, 37 °C
L-5537	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_3	blood agar, 37 °C
L-5538	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_3	UriSelect4 agar, 37 °C
L-5539	*Acinetobacter haemolyticus*	99.18	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_3	UriSelect4 agar, 37 °C
L-5540	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_3	UriSelect4 agar, 37 °C
L-5541	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_3	UriSelect4 agar, 37 °C
L-5542	*Sphingomonas hankookensis*	99.42	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_3	UriSelect4 agar, 37 °C
L-5543	*Acinetobacter haemolyticus*	99.12	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_3	UriSelect4 agar, 37 °C
L-5544	*Staphylococcus warneri*	99.91	1	*Bacillota, Bacilli, Bacillales, Staphylococcaceae*	SC_3	blood agar, 37 °C
L-5545	*Acinetobacter haemolyticus*	99.27	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SC_3	blood agar, 37 °C
L-5547	*Sphingomonas panni*	99.82	1	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SC_R3	blood agar, 37 °C
L-5548	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_R3	blood agar, 37 °C
L-5550	*Acidovorax temperans*	99.65	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	SC_R3	UriSelect4 agar, 37 °C

L-5551	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	SC_R3	UriSelect4 agar, 37 °C
L-5552	*Acinetobacter haemolyticus*	99.26	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	blood agar, 37 °C
L-5553	*Rothia kristinae*	99.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_3	blood agar, 37 °C
L-5554	*Acinetobacter johnsonii*	99.9	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	blood agar, 37 °C
L-5556	*Acinetobacter haemolyticus*	99.2	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	UriSelect4 agar, 37 °C
L-5557	*Rothia amarae*	99.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	FC_3	UriSelect4 agar, 37 °C
L-5559	*Acinetobacter haemolyticus*	99.12	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	FC_3	UriSelect4 agar, 37 °C
L-5560	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	blood agar, 37 °C
L-5561	*Microbacterium zeae*	98.63	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	blood agar, 37 °C
L-5562	*Microbacterium lacus*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	FC_R3	blood agar, 37 °C
L-5563	*Brevibacterium sanguinis*	99.71	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R3	blood agar, 37 °C
L-5564	*Brevibacterium sanguinis*	99.72	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5565	*Pseudomonas alcaligenes*	98.79	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5566	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5567	*Stenotrophomonas maltophilia*	99.91	2	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	FC_R3	blood agar, 37 °C
L-5568	*Rhodococcus corynebacterioides*	99.81	1	*Actinomycetota, Actinomycetes, Mycobacteriales, Nocardiaceae*	FC_R3	blood agar, 37 °C
L-5569	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	blood agar, 37 °C

L-5570	*Brevibacterium sanguinis/ Brevibacterium celere/ Brevibacterium antiquum/ Brevibacterium aurantiacum/ Brevibacterium casei*	100	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R3	blood agar, 37 °C
L-5571	*Pseudomonas peli*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5572	*Brevibacterium sanguinis*	99.7	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	FC_R3	UriSelect4 agar, 37 °C
L-5573	*Brevundimonas mediterranea*	99.91	1	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	BC_3	blood agar, 37 °C
L-5574	*Aquincola tertiaricarbonis*	98.24	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	BC_3	blood agar, 37 °C
L-5575	*Brevundimonas vesicularis/ Brevundimonas nasdae*	99.91	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	BC_3	blood agar, 37 °C
L-5576	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	BC_3	UriSelect4 agar, 37 °C
L-5577	*Acinetobacter lwoffii*	99.48	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_3	blood agar, 37 °C
L-5578	*Acinetobacter johnsonii*	99.91	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_3	blood agar, 37 °C
L-5579	*Microbacterium paraoxydans*	99.52	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_3	blood agar, 37 °C
L-5580	*Aeromonas media*	99.79	1	*Pseudomonadota, Gammaproteobacteria, Aeromonadales, Aeromonadaceae*	RC_3	blood agar, 37 °C
L-5581	*Pseudomonas plecoglossicida*	99.72	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_3	blood agar, 37 °C
L-5582	*Tsukamurella pulmonis*	100	2	*Actinomycetota, Actinomycetes, Mycobacteriales, Tsukamurellaceae*	RC_3	blood agar, 37 °C
L-5583	*Microbacterium foliorum*	99.53	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_3	blood agar, 37 °C
L-5584	*Acinetobacter lwoffii*	99.53	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_3	blood agar, 37 °C

L-5585	*Delftia lacustris*	99.91	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_3	UriSelect4 agar, 37 °C
L-5586	*Acinetobacter johnsonii*	99.31	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_3	UriSelect4 agar, 37 °C
L-5587	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_3	UriSelect4 agar, 37 °C
L-5588	*Microbacterium paraoxydans*	99.82	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_3	UriSelect4 agar, 37 °C
L-5589	*Acinetobacter johnsonii*	99.01	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_3	UriSelect4 agar, 37 °C
L-5591	*Microbacterium schleiferi*	99.16	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_3	blood agar, 37 °C
L-5592	*Micrococcus yunnanensis*	99.72	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_3	UriSelect4 agar, 37 °C
L-5593	*Delftia lacustris*	100	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_3	UriSelect4 agar, 37 °C
L-5594	*Aeromonas salmonicida/ Aeromonas piscicola/ Aeromonas bestiarum*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Aeromonadales, Aeromonadaceae*	RC_3	UriSelect4 agar, 37 °C
L-5595	*Acidovorax soli*	98.6	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_R3	blood agar, 37 °C
L-5596	*Microbacterium phyllosphaerae*	99.72	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R3	blood agar, 37 °C
L-5597	*Microbacterium maritypicum*	99.82	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R3	blood agar, 37 °C
L-5598	*Exiguobacterium mexicanum*	99.82	1	*Bacillota, Bacilli, Bacillales, Bacillales Incertae Sedis XII*	RC_R3	blood agar, 37 °C
L-5599	*Acinetobacter johnsonii*	99.37	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R3	blood agar, 37 °C
L-5600	*Acinetobacter johnsonii*	99.43	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R3	blood agar, 37 °C
L-5601	*Acinetobacter lwoffii*	99.65	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R3	blood agar, 37 °C
L-5602	*Brevibacterium casei*	99.52	2	*Actinomycetota, Actinomycetes, Micrococcales, Brevibacteriaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5603	*Sphingomonas panaciterrae*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	RC_R3	UriSelect4 agar, 37 °C

L-5604	*Acidovorax soli*	98.63	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5605	*Neomicrococcus aestuarii*	99.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	RC_R3	UriSelect4 agar, 37 °C
L-5606	*Acinetobacter johnsonii*	99.71	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5607	*Acinetobacter lwoffii*	99.81	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5608	*Microbacterium maritypicum*	99.82	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5609	*Microbacterium maritypicum*	100	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5610	*Microbacterium paraoxydans*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	RC_R3	UriSelect4 agar, 37 °C
L-5611	*Comamonas testosteroni*	99.64	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	RC_R3	blood agar, 37 °C
L-5612	*Pseudomonas oryzihabitans*	99.45	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	RC_R3	blood agar, 37 °C
L-5613	*Acinetobacter lwoffii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_3	blood agar, 37 °C
L-5614	*Massilia varians*	99.6	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	UC_3	blood agar, 37 °C
L-5615	*Microbacterium maritypicum*	99.73	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_3	blood agar, 37 °C
L-5616	*Pseudomonas peli*	98.82	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_3	blood agar, 37 °C
L-5617	*Acinetobacter lwoffii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_3	blood agar, 37 °C
L-5618	*Rothia amarae*	97.99	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	UC_3	UriSelect4 agar, 37 °C
L-5619	*Sphingomonas olei*	99.82	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	UC_3	UriSelect4 agar, 37 °C
L-5620	*Massilia varians*	99.79	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	UC_3	UriSelect4 agar, 37 °C

L-5621	*Pseudomonas pseudoalcaligenes*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_3	UriSelect4 agar, 37 °C
L-5622	*Massilia timonae*	99.89	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	UC_3	UriSelect4 agar, 37 °C
L-5623	*Acinetobacter lwoffii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_3	UriSelect4 agar, 37 °C
L-5624	*Pseudomonas stutzeri*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_3	blood agar, 37 °C
L-5625	*Sphingomonas olei/ Sphingomonas panaciterrae*	99.9	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	UC_3	blood agar, 37 °C
L-5626	*Massilia timonae*	99.89	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	UC_3	blood agar, 37 °C
L-5627	*Rothia terrae*	99.26	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	UC_3	UriSelect4 agar, 37 °C
L-5628	*Pseudomonas oryzihabitans/ Pseudomonas psychrotolerans*	99.53	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_3	UriSelect4 agar, 37 °C
L-5629	*Microbacterium maritypicum*	99.91	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_3	UriSelect4 agar, 37 °C
L-5630	*Acinetobacter lwoffii*	99.8	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_3	UriSelect4 agar, 37 °C
L-5631	*Massilia varians*	99.79	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	UC_3	UriSelect4 agar, 37 °C
L-5633	*Pseudomonas peli*	99.07	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_R3	blood agar, 37 °C
L-5634	*Sphingomonas olei*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	UC_R3	blood agar, 37 °C
L-5635	*Acinetobacter lwoffii*	100	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_R3	blood agar, 37 °C

L-5636	*Pseudomonas peli*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_R3	blood agar, 37 °C
L-5637	*Brevundimonas vesicularis/ Brevundimonas nasdae*	100	2	*Pseudomonadota, Alphaproteobacteria, Caulobacterales, Caulobacteraceae*	UC_R3	blood agar, 37 °C
L-5638	*Sphingomonas olei/ Sphingomonas panaciterrae*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	UC_R3	UriSelect4 agar, 37 °C
L-5639	*Pseudomonas peli*	100	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_R3	UriSelect4 agar, 37 °C
L-5640	*Acinetobacter schindleri*	98.68	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_R3	UriSelect4 agar, 37 °C
L-5643	*Acinetobacter lwoffii*	99.91	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	UC_R3	blood agar, 37 °C
L-5644	*Sphingomonas olei/ Sphingomonas panaciterrae*	99.9	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	UC_R3	blood agar, 37 °C
L-5645	*Pseudomonas koreensis*	99.91	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	UC_R3	UriSelect4 agar, 37 °C
L-5646	*Rothia terrae*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	UC_R3	UriSelect4 agar, 37 °C
L-5647	*Rothia terrae*	99.72	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	UC_R3	UriSelect4 agar, 37 °C
L-5648	*Microbacterium maritypicum*	99.81	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	UC_R3	UriSelect4 agar, 37 °C
L-5649	*Limnobacter thiooxidans*	99.7	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiaceae*	UC_R3	UriSelect4 agar, 37 °C
L-5650	*Rothia kristinae*	99.54	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	UC_R3	UriSelect4 agar, 37 °C
L-5651	*Hydrogenophaga palleronii*	99.91	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Comamonadaceae*	SI_3	blood agar, 37 °C
L-5653	*Sphingobium hydrophobicum*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_3	blood agar, 37 °C

L-5654	*Sphingomonas olei/ Sphingomonas panaciterrae*	99.91	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	SI_3	blood agar, 37 °C
L-5655	*Aquincola tertiaricarbonis*	98.15	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	SI_3	blood agar, 37 °C
L-5656	*Acinetobacter lwoffii*	99.82	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SI_3	blood agar, 37 °C
L-5657	*Pseudomonas koreensis*	99.72	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_3	blood agar, 37 °C
L-5658	*Aquincola tertiaricarbonis*	98.2	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Burkholderiales incertae sedis*	SI_3	UriSelect4 agar, 37 °C
L-5659	*Chryseobacterium shandongense*	99.27	-	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	SI_3	UriSelect4 agar, 37 °C
L-5660	*Pseudomonas koreensis*	99.55	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_3	UriSelect4 agar, 37 °C
L-5661	*Acinetobacter johnsonii*	99.72	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SI_3	UriSelect4 agar, 37 °C
L-5662	*Chryseobacterium aquaticum*	99.63	1	*Bacteroidota, Flavobacteriia, Flavobacteriales, Weeksellaceae*	SI_3	UriSelect4 agar, 37 °C
L-5663	*Acinetobacter beijerinckii*	99.37	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SI_3	blood agar, 37 °C
L-5667	*Pseudomonas koreensis*	99.72	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_3	UriSelect4 agar, 37 °C
L-5668	*Rothia amarae*	97.9	1	*Actinomycetota, Actinomycetes, Micrococcales, Micrococcineae*	SI_3	UriSelect4 agar, 37 °C
L-5671	*Acinetobacter johnsonii*	99.3	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SI_R3	blood agar, 37 °C
L-5672	*Microbacterium maritypicum*	99.89	1	*Actinomycetota, Actinomycetes, Micrococcales, Microbacteriaceae*	SI_R3	blood agar, 37 °C
L-5673	*Acinetobacter johnsonii*	99.37	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	SI_R3	blood agar, 37 °C

L-5675	*Massilia varians*	99.34	1	*Pseudomonadota, Betaproteobacteria, Burkholderiales, Oxalobacteraceae*	SI_R3	UriSelect4 agar, 37 °C
L-5676	*Stenotrophomonas rhizophila*	99.63	1	*Pseudomonadota, Gammaproteobacteria, Lysobacterales, Lysobacteraceae*	SI_R3	UriSelect4 agar, 37 °C
L-5677	*Pseudomonas rhodesiae*	99.82	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R3	UriSelect4 agar, 37 °C
L-5678	*Bacillus aryabhattai*	99.9	1	*Bacillota, Bacilli, Bacillales, Bacillaceae*	SI_R3	UriSelect4 agar, 37 °C
L-5680	*Serratia quinivorans*	99.53	1	*Pseudomonadota, Gammaproteobacteria, Enterobacteriales, Yersiniaceae*	SI_R3	UriSelect4 agar, 37 °C
L-5681	*Pseudomonas rhodesiae*	99.73	1	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae*	SI_R3	blood agar, 37 °C
L-5682	*Acinetobacter johnsonii*	99.24	2	*Pseudomonadota, Gammaproteobacteria, Pseudomonadales, Moraxellaceae*	VC_3	UriSelect4 agar, 37 °C
L-5683	*Sphingobium hydrophobicum*	99.81	-	*Pseudomonadota, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae*	DC_3	blood agar, 37 °C

Table S2: List of the isolates selected for the antimicrobial susceptibility testing with the corresponding antimicrobial profile. + indicates resistance; - indicates susceptibility.
Ampicillin (AMP) 100 mg/l; Chloramphenicol (CHL) 25 mg/l; Cefotaxime (CTX) 2 mg/l; Colistin (COL) 3.5 mg/l; Enrofloxacin (ENR) 0.5 mg/l; Erythromycin (ERY) 15 mg/l; Imipenem (IPM) 4 mg/l; Kanamycin (KAN) 50 mg/l); Tetracycline (TET) 10 mg/l.

Tabela S2: Seznam izolatov, izbranih za testiranje odpornosti proti antibiotikom, s protimikrobnim profilom. + označuje odpornost; - označuje občutljivost.

Mycosmo culture collection No. (EXB)	Bacterial strain	AMP 100 mg/l	TET 12,5 mg/l	IPM 4 mg/l	ERY 15 mg/l	CHL 25 mg/l	KAN 50 mg/l	CTX 2 mg/l	ENR 0.5 mg/l	COL 3.5 mg/l	LB
L-5122	*Acinetobacter beijerinckii*	-	-	-	-	-	-	-	-	+	+
L-5171	*Acinetobacter beijerinckii*	-	-	-	-	-	-	-	-	+	+
L-5091	*Acinetobacter haemolyticus*	-	-	-	-	-	-	+	-	+	+
L-5256	*Acinetobacter haemolyticus*	-	-	-	-	-	-	+	-	+	+
L-5279	*Acinetobacter haemolyticus*	-	-	-	-	-	-	+	-	+	+
L-5539	*Acinetobacter haemolyticus*	-	-	-	-	-	-	+	-	+	+
L-5559	*Acinetobacter haemolyticus*	-	-	-	-	-	-	+	-	+	+
L-5094	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5125	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5165	*Acinetobacter johnsonii*	-	-	-	+	-	-	+	+	+	+
L-5183	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5217	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5232	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5286	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5296	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5470	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5523	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5578	*Acinetobacter johnsonii*	-	-	-	-	-	-	+	-	-	+
L-5522	*Acinetobacter lwoffii*	-	-	-	-	-	-	-	-	-	+
L-5577	*Acinetobacter lwoffii*	-	-	-	-	-	-	+	-	-	+
L-5397	*Acinetobacter lwoffii/ Prolinoborus fasciculus*	-	-	-	-	-	-	+	-	-	+
L-5401	*Acinetobacter lwoffii/ Prolinoborus fasciculus*	-	-	-	-	-	-	+	-	-	+
L-5072	*Brevibacterium casei*	-	-	-	-	+	-	+	+	+	+

L-5563	*Brevibacterium sanguinis*	-	-	-	-	-	-	-	-	+	+
L-5032	*Brevundimonas aurantiaca*	-	-	-	-	-	-	+	+	+	+
L-5168	*Brevundimonas aurantiaca*	-	-	-	-	-	+	+	-	+	+
L-5266	*Brevundimonas aurantiaca*	-	-	-	-	-	-	+	+	+	+
L-5226	*Brevundimonas diminuta*	-	-	-	-	-	-	+	+	+	+
L-5081	*Brevundimonas mediterranea*	-	-	-	-	-	-	+	-	+	+
L-5179	*Brevundimonas mediterranea*	-	-	-	-	-	-	+	-	+	+
L-5492	*Brevundimonas olei*	-	-	-	-	-	-	+	+	+	+
L-5050	*Brevundimonas vesicularis/ Brevundimonas nasdae*	-	-	-	-	-	-	-	-	+	+
L-5083	*Brevundimonas vesicularis/ Brevundimonas nasdae*	-	-	-	-	-	-	-	-	+	+
L-5182	*Brevundimonas vesicularis/ Brevundimonas nasdae*	-	-	-	-	-	-	-	-	+	+
L-5421	*Brevundimonas vesicularis/ Brevundimonas nasdae*	-	-	-	-	-	-	+	-	+	+
L-5321	*Chryseobacterium echinoideorum*	-	-	-	-	-	+	+	-	+	+
L-5201	*Chryseobacterium sediminis*	+	+	+	-	-	+	+	-	+	+
L-5071	*Chryseobacterium shandongense*	-	-	-	-	-	+	+	-	+	+
L-5398	*Chryseobacterium shandongense*	-	-	-	-	-	+	-	-	+	+
L-5364	*Citrobacter freundii*	-	-	-	+	-	-	-	-	-	+
L-5593	*Delftia lacustris*	+	-	-	+	-	-	-	-	+	+
L-5494	*Enterobacter cloacae*	-	-	-	+	-	-	-	-	+	+
L-5108	*Enterococcus silesiacus/ Enterococcus caccae/ Enterococcus ureilyticus*	-	-	-	-	-	-	+	+	+	+
L-5029	*Kocuria arsenatis/ Kocuria rhizophila*	-	-	-	-	-	-	-	+	+	+
L-5114	*Kocuria carniphila*	-	-	-	-	-	-	-	+	+	+
L-5129	*Kocuria carniphila*	-	-	-	-	-	-	-	-	+	+

L-5181	*Kocuria uropygioeca*	-	-	-	-	-	-	-	+	+	+
L-5223	*Microbacterium lacus*	-	-	-	-	-	-	-	-	-	+
L-5587	*Microbacterium maritypicum*	-	-	-	+	-	-	-	-	+	+
L-5205	*Microbacterium paraoxydans*	-	-	-	+	-	+	+	-	+	+
L-5483	*Microbacterium paraoxydans*	-	-	-	+	-	+	+	-	+	+
L-5579	*Microbacterium paraoxydans*	-	-	-	-	-	-	+	-	+	+
L-5099	*Microbacterium testaceum*	-	-	-	-	-	-	-	+	+	+
L-5268	*Pantoea dispersa*	-	-	-	+	-	-	-	-	-	+
L-5292	*Pseudomonas aeruginosa*	+	+	+	+	+	+	+	-	-	+
L-5565	*Pseudomonas alcaligenes*	+	-	+	+	-	-	+	-	-	+
L-5243	*Pseudomonas chloritidismutans*	-	-	-	-	-	-	-	-	-	+
L-5293	*Pseudomonas chloritidismutans*	-	-	-	-	-	-	-	-	-	+
L-5344	*Pseudomonas chloritidismutans*	-	-	-	-	-	-	-	-	-	+
L-5521	*Pseudomonas chloritidismutans*	-	-	-	-	-	-	-	-	-	+
L-5527	*Pseudomonas koreensis*	+	-	-	+	+	-	+	-	-	+
L-5275	*Pseudomonas oryzihabitans*	-	-	-	-	-	-	-	-	-	+
L-5390	*Pseudomonas oryzihabitans*	-	-	-	+	-	-	+	+	-	+
L-5467	*Pseudomonas oryzihabitans*	-	-	-	+	-	-	+	-	-	+
L-5242	*Pseudomonas putida*	-	-	-	+	-	-	+	-	-	+
L-5474	*Pseudomonas putida*	+	-	-	+	-	-	+	-	-	+
L-5055	*Rothia amarae*	-	-	-	-	-	-	-	+	+	+
L-5204	*Rothia amarae*	-	-	-	-	-	-	-	+	+	+
L-5049	*Rothia kristinae*	-	-	-	-	-	-	-	+	+	+
L-5553	*Rothia kristinae*	-	-	-	-	-	-	-	+	+	+
L-5487	*Sphingobacterium cellulitidis*	-	-	-	-	-	+	+	-	+	+

L-5469	*Sphingobacterium multivorum*	-	-	-	-	-	-	-	+	+	+
L-5488	*Sphingobacterium multivorum*	-	-	+	-	-	+	+	-	+	+
L-5177	*Sphingomonas paucimobilis*	-	-	-	-	-	-	-	-	+	+
L-5531	*Staphylococcus epidermidis*	-	-	-	+	-	-	-	-	+	+
L-5057	*Staphylococcus haemolyticus*	-	-	-	-	-	-	-	-	+	+
L-5207	*Staphylococcus haemolyticus*	-	-	-	+	-	-	-	-	+	+
L-5412	*Staphylococcus lentus*	-	-	-	-	-	-	+	-	+	+
L-5028	*Staphylococcus lugdunensis*	-	-	-	-	-	-	-	-	+	+
L-5076	*Stenotrophomonas maltophilia*	-	-	+	+	-	+	+	-	-	+
L-5250	*Stenotrophomonas maltophilia*	+	-	+	+	-	+	+	-	+	+
L-5259	*Stenotrophomonas maltophilia/ Pseudomonas hibiscicola*	-	-	+	+	-	+	+	-	-	+
L-5269	*Stenotrophomonas pavanii/ Stenotrophomonas maltophilia*	+	-	+	+	-	+	+	+	+	+

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Phylogenetic study of *Aliinostoc* species (Cyanobacteria) using *pc-igs*, *nifH* and *mcy* as markers for investigation of horizontal gene transfer

Filogenetska študija vrst *Aliinostoc* (Cyanobacteria) z uporabo označevalcev *pc-igs*, *nifH* in *mcy* za ugotavljanje horizontalnega genskega prenosa

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Abstract: Selection of genes that have not been horizontally transferred for prokaryote phylogenetic studies is regarded as a challenging task. Internal transcribed spacer of ribosomal genes (16S–23S ITS), microcystin synthetase genes (*mcy*), nitrogenase (*nifH*) and phycocyanin intergenic spacer (*PC-IGS*) are among the most used markers in cyanobacteria. The region of the ribosomal genes has been considered stable, whereas the *nifH*, *mcyG* and *PC-IGS* may have undergone horizontal transfer. To investigate the occurrence of horizontal transfer of *nifH*, *mcyG* and *PC-IGS*, phylogenetic trees of *Aliinostoc* strains Ay1375 and Me1355 were generated and compared. Phylogenetic trees based on the markers were mostly congruent for *PC-IGS*, indicating a common evolutionary history among ribosomal and phycocyanin genes with no evidence for horizontal transfer of *PC-IGS*. Phylogenetic trees constructed from the *nifH* and 16S rRNA genes were incongruent. Our results suggest that *nifH* has been transferred from one cyanobacterium to another. Moreover, the low non-synonymous/synonymous mutation ratio (Ka/Ks) was consistent with an ancient origin of the *mcyG*.

Keywords: 16S–23S ITS, cyanobacteria, horizontal gene transfer, molecular phylogeny, phycocyanin, ribosomal genes

Izvleček: Za filogenetske študije prokariontov velja, da je izbira genov, ki niso bili horizontalno preneseni, zahtevna naloga. Notranji prepisani vmesnik ribosomskih genov (16S–23S ITS), geni mikrocistin sintetaze (*mcy*), nitrogenaze (*nifH*) in fikocianinski medgenski vmesnik (*PC-IGS*) so med najpogosteje uporabljenimi označevalci pri cianobakterijah. Območje ribosomskih genov velja za stabilno, medtem ko so zaporedja *nifH*, *mcyG* in PC-IGS lahko bila prenesena s horizontalnim genskim prenosom. Da bi raziskali pojav horizontalnega prenosa *nifH*, *mcyG* in PC-IGS, smo ustvarili filogenetska drevesa sevov Ay1375 in Me1355 vrste *Aliinostoc* ter jih med seboj primerjali. Filogenetska drevesa na podlagi označevalcev so bila večinoma skladna za *PC-IGS* in niso razkrila morebitnih horizontalnih genskih prenosov, kar kaže na skupno evolucijsko zgodovino med ribosomskimi in fikocianinskimi geni. Primerjava filogenetskih dreves, pridobljenih na podlagi gena *nifH* s filogenetskimi drevesi, pridobljenimi na podlagi gena za 16S rRNA, je razkrila neskladja. Naši rezultati tako nakazujejo, da je bil *nifH* prenesen iz ene cianobakterije v drugo s horizontalnim genskim prenosom. Poleg tega

se nizko razmerje med nesinonimnimi/sinonimnimi mutacijami (Ka/Ks), ki smo ga razkrili v študiji, sklada s starodavnim izvorom gena *mcyG*.

Ključne besede: 16S–23S ITS, cianobakterije, horizontalni genski prenos, molekularna filogenija, fikocianin, ribosomski geni

Introduction

The morphological characteristics of cyanobacteria do not always correspond to their taxonomic diversity (Komárek et al. 2016) and therefore the use of molecular markers for phylogenetic studies have become essential (Han et al. 2009).

Aliinostoc species is a cosmopolitan, nitrogen (N_2)-fixing cyanobacterial species found in temperate to tropical freshwater or terrestrial habitats. The widespread proliferation of *Aliinostoc* species in paddy fields has increased the nitrogen in soils. Molecular approaches are particularly useful in the detection and identification of specific strains, especially those that are morphologically identical at the species level. Genetic identification can also be used to characterize the degree of genetic similarity among populations (Kabirnataj et al. 2020; Nowruzi et al., 2021; Nowruzi and Shalygin 2021).

One of the genes utilized for genetic differences between *Aliinostoc* cultures was *nifH*, a highly conserved gene that encodes dinitrogenase reductase, a protein subunit in the nitrogenase complex involved in N_2 fixation. Common to all N_2 fixers, the 324-bp *nifH* fragment is useful in characterizing diazotrophic communities and for differentiating cyanobacterial genera (Foster and Zehr, 2006). The other genetic locus used was *cpcBA*-IGS, which includes the highly variable intergenic spacer (IGS) region between two phycobilisome subunits (*cpcB* and *cpcA*) within the phycocyanin operon (Dyble et al., 2002; Brient et al., 2008). Both *cpcA*-IGS (Bastien et al., 2011) and *nifH* appear to be more useful in discriminating between strains than the commonly employed 16S rRNA gene, which exhibits low intrageneric variability in many cyanobacteria (Teneva et al., 2012). Moreover, microcystins, cyclic heptapeptide hepatotoxins, are by far the most prevalent of the cyanobacterial toxins and are produced by microcystin synthetase gene cluster (Jungblut et al. 2006; Nowruzi et al., 2022).

One of the greatest challenges in the selection of markers for phylogenetic studies in cyanobacteria is targeting markers that have not undergone horizontal gene transfer (HGT) (Yerrapragada and Siefert, 2009; Piccin-Santos et al., 2014). HGT and orthologous gene substitutions are relatively common among cyanobacteria and have been important processes in the evolution of this group (Piccin-Santos et al., 2014). However, HGT events in cyanobacteria may still be underestimated, and genes with several functions could have been subjected to this process (Zhaxybayeva et al. 2006). There is no reported evidence that the operons of ribosomal genes have undergone HGT among cyanobacteria. However, the variability observed among the multiple copies of the ribosomal operon found within a single individual can hinder their use in phylogenetic studies (Iteman et al. 2002).

The construction and comparison of phylogenetic trees are perhaps the best ways to assess the contribution of HGT to the evolutionary history of a gene family (Koonin et al., 2002). Incongruence is taken to indicate a role for HGT, whereas congruence is consistent with descent through common ancestry. Therefore, to resolve the relationship between microcystin synthetase genes, *PC-IGS*, *nifH*, 16S rRNA and the role of HGT in the evolutionary history, we undertook a molecular phylogenetic study. We analyzed and tested for congruence two data sets comprised of genes involved in primary metabolism and genes involved directly in the synthesis of microcystins and nodularins.

Our goal, using strains of *Aliinostoc* species as models, was to evaluate the possible occurrence of HGT by comparing phylogenetic trees built with *mcy*, *PC-IGS*, *nifH* and 16S rRNA. This is the first study to compare the different molecular markers in characterizing two *Aliinostoc* isolates originating from paddy fields of Iran.

Abbreviations: 16S–23S ITS, internal transcribed spacer of ribosomal genes marker; HGT, horizontal gene transfer; PC-IGS, phycocyanin intergenic spacer marker

Material and methods

Strains and cultivation conditions

The clonal and axenic strains (strain designations Ay1375 and Me1355) of *Aliinostoc* belonged to the Cyanobacteria Culture Collection (CCC) and ALBORZ herbarium. Strains were maintained in climate chambers with controlled conditions of continues light and temperature (25 ± 5°C) in BG-11 cultivation medium (Rippka et al. 1979), of pH value 7.4.

Molecular and sequence analysis

Genomic DNA was isolated from 16-18 days old log phase cultures using the Himedia Ultrasensitive Spin Purification Kit (MB505) following the instructions of the manufacturer, except the increase of incubation time for the lysis solutions AL and C1, which were set to 60 and 20 min, respectively. DNA fragments within the following genes were amplified using the oligonucleotide primers and PCR programs listed in Table 1: 16S rRNA gene, ITS, *nifH*, *PC-IGS, mcyG and mcyD.* PCR reactions were performed using a thermal cycler 5.9 and the following procedure: 25 µl aliquots containing 10-20 ng DNA template, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 1U/µl Taq DNA polymerase (Robertson et al., 2001; Dyble et al., 2002; Nowruzi and Lorenzi, 2021). PCR products were analyzed by electrophoresis on 1% agarose gels (SeaPlaque® GTG®, Cambrex Corporation), using standard protocols. The products were purified directly using the Geneclean® Turbo kit (Qbiogene, MP Biomedicals) and sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies).

The partial sequences were compared with the ones available in the NCBI database (March, 2022) using BLASTn. The BLAST X tool (blast.ncbi.nlm.nih.gov/Blast.cgi) was used for *cpc*A-IGS, *nifH, mcy D* and *mcyE genes.* The sequences were annotated with the NCBI ORF Finder and the ExPASY (https://www.ncbi.nlm.nih.gov/orffinder/) proteomics tools.

Table 1. Target genes, oligonucleotide primers and PCR programs used in this study.
Tabela 1: Tarčni geni, začetni oligonukleotidi in programi PCR, uporabljeni v raziskavi.

Target gene/ sequence	Primer designation (sequence 5´→3´)	PCR program (reference in superscript)
16S rRNA	PA (5'-AGAGTTTGATCCTGGCTCAG-3') B23S (5'-CTTCGCCTCTGTGTGCCTAGGT-3')	[1]94˚C, 3 min [1]30 × (94˚C, 30 s; 55˚C, 40 s; 72˚C, 1.30 min) [2]72˚C, 3 min [2]4˚C, ∞
16S-23S rRNA ITS	ITS-F (5'-TGTACACACCGCCCGTC-3') ITS-R (5'-CTCTGTGTGCCTAGGTATCC-3')	
***cpc*A-IGS**	Cpc F (5'-GGCTGCTTGTTTACGCGACA-3') Cpc R (5'-CCAGTACCACCAGCAACTAA-3')	[3]94˚C, 5 min [3]30 × (92˚C, 1 min; 55˚C, 1 min; 72˚C, 2 min) [3]72˚C, 6 min [3]4˚C, ∞
psbA	PSBA86F (5'-TTTATGTGGGTTGGTTCGG-3') PSBA980R4 (5'-TGAGCATTACGCTCGTGC-3')	[4]94˚C, 5 min [4]35 × (94˚C, 60 s; 56˚C, 60 s; 72˚C, 60 s) [5]72˚C, 10 min [5]4˚C, ∞
***nif*H**	*nif*H F (5'-CGTAGGTTGCGACCCTAAGGCTGA-3') *nif*H R (5'-GCATACATCGCCATCATTTCACC-3')	
mcyG	*mcyG* F (5'-GAAATTGGTGCGGGAACTGGAG-3') *mcyG* R (5'-TTTGAGCAACAATGATACTTTGCTG-3')	[6]95˚C, 5 min [6]34 × (95˚C, 30 s; 53˚C, 30 s; 72˚C, 60 s) [7]72˚C, 5 min [7]4˚C, ∞
***mcy*D**	*mcy*D F (5'-GCTCAAGAAAAATTACATCAAG-3') *mcy*D R (5'-TTAAAGGAGAATGAAAAGCATGAGA-3')	

References: [1]Taton et al. 2003; [2]Iteman et al. 2000, [3]Neilan et al. 1997, [4] Junier et al. 2007, [5]Gaby and Buckley 2012, [6] Fewer et al. 2007, [7] Rantala et al. 2004

Nucleotide sequence accession numbers

Sequence data were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers showed in Table 2.

Table 2: Accession numbers of sequence data deposited in the DNA Data Bank of Japan. *mcyG* was not found in *Aliinostoc* sp. Ay1375.
Tabela 2: Oznake v japonski podatkovni bazi DNA deponiranih nukleotidnih zaporedij *Aliinostoc* sp. *mcyG* pri *Aliinostoc* sp. Ay1375 ni bil določen.

Nucleotide ID	Target gene	Strain	Number of nucleotides	Number of amino acids	Tree model
ON751925	*16S rRNA*	Ay1375	1442	-	TVM+F+I+G4
ON751926		Me1355	1442		
ON755128	*nifH*	Ay1375	286	80	TIM2e+I+G4+F
ON755129		Me1355	282	79	
ON755126	*cpcA*-IGS	Ay1375	589	98	LG+G4
ON755127		Me1355	612	98	
OM801556	*mcyG*	Me1355	494	164	TPM3U+G4+F

Phylogenetic analysis

The 16S rRNA, ITS, *cpc*A-IGS, *nifH, mcyG* and *mcyD* genes sequences obtained in this study, as well as the best hit sequences (> 94% identity) retrieved from GenBank, were first aligned using MUSCLE (Edgar 2004), and then maximum likelihood phylogenetic trees were inferred in IQ-Tree (multicore v1.5.5) (Nguyen et al. 2015). Different models were used as suggested (BIC criterion) after employing model test implemented in IQ-tree (Table 2). Tree robustness was estimated with bootstrap percentages using 100 standard bootstrap and 10,000 ultrafast bootstrap to evaluate branch supports (Guajardo-Leiva et al. 2018).

16S-23S rRNA ITS region secondary structure analysis

The sequences corresponding to the D1-D1' helix, D2, D3, Box-B and Box-A regions of the 16S-23S ITS of the studied strains were characterized according to the Johansen et al. (2011), and $trRNA^{Ile}$ and $trRNA^{Ala}$ were determined according to the tRNAscan-SE 2.0 (Chan et al., 2021). Comparison of the ITS secondary structures of studied strains and the reference strains were generated using the M-fold web server (version 2.3) (Zuker 2003) under ideal conditions of untangled loop fix and the temperature set to default (37 °C).

Sequence divergence

We calculated the number of non-synonymous substitutions per non-synonymous site (Ka) and the number of synonymous substitutions per synonymous site (Ks) by using MEGA X (Nowruzi and Blanco, 2019). A Ka/Ks ratio >1 indicates positive selection for advantageous mutations, whereas a Ka/Ks ratio <1 indicates purifying selection to prevent the spread of detrimental mutations (Leikoski et al., 2009).

Results

Phylogenetic analyses

Phylogenetic trees based on different gen markers are shown in Figs. 1 to 3. The *nifH* gene fragment and the fragment of the phycocyanin operon (*cpcA*-IGS) were amplified from both studied strains, however *mcyG* was only detected in *Aliinostoc* Me1355 strain.

The *Aliinostoc* phylogenetic trees based on the markers *PC-IGS* and 16S–23S ITS (Fig. 1) showed similar topologies. From the phylogenetic analysis based on 16 rRNA gene sequences, it is possible to observe that the studied strain is within a cluster composed by other *Nostoc* strains and its closest one is *Nostoc_elgonense*_TAU-MAC_0299 (MN062664). However, the phylogenetic trees obtained using *nifH* and 16S–23S ITS (Fig. 2) have differences in the branch positions of some strains. In the phylogeny based on the gene 16S–23S ITS, the studied strains were placed with *Nostoc_calcicola*_Ind32 (N216874) in the same cluster, However, when we look into the phylogeny based on *nifH* gene, the studied strains fall into separate clades and its closest one is *Nostoc*_sp. NQAIF320 (KJ636979), indicating that this gene probably could be the best marker for a high resolution at species level.

The highest *mcyG* sequence similarity was found to be 100% identical with *Nostoc* sp. CENA88 (Q259210) (Fig. 3). Moreover, The *Aliinostoc* phylogenetic trees based on the markers *mcyG* and 16S–23S ITS (Fig. 3) showed similar topologies. We have also compared the 16S rRNA *p*-distances of our strains with related genera, namely *Aliinostoc_morphoplasticum* NOS (KY403996_1), *Aliinostoc* sp. SA46 (MK503795), *Aliinostoc* sp. SA9 (MK503790) and *Aliinostoc magnakinetifex* SA18 (MK503791). Results showed that *Aliinostoc* sp. strain Ay1375 shared a 16S rRNA sequence similarity of 97.22% with *Aliinostoc morphoplasticum* NOS (KY403996_1), 96.52% with *Aliinostoc* sp. SA46 (MK503795), 96.80% with *Aliinostoc* sp. SA9 (MK503790) and 93.73% with *Aliinostoc magnakinetifex* SA18 (MK503791), while *Aliinostoc* sp. strain Me1355 shared a 16S rRNA sequence similarity of 97.22% with *Aliinostoc morphoplasticum* NOS (KY403996_1), 96.38% with *Aliinostoc* sp. SA46 (MK503795), 96.80% with *Aliinostoc* sp. SA9 (MK503790) and 93.59% with *Aliinostoc magnakinetifex* SA18 (MK503791) (Tab. 3).

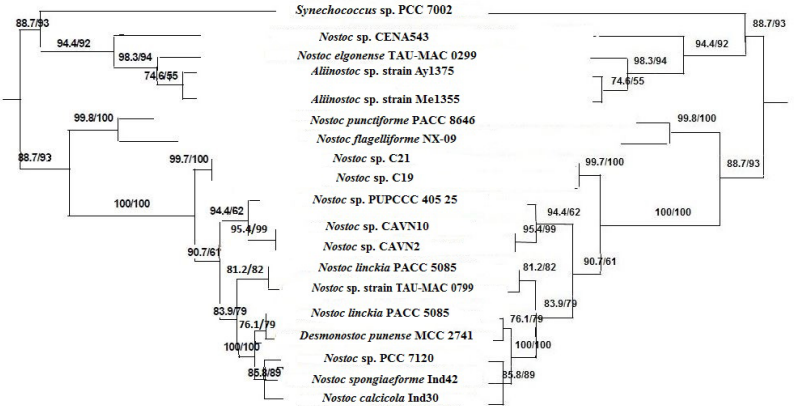

Figure 1: Congruence between phylogenies inferred from the 16S rRNA and *cpc*A-IGS sequences. A maximum-likelihood tree based on the 16SrRNA data set (left). A maximum-likelihood tree based on the *cpc*A-IGS data set (right). Numbers near nodes indicate standard bootstrap support (%) / ultrafast bootstrap support (%) for ML analyses.

Slika 1: Skladnost med nizoma podatkov nukleotidnih zaporedij 16S rRNA in *cpc*A-IGS. Drevo, ocenjeno po metodi največjega verjetja na osnovi 16S rRNA (levo). Drevo, ocenjeno po metodi največjega verjetja na osnovi *cpc*A-IGS (desno). Številke ob razvejitvah prikazujejo podporo izračunano z običajno metodo samovzorčenja (%) / ultrahitro metodo samovzorčenja (%) za analize največjega verjetja.

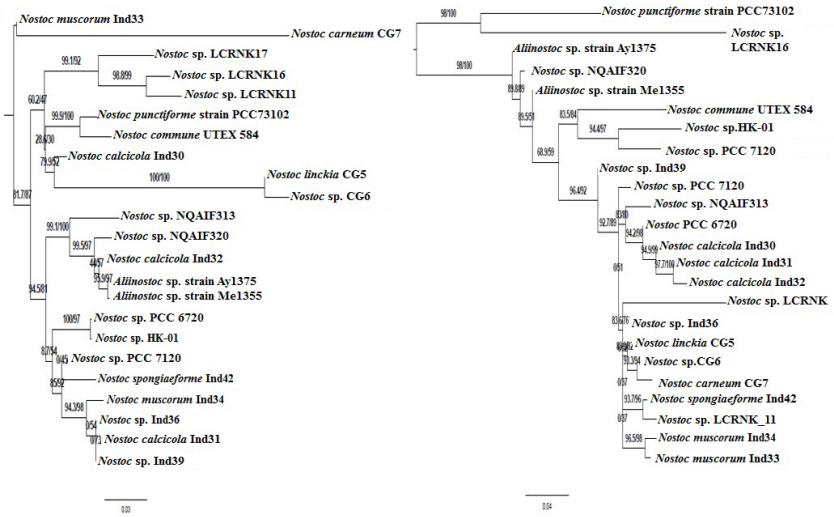

Fig. 2. Congruence between phylogenies inferred from the 16S rRNA and *nifH* sequences. A maximum-likelihood tree based on the 16SrRNA data set (left). A maximum-likelihood tree based on the *nifH* data set (right). Numbers near nodes indicate standard bootstrap support (%) / ultrafast bootstrap support (%) for ML analyses.

Slika 2: Skladnost med nizoma podatkov nukleotidnih zaporedij 16S rRNA in *nifH*. Drevo, ocenjeno po metodi največjega verjetja na osnovi 16S rRNA (levo). Drevo, ocenjeno po metodi največjega verjetja na osnovi *nifH* (desno). Številke ob razvejitvah prikazujejo podporo izračunano z običajno metodo samovzorčenja (%) / ultrahitro metodo samovzorčenja (%) za analize največjega verjetja.

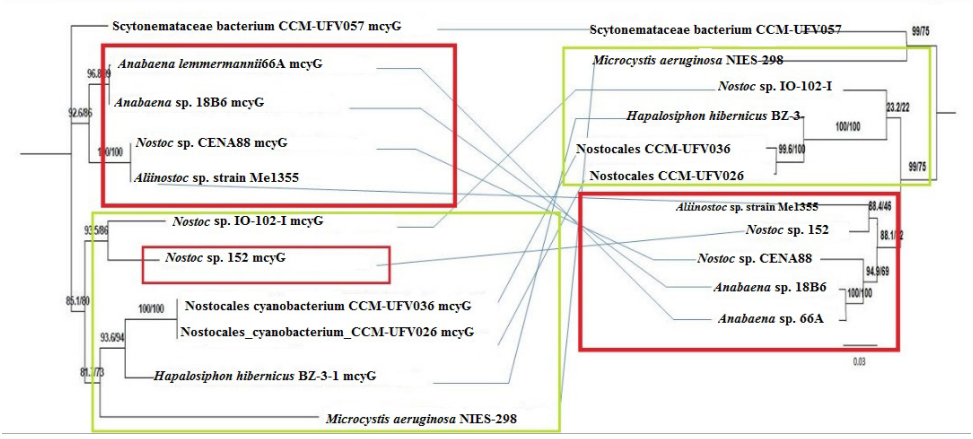

Fig. 3. Congruence between phylogenies inferred from the 16S rRNA and *mcyG* sequences. A maximum-likelihood tree based on the *mcyG* data set (left). A maximum-likelihood tree based on the 16SrRNA data set (right). Numbers near nodes indicate standard bootstrap support (%) / ultrafast bootstrap support (%) for ML analyses.

Slika 3: Skladnost med nizoma podatkov nukleotidnih zaporedij 16S rRNA in *mcyG*. Drevo, ocenjeno po metodi največjega verjetja na osnovi 16S rRNA (levo). Drevo, ocenjeno po metodi največjega verjetja na osnovi *mcyG* (desno). Številke ob razvejitvah prikazujejo podporo izračunano z običajno metodo samovzorčenja (%) / ultrahitro metodo samovzorčenja (%) za analize največjega verjetja.

Table 3. 16S rRNA gene sequence similarity matrix of studied strains and related taxa.
Tabela 3: Matrika podobnosti za nukleotidno zaporedje gena 16S rRNA pri preučevanih sevih in sorodnih taksonih.

Strain	*Aliinostoc* sp. strain Ay1375	*Aliinostoc* sp. strain Me1355	KY403996_1_ *Aliinostoc_ morphoplasticum_* NOS	MK503795_1_ *Aliinostoc_* sp_SA46	MK503790_1_ *Aliinostoc_* sp_SA9
***Aliinostoc* sp. strain Ay1375**					
***Aliinostoc* sp. strain Me1355**	0				
***Aliinostoc_morphoplasticum*_NOS**	97.22	97.22			
***Aliinostoc*_sp_SA46**	96.52	96.38	95.80		
***Aliinostoc*_sp_SA9**	96.80	96.80	95.52	99.33	
***Aliinostoc_magnakinetifex*_SA18**	93.73	93.59	94.16	95.79	95.50

16S-23S rRNA ITS secondary structure

Four reference sequences were used to search for ITS secondary structure. According to Johansen et al. (2011), nine different areas (D1-D1' helix, D2, D3, trRNA^{Ile}, trRNA^{Ala}, Box-B, Box-A and D4) were found in the ITS secondary structure of studied strain. The D1-D1' and Box-B regions of all studied strains were revealed to be very different in terms of length and shape (Fig. 4, Tab. 4).

The D1-D1' region included a terminal bilateral bulge (A), bilateral bulge (B), unilateral bulge (C), and basal clamp (D) (Fig. 4). The lengths of D1-D1' helix varied from 93 nt (*Aliinostoc* sp. strain Ay1375, *Aliinostoc* sp. strain Me1355, KY403996.1 *Aliinostoc morphoplasticum* NOS) to 60 nt (*Aliinostoc magnakinetifex* SA18) (Tab. 4). The basal stem revealed to be the same for all studied strains (5'- GACCUA- UAGGUC - 3') (Fig. 4).

Box-B was nominated by a terminal bilateral bulge (A) and bilateral bulge (B). Box-B helix was not found for *Aliinostoc magnakinetifex* SA18. As to the Box-B + spacer, lengths varied from 39 nt (*Aliinostoc morphoplasticum* NOS) to 55 nt (*Aliinostoc* sp. SA46), with studied strains showing a length of 44 nt (Fig. 4) (Tab. 5).

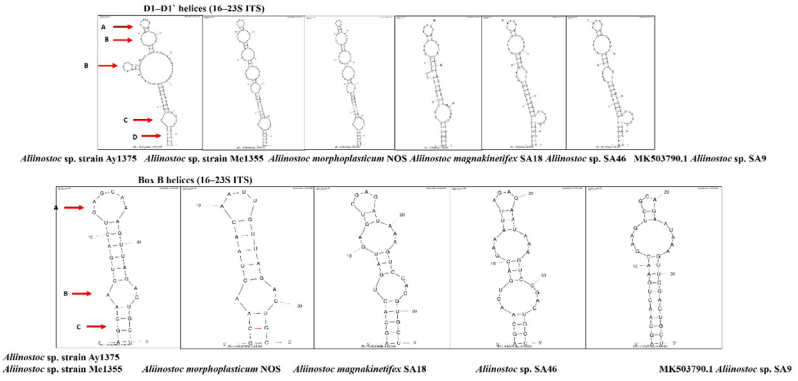

Figure 4. Comparison of secondary structures of D1–D1′ helices (upper row) and Box-B helices and V3 helices (lower row), both from 16S–23S intergenic spacers between studied strains with reference strains. Marks: A - Terminal bilateral bulge, B - bilateral bulge, C - Unilateral bulge, D - Basal clamp, arrows - bulges and basal clamp.
Slika 4: Primerjava sekundarnih struktur D1-D1' vijačnic (zgornja vrstica) ter Box-B vijačnic in V3 vijačnic (spodnja vrstica) iz 16S–23S medgenskih vmesnikov med preučevanimi in referenčnimi sevi. Oznake: A - Terminalna bilateralna izboklina, B - Bilateralna izboklina, C - Unilateralna izboklina, D – Bazalna spona, puščice – izbokline in bazalna spona.

Table 4: Nucleotide lengths of the 16S–23S ITS regions of the studied strains.
Tabela 4: Dolžine nukleotidov za območja 16S–23S ITS pri preučevanih sevih.

Strain	D1-D1' helix	spacer+D2+spacer	D3	D3 + spacer	trRNA^{Ile} gene	spacer+V2+spacer	TrRNA^{Ala} gene	BoxB+spacer	Box A	D 4
***Aliinostoc* sp. strain Ay1375**	93	39	3	30	-	-	-	44	11	9
***Aliinostoc* sp. strain Me1355**	93	39	3	30	-	-	-	44	11	9
***Aliinostoc morphoplasticum* NOS**	93	38	3	22	-	-	-	39	11	9
***Aliinostoc magnakinetifex* SA18**	60	34	3	37	-	-	-	-	-	9
***Aliinostoc* sp. SA46**	66	38	3	43	-	-	-	55	10	10
***Aliinostoc* sp. SA9**	66	40	3	46	-	-	-	54	11	9

Table 5: Comparison of secondary structure of 16S-23S rRNA (D1-D1'helix and Box-B helix) between the studied strains with reference strains.
Tabela 5: Primerjava sekundarne zgradbe 16S-23S rRNA (vijačnica D1-D1' in Box-B) med preučevanimi in referenčnimi sevi.

Strain	D1-D1'helix				Box-B		
	Terminal bilateral bulge (A)	Bilateral bulge (B)	Unilateral bulge (C)	Basal clamp (D)	Terminal bilateral bulge (A)	Bilateral bulge (B)	Basal clamp (C)
	Number of nucleotides	Number of loops	Number of loops	Number of nucleotides	Number of nucleotides	Number of loops	Number of nucleotides
***Aliinostoc* sp. strain Ay1375**	7	3	1	12	6	1	8
***Aliinostoc* sp. strain Me1355**	7	4	1	12	6	1	8
***Aliinostoc morphoplasticum* NOS**	6	4	1	12	8	1	6
***Aliinostoc magnakinetifex* SA18**	7	2	1	12	6	2	10
***Aliinostoc* sp. SA46**	7	1	2	12	6	2	8
***Aliinostoc* sp. SA9**	7	1	2	12	5	1	22

Sequence divergences

Sequence divergences in the *mcyG* gene data set were much higher than expected in an evolutionary scenario, favoring recent horizontal gene transfer as a mechanism to explain the sporadic distribution of microcystin producers among cyanobacteria. To determine whether the *mcyG* gene is under positive

or negative selection pressure, we compared the number of nonsynonymous substitutions per non-synonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks). The Ka/Ks ratio was well below 1 in pairwise comparisons from representative strains of each genus. A low Ka/Ks ratio is indicative of purifying selection in which deleterious mutations affecting the protein sequence are selected against and is consistent with an ancient origin of the *mcyG* gene.

Discussion

HGT is relatively common among cyanobacteria, but it does not affect all genes in the same way. For some genomes, gene clusters have a lower probability of being transferred (Rantala et al., 2004).

The phylograms based on *PC-IGS* and *mcyG* were mostly congruent and no clear HGT signal was found for these genes, indicating a common evolutionary pathway for the phycocyanin, *mcyG* and ribosomal genes. This result is consistent with those of Sanchis et al. (2005) and Dadheech et al. (2010), who found that *PC-IGS* and 16S–23S ITS regions of *Microcystis* and *Arthrospira* strains also showed a high similarity between marker topologies. Phylogenetic analysis of this region was largely consistent with that obtained from 16S rDNA sequence analysis and revealed a relationship between the 16S rDNA sequence and the phycobilin content of cells.

However, phylogenetic trees constructed from the *nifH* and 16S rRNA genes were incongruent. Our results suggest that the *nifH* gene encoding the dinitrogenase reductase has been transferred from one cyanobacterium to another. However, the phylogenetic incongruence detected is likely to be a result of ancient horizontal transfers of the *nifH* biosynthetic genes since the sequence divergence of the dinitrogenase reductase genes was high. The main point of discordance between in the *nifH* phylogenetic tree was the location of two studied strains, in the phylogeny based on the gene 16S–23S ITS, they were placed in the same cluster, however into the phylogeny based on *nifH* gene, the studied strain falls into separate clades. In addition, it is noteworthy that in the 16S–23S ITS proposed phylogeny, there is a high Bayesian posterior probability to support its location.

Morphological studies showed that both studied strains were morphologically similar to each other, but that two of them formed an isolated clade in the *nifH* phylogram, indicating that despite the morphological similarity, they represent genetically divergent strains. Thus, the hypothesis that the divergence of the strains observed in the *nifH* tree could have been due to HGT was confirmed.

Moreover, our analyses do not corroborate the presence of HGT in *PC-IGS* and *mcyG*, but this event cannot be neglected as a hypothesis for explaining divergences in phylogenies. A study on the genome of Synechococcus spp. indicated that genes encoding phycocyanin may have evolved independently from genes of the core genome such as the *allo-PC* gene or the ribosomal regions (Six et al. 2007).

The search for more stable markers, not biased by HGT, has become essential for understanding the phylogeny and taxonomy of cyanobacteria (Gribaldo and Brochier 2009). The results presented herein strongly support *nifH* as a marker of choice for cyanobacterial phylogenetic studies and emphasize the importance of using multiple molecular markers to prevent erroneous conclusions based on HGT.

Summary

Horizontal gene transfer (HGT), potentially followed by recombination with or replacement of resident homologues, represents an important factor in the phylogeny of prokaryotic organisms such as cyanobacteria, and shapes their evolutionar history. Nowadays, HGT seems to be a major factor in species delimitation in cyanobacteria and plays a key selection pressure leading to cyanobacterial diversification. In this study, *PC-IGS*, *nifH*, *mcyD, mycG* and the ribosomal gene spacer 16S–23S ITS as molecular markers were compared to investigate the occurrence of horizontal transfer. The phylograms based on *PC-IGS* and *mcyG* were mostly congruent and no clear HGT signal was found for these genes. However, phylogenetic trees constructed from the *nifH* and 16S rRNA genes were incongruent. The exploration for more

steady markers, not biased by HGT, has become important for detection of the phylogeny and taxonomy of cyanobacteria.

Povzetek

Horizontalni genski prenos (HGT), ki mu lahko sledi rekombinacija ali zamenjava obstoječih homolognih zaporedij, predstavlja pomemben dejavnik v filogeniji prokariontskih organizmov, kot so cianobakterije, in oblikuje njihovo evolucijsko zgodovino. Danes se zdi, da je HGT glavni dejavnik pri razmejitvi vrst pri cianobakterijah in je ključni selekcijski pritisk, ki vodi v diverzifikacijo cianobakterij. V tej študiji smo primerjali nukleotidna zaporedja *PC-IGS*, *nifH*, *mcyD*, *mycG* in ribosomski medgenski vmesnik 16S–23S ITS kot molekularne označevalce, da bi raziskali pojav HGT. Filogenetska drevesa, ki temeljijo na nukleotidnih zaporedijh *PC-IGS* in *mcyG* so si med seboj bila večinoma skladna, tzato lahko za ta nukleotidna zaporedja predvidevamo, da se niso prenašala s HGT. Filogenetska drevesa, ki so bila narejena na podlagi nukleotidnih zaporedij *nifH* in genov za 16S rRNA, so bila med seboj neskladna, kar nakazuje na HGT. Raziskovanje bolj stabilnih označevalcev, na katere HGT ne vpliva, je postalo pomembno za odkrivanje filogenije in taksonomije cianobakterij.

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Plants in changing evironment – mednarodna konferenca Slovenskega društva za biologijo rastlin

Plants in changing evironment – International conference of the Slovenian Society of Plant Biology

Prejemnika nagrade »nada«, prof. dr. Marina Dermastia in prof. dr. Dominik Vodnik (foto: Aleš Kladnik).

Slovensko društvo za biologijo rastlin je 15. in 16. septembra 2022 v Biološkem središču v Ljubljani že osmič organiziralo mednarodno srečanje na temo biologije rastlin. Letos smo konferenco naslovili »Plants in Changing Environment« (slo. »Rastline v spreminjajočem se okolju«) in tako še posebej izpostavili pomen rastlin v luči zaskrbljujočih okoljskih razmer. Rastline so namreč temelj prehranske varnosti in hkrati blažijo negativne vplive klimatskih sprememb v okolju. Zanimiv program z enajstimi vabljenimi predavatelji je privabil skoraj sto udeležencev iz kar 11 različnih držav, ki so svoje delo predstavili še v okviru 18 kratkih predavanj in 38 posterjev.

Konferenco je otvorila Maria J. Pozo iz CSIC Granada (Španija) s predavanjem o ugodnih vplivih mikroorganizmov na rast rastlin in njihovo odpornost na okoljske dejavnike. Predavanje je bilo odličen uvod v naslednjo sekcijo, kjer so bile predstavljene različne aplikativne in agronomske raziskave, od vpliva klimatskih sprememb na razširjanje povzročiteljev bolezni, njihove detekcije v različnih okoljih in razvoja alternativnih sredstev za zaščito rastlin. V sekciji »Metabolizem struktura in funkcija rastlin« so bile predstavljene različne visokozmogljive tehnike, od rentgenskih tehnik za odkrivanje elementov v sledovih v ratslinskih tkivih, fenotipizacije ter raziskave metabolizma glutationa. V najobsežnejši sekciji »Interakcije rastlin z drugimi organizmi« so bile predstavljene raziskave mikrobioma rastlin, endofitov ter virusnih povzročiteljev bolezni; po drugi strani pa različni vidiki imunskega odziva krompirja in vinske trte ter alelopatsko delovanje dresnika. V luči spreminjajočega okolja smo obravnavali tudi naravne ekosisteme, evolucijski potencial in fenotipsko plastičnost v gozdnih ekosistemih, vpliv abiotskih dejavnikov na mikorizne glive hrasta in razvoja lesa ter diferenciacijo in razširjenost bekic. V zadnji sekciji, »Interakcije rastlin z okoljem« so bile predstavljeni različni vidiki oksidativnega stresa rastlin ter raziskave odziva vinske trte na stresne dejavnike. Slednja tema je bila odličen uvod v zaključno predavanje, ko je Mario Pezotti z Univerze v Veroni (Italija) predstavil različne primere »omskih« raziskav vinske trte, ki je zaradi klimatskih sprememb še posebej prizadeta. Knjiga povzetkov konference je dostopna na spletni strani društva https://www.plantslo.org/wp/conference2022/.

Slovensko društvo za biologijo rastlin, ki je bilo sicer ustanovljeno kot Slovensko društvo za rastlinsko fiziologijo, je aktivno od leta 1993 in združuje rastlinske biologe, ki delujejo na različnih področjih. Društvo mednarodna srečanja organizira vsake 4 leta, prvo je bilo organizirano leta 1993. Na tokratnem srečanju smo obeležili 40-letnico neprekinjenega delovanja društva in prvič podelili nagrade »nada«, ki nas po eni strani spominjajo na prof. dr. Nado Gogala, po drugi strani pa simbolizirajo upanje. Nagradi sta za dolgoletno in požrtvovalno delovanje v društvu, s katerim sta trajno prispevala k njegovemu delovanju in prepoznavnosti, prejela prof. dr. Marina Dermastia in prof. dr. Dominik Vodnik, ki sta izvedla tudi priložnostni predavanji o zgodovini društva in o vplivu atmosferskega sušenja na rastline.

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Ponovno je bila konferenca, poleg obravnave aktualnih tematik, odlična priložnost za povezovanje strokovnjakov z različnih področij biologije rastlin v Sloveniji in okolici, in ne dvomimo, da bo krepitev obstoječih navezav in vzpostavitev novih vodila v nova sodelovanja.

Špela Baebler
predsednica organizacijskega odbora

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