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Genome analysis of manure and soil-dwelling *Acinetobacter* strains indicates potential health risks associated with antibiotic resistance and virulence factors

Alper Dede [a,b,c,*], Eduardo Pérez-Valera [a,d], Dana Elhottová [a,**]

[a] Biology Centre of the Czech Academy of Sciences, Institute of Soil Biology and Biogeochemistry, Na Sádkách 7, 37005, České Budějovice, Czech Republic
[b] University of South Bohemia, Faculty of Science, Branišovská, 37005, České Budějovice, Czech Republic
[c] Biology Centre of the Czech Academy of Sciences, Institute of Parasitology, Branišovská 1160/31, 37005, České Budějovice, Czech Republic
[d] INRAE, Université de Bourgogne, Institut Agro Dijon, Agroécologie, 21000, Dijon, France

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ABSTRACT

The genus *Acinetobacter* includes opportunistic pathogenic species of increasing clinical importance due to their ability to resist multiple antibiotics and to face multiple environmental stresses. Here, comprehensive whole-genome analysis was used to reveal the presence of genes potentially related to pathogenicity in 11 tetracycline-resistant *Acinetobacter* isolates from manure of dairy cows under tetracycline and amoxicillin prophylaxis and from soils affected by this manure. The isolates were identified as *Acinetobacter pseudolwoffii*, *A. gerneri*, *A. gandensis*, and *A. amyesii* and screened their genomes for the presence of genes related to host colonization, infection, or environmental persistence. We detected 164 genes of antimicrobial resistance, virulence factors, mobile genetic elements, or biofilm formation. All isolates were predicted to be human pathogens with high probability (0.7–0.8) and the manure isolates identified as *A. pseudolwoffii* carried a top-risk human-associated aminoglycoside resistance gene. The phenotypic characterization of the isolates showed that they could all grow at 37 °C, some up to 41 °C, and that they showed differential susceptibility to several antibiotics, with one isolate identified as *A. gandensis* being multi-resistant. Our analyses suggest that environmental *Acinetobacter* strains from fresh manure and manured soils possess risky antibiotic-resistance genes and phenotypic traits of clinical relevance indicating potential pathogenicity.

1. Introduction

Acinetobacter is a genus of the family Moraxellaceae (Gammaproteobacteria) comprising strictly aerobic, Gram-stain-negative, and non-spore forming bacteria [1], which in April 2025 included 85 species with correct binominal names (https://lpsn.dsmz.de/genus/acinetobacter, accessed April 08, 2025). Some *Acinetobacter* species are responsible for infections of the blood [2], wounds [3], or urinary tract [4] as well as for lung infections (pneumonia) [5]. The main reservoirs of culturable *Acinetobacter* are soil and aquatic environments such as natural and waste water [6,7], although they can also be isolated from various animal and plant hosts, and food products [1,4,8–10]. Garcia-Garcera et al [11] showed that phylogenetically close *Acinetobacter* species were more frequently found in similar environments than distantly related taxa and revealed the distinctness of three main *Acinetobacter* clades. Two clades were associated with (i) aquatic environments and (ii) waste water together with marine sediments. The third clade including the *A. calcoaceticus - A. baumannii (ACB)* complex has been mostly found in soil and human-associated environments [11].

Although the genus *Acinetobacter* is not found in extreme

Abbreviations: tet, Tetracycline; AMR, Antimicrobial resistance; VF, Virulence factor; gDNA, Genomic DNA; *bla*OXA, OXA β-lactamase; ATB, Antibiotic; ANI, Average nucleotide identity; GTDB, Genome taxonomy database; BCCO, Biology Centre Collections of Organisms; PFDB, protein family database; PNAG, polyglycolic acid; WGS, whole genome sequencing; MFS, major facilitator superfamily; SMR, small multidrug resistance; MDR or MATE, multidrug and toxin extrusion; ABC, ATP-binding cassette; RND, resistance-nodulation-division; PCA, principal component analysis; HGT, horizontal gene transfer.

* Corresponding author. Biology Centre of the Czech Academy of Sciences, Institute of Soil Biology and Biogeochemistry, Na Sádkách 7, 37005, České Budějovice, Czech Republic.
** Corresponding author.
E-mail addresses: dede.alper@gmail.com (A. Dede), danael@upb.cas.cz (D. Elhottová).

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environments, they are well equipped to overcome environmental obstacles [12]. This is at least partly due to their ability to withstand drying and disinfection as well as their resistance to numerous antibiotic compounds in the environment. Antimicrobial resistance (AMR) can be intrinsic or acquired [13]. Intrinsic antibiotic resistance mechanisms are mostly conserved in chromosomal DNA and include non-specific efflux pumps evolved as a general response to environmental toxins, antibiotic inactivating enzymes, or mechanisms that serve as permeability barriers [14]. Acquired resistance is related to horizontal gene transfer (HGT) mechanisms through mobile genetic elements such as transposons, plasmids, integrons and phages [15]. This mechanism includes plasmid-encoded specific efflux pumps, enzymes that modify the antibiotic, and enzymes that mediate the protection of the antibiotic's target site [14]. Antibiotic resistance in *Acinetobacter* encompasses all the AMR mechanisms listed, including the acquisition of mobile genetic elements and the ability to survive in the environment, which is most likely associated with biofilm production. These factors have contributed to the emergence of multidrug-resistant, extensively drug-resistant, and pandrug-resistant *A. baumannii* strains [1,16]. Other traits important for dispersal and, depending on the host and conditions, pathogenicity [17] include virulence factors [18], biofilm formation, motility [19], quorum sensing [20], accumulation of reserve compounds, production of biopolymers [21] and synthesis of secondary metabolites [22]. These traits contribute to effective stress response mechanisms and are important for adaptation to diverse conditions. The ability to acquire genes responsible for antibiotic resistance, alongside factors related to environmental persistence and spread, may have driven the transformation of previously harmless *Acinetobacter* representatives into opportunistic pathogens. Such an example is represented by *A. baumannii,* originally considered harmless but now recognized as one of the most important nosocomial pathogens in intensive care units [23]. Additionally, other *Acinetobacter* species are emerging as potential nosocomial agents [24], and therefore a better understanding of the genomic potential of environmental *Acinetobacter* strains to carry genes related to antibiotic resistance and virulence is needed. This understanding is currently still insufficient.

The overuse of antimicrobial agents is considered to be one of the main causes of the increasing resistance of pathogenic bacteria worldwide [25]. The use of antimicrobials in agricultural production contributes to the spread of antibiotic-resistant and potentially pathogenic bacteria [26]), including the genus *Acinetobacter* [14,19,27,28]. Antibiotics derived from tetracycline and penicillin represent the largest proportion of all used antimicrobial agents in animal health globally [29] and contribute to the spreading of antimicrobial resistance in livestock. From the agricultural perspective, land treated or exposed with manure from animals treated by antimicrobial agents loads the soil with antimicrobial residues [30] and spreads AMR-related bacteria and genes to the environment [31]. Pulami et al. [32,33] showed that raw as well as digested manure is considered as a source of pathogenic and antibiotic resistant *Acinetobacter* strains. Nevertheless, information on the spread, persistence and potential health risks of *Acinetobacter* in manure-affected soils remains limited. Pérez-Valera et al. [34] demonstrated that cattle manure application triggers a short-term but significant dominance of *Acinetobacter* within both total and cultivable soil microbial communities. They also observed that soil microbes prevent the spread of *Acinetobacter* and other potentially risky taxa [34,35]. Sardar et al. [36] confirmed the potential of culturable *Acinetobacter* spp. to accumulate antimicrobial resistance in pasture soil receiving fresh manure.

The purpose of this study was to assess the potential pathogenicity risk of 11 tetracycline-resistant *Acinetobacter* isolates obtained from the manure of dairy cows under tetracycline and amoxicillin prophylaxis, as well as from soils affected by this manure. A comprehensive genome-wide analysis was conducted, including taxonomic classification, detection and identification of genes associated with risky characters such as antibiotic resistance, virulence or quorum sensing/quenching, and pathogenicity prediction. We also evaluated the ability of the isolates to grow at temperatures of 37 °C 40 °C, 41 °C, and 44 °C, as well as the resistance to clinically important antibiotics as indicators of risky phenotypes.

2. Methods

2.1. Isolation of Acinetobacter from raw manure and manure-soil microcosms

Eleven *Acinetobacter* isolates were obtained from soil microcosms established to analyze the effects of cattle manure on the resistome of antibiotic-free pasture soils, as described by Pérez-Valera et al. [35]. Briefly, fresh manure from cattle under antibiotic prophylaxis with chlortetracycline and amoxicillin [31] was applied to soils from organic farms in laboratory microcosms [35]. The bacteria were isolated using the serial plate dilution method from fresh manure at the start of the experiment and from the soil beneath the top manure layer after incubating the microcosms for seven days. Plates containing CHROMagar Acinetobacter (CHROMagar, France), tryptic soya agar (TSA) (BD, France) and ENDO agar (Difco™, USA) were inoculated with 0.1 mL of manure or soil suspensions diluted to concentrations ranging from 10^{-1} to 10^{-6}. CHROMagar and TSA plates were incubated at 28 °C for 24 h and seven days, and ENDOagar plates at 37 °C for 24 h. A total of 1377 bacterial colonies were isolated and tested for growth on fresh plates supplemented with tetracycline (Sigma, USA) (30 mg L^{-1}). 135 tetracycline-resistant isolates were selected and further identified [35]. Of these, three isolates from fresh manure and eight isolates from soil, classified as *Acinetobacter* using Sanger sequencing of 16S rRNA amplicons [35], were further selected for downstream analyses (Table 1).

2.2. Genomic DNA extraction and whole-genome sequencing

Genomic DNA from the eleven isolates was isolated from freshly grown pure cultures on TSA at 28 °C for 72 h using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's recommendations. DNA quality was checked by electrophoresis in 1 % agarose gels in 0.5X Tris-acetate–EDTA buffer and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Whole-genome sequencing was conducted on the Illumina NovaSeq 6000 platform with 150-bp paired-end technology using the services of a commercial provider (Novogene, Beijing, China). Sequencing libraries were prepared from 1.0 μg DNA per isolate using the NEBNext DNA Library Prep Kit (NEB, USA) according to the manufacturer's recommendations. Indexes were added to each sample. Genomic DNA was randomly fragmented by sonication to a size of 350 bp. DNA fragments were end-polished, A-tailed, and ligated using the NEBNext adapter for Illumina sequencing and further enriched by PCR with P5 and indexed P7 oligos. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Inc). The resulting libraries were analyzed for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and quantified using real-time PCR (to meet the criteria of 3 nM).

2.3. Sequence processing and taxonomic identification

DNA sequencing produced 3 to 6 million reads per isolate. DNA reads were processed to trim Illumina adapters and remove low-quality reads using fastp v0.23.3 [37]. Contaminants (masked against contaminant references such as human DNA, according to https://www.seqanswers.com/forum/bioinformatics/bioinformatics-aa/37175-introducing-removehuman-human-contaminant-removal?t=42552) were removed using BBmap and BBduk v39.00 [38]. DNA reads were assembled with Unicycler v3.0.8 [39]. 16S rRNA genes were retrieved from the assembled genomes with ContEst16S [40] and RASTtk [41], and uploaded to the EzTaxon server [42] for taxonomic identification. Contigs shorter than 200 bp were excluded from further analyses. Assembled genomes were

Table 1
Genome features and taxonomic identification based on the genomes and 16S rRNA genes of 11 *Acinetobacter* isolates used in this study. Additionally, seven reference *Acinetobacter* genomes (5 type strains and 2 host-associated *A. baumannii* strains) used for comparative purposes.

Microorganism	Short name	Isolation source	GTDB closest organism	GTDB ANI (%)	Genome size (bp)	G + C rate (%)	Completeness (%)	Contamination (%)	Contig N50 (bp)	Protein encoding genes	Plasmid contigs	16S rDNA closest organism	16S similarity (%)
BCCO 40_1098	M1098	Manure (cattle)	*A. pseudolwoffii*	97.55	2,974,876	43.20	100	0.1	117,855	2886	10	*A. pseudolwoffii*	100
BCCO 40_1099	M1099	Manure (cattle)	*A. pseudolwoffii*	97.72	2,939,610	43.28	100	0.1	111,378	2836	9	*A. pseudolwoffii*	100
BCCO 40_1100	M1100	Manure (cattle)	*A. gerneri*	98.09	4,108,232	37.47	100	0.2	132,814	4011	8	*A. gerneri*	99.76
BCCO 40_1137	S1137	Soil under manure	*A. amyesii*	97.37	3,487,629	40.34	100	0.0	302,965	3527	6	*A. amyesii*	99.93
BCCO 40_1141	S1141	Soil under manure	*A. gandensis*	98.80	3,196,505	39.79	100	0.0	148,012	3125	1	*A. gandensis*	99.86
BCCO 40_1153	S1153	Soil under manure	*A. gandensis*	98.81	3,078,895	39.88	100	0.3	142,734	2974	2	*A. gandensis*	99.86
BCCO 40_1199	S1199	Soil under manure	*A. gandensis*	98.91	3,260,042	39.82	100	0.1	163,304	3224	4	*A. gandensis*	99.86
BCCO 40_1202	S1202	Soil under manure	*A. amyesii*	97.38	3,453,823	40.33	100	0.0	299,103	3463	6	*A. amyesii*	99.93
BCCO 40_1213	S1213	Soil under manure	*A. gandensis*	98.80	3,154,271	39.81	100	0.2	124,686	3061	1	*A. gandensis*	99.27
BCCO 40_1214	S1214	Soil under manure	*Acinetobacter* sp	98.03	2,628,783	41.54	100	0.0	113,977	2553	1	*A. towneri*	98.92
BCCO 40_1215	S1215	Soil under manure	*A. gandensis*	98.89	3,154,556	39.85	100	0.1	128,664	3117	2	*A. gandensis*	100
***A. gerneri* CIP 107464^T**		Sludge plant	*A. gerneri*	100	4,595,172	37.74	100	0.2	564,812	4420	21	*A. gerneri*	100
***A. gandensis* ANC 4275^T**		Feces (Horse)	*A. gandensis*	100	3,176,356	39.68	100	0.1	266,198	3062	2	*A. gandensis*	99.86
***A. pseudolwoffii* ANC 5044^T**		Water sediment	*A. pseudolwoffii*	100	3,105,311	43.25	100	0.5	452,618	2982	3	*A. pseudolwoffii*	100
A. amyesii ANC 5579^T		Feces (cow), soil	*A. amyesii*	100	3,454,033	40.46	100	0.3	248,864	3373	2	*A. amyesii*	100
***A. baumanii* ATCC 19606^T**		Urine (human)	*A. baumanii*	100	4,026,493	39.32	100	0.1	1,115,199	3915	3	*A. baumannii*	100
A. baumanii		Manure (cattle)	*A. baumanii*	97.87	3,743,112	38.84	100	0.4	349,793	3531	1	*A. baumannii*	99.86
A. baumanii		Unknown (human)	*A. baumanii*	97.88	3,932,447	39.25	100	0.1	3,752,576	3829	3	*A. baumannii*	100

identified using GTDB-tk v2.4.0 [43] and GTDB R220 [44]. Genome completeness and contamination were estimated by CheckM2 v1.0.2 [45]. A phylogenetic tree encompassing all *Acinetobacter* genomes was computed on the TYGS website [46]. The phylogenetic tree was visualized using iTOL [47]. Similarity and clonal differences between the isolates and the reference strains were analyzed according to digital DNA: DNA hybridization (dDDH)-d4 values [48] and average nucleotide identity (ANIb) values by JSpeciesWS (using BLAST+) [49].

Genomes of the reference strains *A. gerneri* CIP 107464^T (accession number GCA_000368565.1), *A. gandensis* ANC 4275^T (GCA_001678755.1), *A. pseudolwoffii* ANC 5044^T (GCA_002803605.1), *A. amyesii* ANC 5579^T (GCA_023499985.1) and *A. baumannii* ATCC 19606^T (GCA_009759685.1) were retrieved from NCBI and incorporated in our study for comparative purposes. Two host-associated *A. baumannii* strains from cattle (GCA_023499155.1) and human hosts (GCA_004564115.1) were also added.

2.4. Genome annotation and comprehensive genome analysis

Whole genome annotation and annotation specific to potential risk genes were performed for our isolates and the *Acinetobacter* reference genomes using the following tools with default parameters, unless otherwise stated. Briefly, PATRIC v.3.22.0 [50], RASTtk v.1.073 [41] and Prokka v.1.14.5 [51] were used for whole genome annotation. Features related to AMR and virulence were further detected using RGI v.6.0 [52] with the CARD v.3.2.5 database [53], ResFinder CGE v.4.1 [54] and abricate v.5.1.16(1) (with the CARD database for AMR and the vfdb database for virulence genes) (https://github.com/tseemann/abricate). Moreover, the detection of AMR genes and their locations was evaluated using VRprofile v.2.0 [55]. Potential health risk traits associated with AMR and its spreading in the soil by MGE were evaluated using MGEfinder v.1.0.3 with 80 % sequence identity [56]. Health risk of AMR genes was assessed using arg_ranker v2.0 [57], which identifies and classifies genes into four risk categories, based on whether the AMR genes are enriched in human-associated environments, their mobility and presence in ESKAPE pathogens (top risk, category I) to genes that do not represent a current or future threat (low risk, category IV) [57]. Genes involved in biofilm production, quorum sensing, quorum quenching, accumulation of reserve compounds, and potential biopolymer were screened with the protein and gene databases and examined manually from abricate, ResFinder, CARD (card.mcmaster.ca) v.rgi 5.2.1 (web), antiSMASH v. 6.1.1 [58], Snapgene v.6.1.2 (www.snapgene.com) and ARTS v.2.0 [59]. The chromosomal or plasmid origin of contigs was predicted using Deeplasmid [60] and VRprofile2 [55].

Pathogenicity potential of the isolates was predicted using PathogenFinder [61]. This tool detects and compares proteins of each *Acinetobacter* isolate to a protein family database (PFDB) composed of groups of proteins that are associated with pathogenic and non-pathogenic organisms. Then, PathogenFinder predicts pathogenicity by assigning a probability, which ranges from 0 (non-pathogenic) to 1 (pathogenic). The origin of replication (*oriC*) was predicted using Ori-Finder 2022 [62] and resistance islands using IslandViewer 4 [63].

2.5. Susceptibility tests to antibiotics and growth at 37 °C, 40 °C, 41 °C and 44 °C

Antibiotic susceptibility of the isolates was assessed using the disk diffusion method [64]. The isolates. The isolates were first grown on TSA agar at 28 °C for 24 h. A homogeneous bacterial suspension was prepared by vortexing (Vortex-Genie2; Mo Bio Laboratories, Inc., Carlsbad, California) of several colonies in 4 mL of sterile 0.9 % NaCl. The turbidity of the suspension was adjusted with sterile 0.9 % NaCl to match the McFarland standard 0.5 (densitometer DEN-1, Biosan, Riga, Latvia). The suspension was spread onto Mueller-Hinton agar medium (Bio-Rad, Hercules, California) supplied with antibiotic disks (Bio-Rad; The FEP – Cefepime 30 μg, AKN – Amikacin 30 μg, GME – Gentamicin 30 μg, DOX – Doxycycline 30 μg, MNO – Minocycline 30 μg, CIP – Ciprofloxacin 5 μg, SXT – Trimethoprim + sulfamethoxazole 1.25 + 23.75 μg, COL – Colistin 10 μg, PTZ – Piperacillin + Tazobactam 30 + 6 μg). Inhibition zone diameters were recorded after 24 h of incubation at 28 °C. *E. coli* ATCC 25922 was used as a quality control; zone diameters for QC were in the recommended range. The criteria specified by the CLSI (https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf) were used to interpret the zones of inhibition for antibiotics. The ability of the isolates to grow at 37 °C and 40 °C was tested on TSA plates for 24 h in thermostat-controlled incubators. The isolates that grew at 40 °C were further tested using the standard clinical method, i.e., growth in liquid medium in an electronically controlled water bath on brain-heart infusion broth (Oxoid) at 41 °C and 44 °C for 48 h [65].

2.6. Statistical analysis

Hierarchical clustering of all isolates and reference *Acinetobacter* genomes was performed based on the presence and absence of detected genes, visualized as a heatmap with a coupled dendrogram using the heatmap.2 function of gplots v3.1.3.1 [66] in R 4.2.2 [67]. In addition, Principal Component Analysis (PCA) was conducted with the prcomp function in R, using features from all genomes. Features with low loadings in the analysis were excluded from the visualization.

3. Results

3.1. Identification and genome-based characterization of Acinetobacter isolates

The results of the identification of isolates based on 16S rRNA sequences (retrieved from the genomes) and whole genomes using the GTDB and TYGS databases were consistent (Fig. 1; Table 1). Manure isolates M1098 and M1099 were assigned to *A. pseudolwoffii*, while M1100 was identified as *A. gerneri* (all ANI>97 %, Supplementary Table S1; dDDH D4 value 74.4 and 81.4 % respectively, Supplementary Table S2). Soil isolates S1141, S1153, S1199, S1213, and S1215 were identified as *A. gandensis* (dDDH d4 values between 87.2 % and 88.7 %), while S1137 and S1202 were allocated to *A. amyesii* (all ANI>97 %; dDDH d4 value 74.8 %). The isolate S1214 remained unclassified according to GTDB and TYGS. All genomes had completeness values of 100 % and contamination <0.5 %. The G + C content varied between 37 and 43 %. The genomes identified as *A. pseudolwoffii* were approximately 2.9 Mb in size, while the one identified as *A. gerneri* (M1100) was approximately 4.1 Mb. Protein-encoding genes ranged from 2553 of S1214 to 4011 of M1100. tRNA counts varied from 65 of S1214 to 84 of M1100 and rRNA counts from 2 of S1153 and S1213 to 5 of M1100. The number of contigs predicted as plasmids varied between 1 and 10, with higher numbers (8−10 per isolate) in manure isolates compared to soil isolates. Replication origins (*oriC* regions) were predicted for all *Acinetobacter* isolates; the *oriC* positions in the genome and the distribution of functional elements (i.e., *dnaA* boxes, *dnaA*-trio, and binding sites of *ctrA*, *fis*, and IHF) are shown in Supplementary Fig. S1 and Supplementary Table S3.

3.2. Presence of genes associated with AMR, virulence, persistence, and dissemination in the environment

A total of 164 genes related to potentially risky traits were identified (Supplementary Table S4). Among these, 60 genes were related to antimicrobial resistance (AMR) (Supplementary Table S4, section A). Most genes involved in antibiotic resistance through alteration of the target site were shared across all isolates (24 out of 28 genes), while those encoding efflux pumps showed greater variability among isolates, with only 5 out of 17 genes being common. AMR genes related to antibiotic inactivation (15 genes) were isolate-specific, with no single gene present in all genomes.

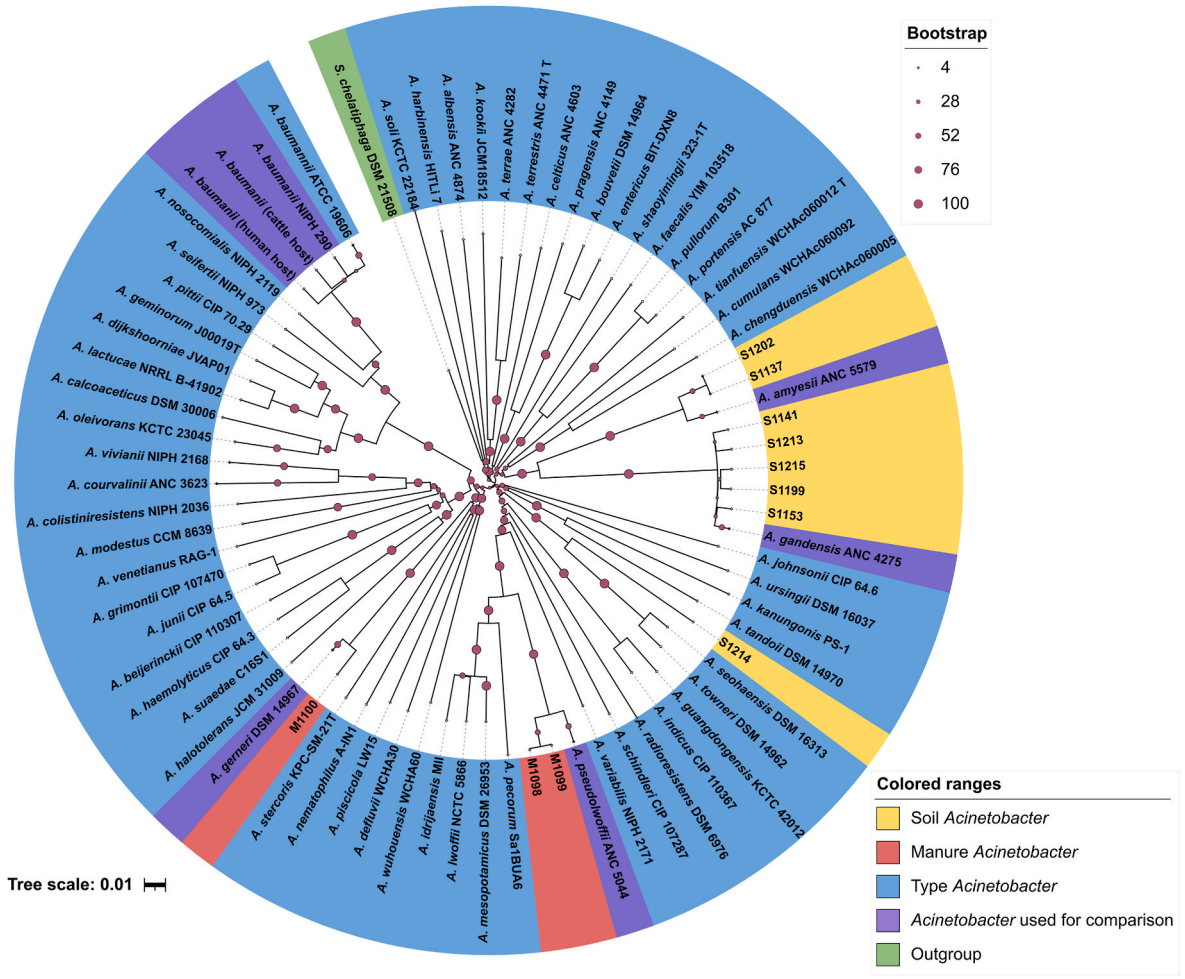


Fig. 1. Phylogenetic tree at the genomic level of all *Acinetobacter* isolates and their closest relatives. The genomic phylogenetic relationships [96] of *Acinetobacter* species were plotted using iTOL [47]. *Stenotrophomonas chelatiphaga* was selected as the outgroup.

The genes identified as virulence factors (VF) were found to be associated with numerous mechanisms, including adherence, modulation of host immunity, release of toxins, ability to form biofilms, and desiccation tolerance (Supplementary Table S4, section B). All genomes had the same four genes related to virulence adherence mechanisms. Thirty genes related to mobile genetic elements (MGE) were detected, but none were common to all *Acinetobacter* genomes (Supplementary Table S4, section C). According to the VR2 database, several AMR genes associated with tetracycline, aminoglycoside, sulfonamide, and carbapenem resistance were found. The genes related to tetracycline, aminoglycoside, and sulfonamide resistance were encountered in contigs either predicted to be of plasmid or chromosomal origin (Fig. 2, Supplementary Table S5). Antibiotic-resistance genes tended to be located within genomic regions predicted as resistance islands (Supplementary Table S6). Carbapenem resistance genes (*bla*) were only found in contigs of chromosomal origin. Several MGE (e.g. IS1 transposon) were located near genes related to tetracycline –*tet*(Y), *tet*(H) and *tet*(39)-, aminoglycoside -*aph*(3), *aph*(6)-, and sulfonamide -*sul*2- resistance.

The potential ability to form biofilms was represented by twenty-nine different genes, nine of which were common to all genomes (Supplementary Table S4, section D). Genes related to motility and chemotaxis, quorum quenching, and quorum sensing are summarized in Supplementary Table S4, sections E, F, and G, respectively. Four genes (*pilC*, *pilG*, *pilH*, and *pilT*) related to pili-movement mechanisms in all *Acinetobacter* genomes were also detected as virulence factors (Supplementary Table S4, section B). All genomes included genes linked to the production of reserve compounds such as peptidoglycan, wax esters, polyhydroxyalkanoates, and cyanophycin (Supplementary Table S4, section H). Conversely, the presence of genes related to the production of the polyglycolide (PNAG), as well as synthesis of secondary metabolites (fengycin, mycosubtilin, plipastatin, berninamycin K, APE Vf, lankacidin C, acinetoferrin, TP-1161, acinetobactin, propanochelin) depended on the isolate (Supplementary Table S4, section Iand Section J, respectively).

3.3. Genome clustering according to the presence of risky traits

Hierarchical clustering based on the presence or absence of risky traits resulted in a grouping similar to the phylogenetic relationships of the isolates (Fig. 3). Three major clusters were identified according to the *Acinetobacter* species detected. The cluster that most differed from the others was formed by manure isolates M1098 and M1099 with the reference strains *A. pseudolwoffii* and the soil isolate S1214 (Group 1). The second big cluster consisted of all *A. baumannii* genomes (Group 2) and was separated from the third cluster (Group 3) which contained the remaining isolates and reference strains. Group 3 consisted of three subgroups determined by the taxonomic species: soil isolates S1153, S1213, S1199, S1141, and S1215 together with the reference strain *A. gandensis* (subgroup 3.1), manure isolate M1100 and the reference strain *A. gerneri* (subgroup 3.2) and soil isolates S1137 and S1202 and the reference strain *A. amyesii* (subgroup 3.3). The most relevant traits related to the hierarchical clustering are described in the Supplementary results and shown in the heatmap (Fig. 3) and the PCA plot (Supplementary Fig. S2).

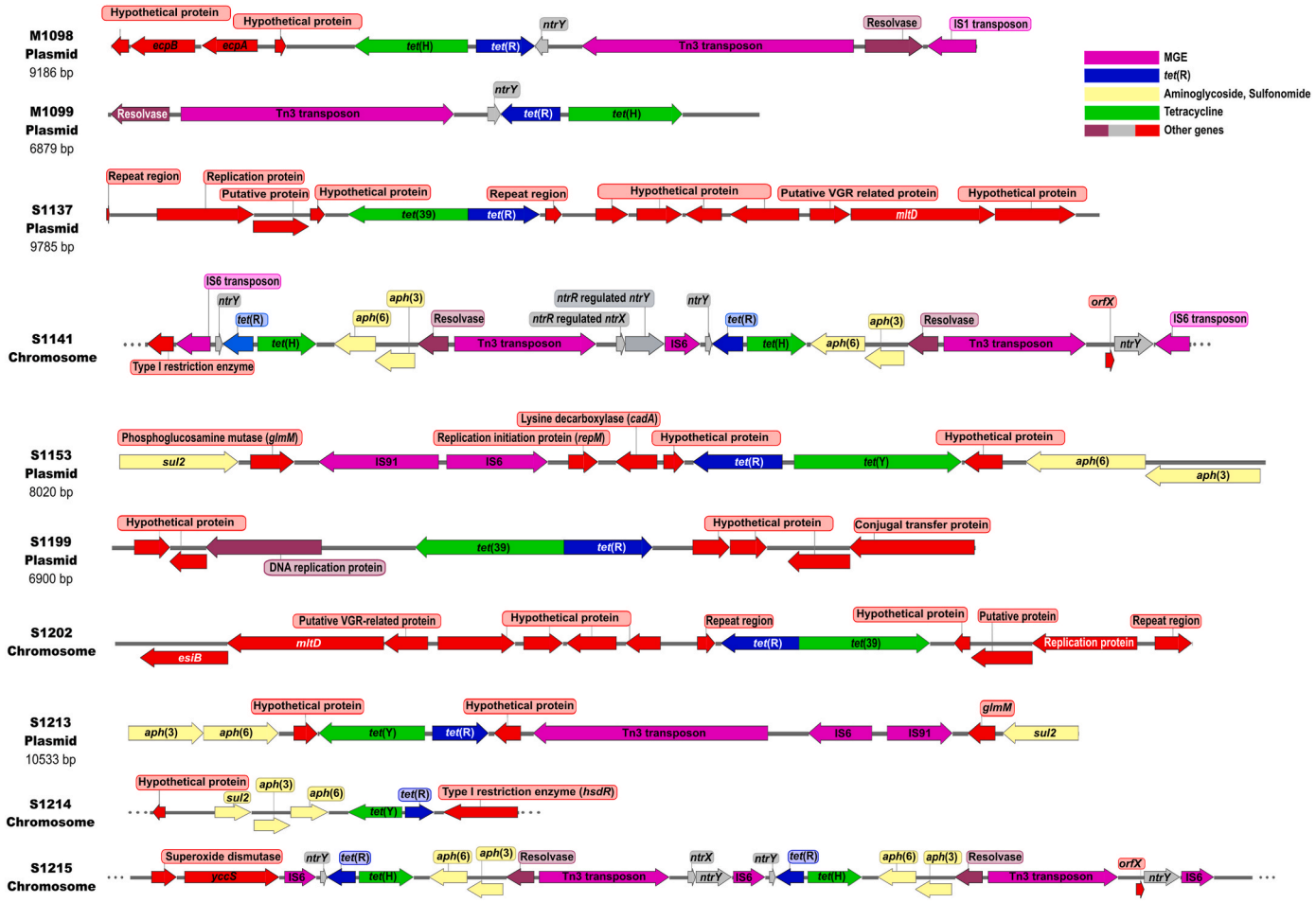


Fig. 2. Genetic maps of contigs carrying the *tet*(Y), *tet*(H), *tet*(39) genes and MGE compositions in the different *Acinetobacter* isolates. The represented contigs are all part of predicted resistance islands (see the highlighted resistance island in Supplementary Table S6).

3.4. Evaluation of potential health risks of environmental Acinetobacter isolates

According to CGE PathogenFinder, all *Acinetobacter* isolates and two of the reference strains, i.e., *A. gerneri* (isolated from sludge plant) and *A. amyesii* (isolated from cow feces), were predicted as human pathogens (Table 2). All predictions of pathogenicity were confirmed by at least 14 pathogenic gene families, most commonly found in *A. baumannii*. The other two reference strains, i.e., *A. gandensis* (isolated from horse feces) and *A. pseudolwoffii* (isolated from soil sediment), were not predicted as human pathogens.

A top risky mobile human-associated variant of the gene *aph*(6)-Id, related to aminoglycoside resistance (Rank I according to Ref. [57]), was found in the manure isolates M1098 and M1099 (*A. pseudolwoffii*). Two other aminoglycoside resistance genes, *aph*(3')-VIa and *aac*(3)-IId, also from Rank I were detected in the reference strains *A. baumannii* and *A. amyesii*, respectively, while the remaining AMR genes were categorized in the lowest risk rank IV, non-human associated genes.

3.5. Growth at 37 °C, 40 °C, 41 °C and 44 °C

All isolates grew strongly at 37 °C. In addition, S1214, S1202, and S1137 also grew at 40 °C and 41 °C (Table 3). No strain was confirmed to grow at 44 °C.

3.6. Susceptibility to antibiotics

The susceptibility of the isolates was tested to 9 antibiotics (Table 4). We observed full susceptibility (100 %) to gentamicin and minocycline. High susceptibility (91 %) was also found for amikacin, doxycycline, colistin and (82 %) for ciprofloxacin. Nine isolates were susceptible to cefepime and trimethoprim-sulfamethoxazole and only one isolate (S1215) was susceptible to piperacillin + tazobactam.

The isolate S1153 was classified as multi-resistant as it showed resistance in at least three antimicrobial categories [68]. Specifically, it showed resistance to 1) tetracyclines (DOX), 2) polymyxins (COL), 3) foliate pathway inhibitors (SXT), 4) antipseudomonal penicillins + beta-lactamase inhibitors (PTZ). An intermediate level of resistance was also found to CIP, which represented antipseudomonal fluoroquinolones.

4. Discussion

Our study provided the genotypic and phenotypic characteristics of *Acinetobacter* strains isolated from the manure of dairy cows under antibiotic prophylaxis and from the soil affected by this manure to reveal potential health risks associated with the isolates. A wide range of virulence factors of the isolates were compared with those of *A. baumannii* and other *Acinetobacter* species, and the pathogenic predisposition of the isolates is critically analyzed in the discussion.

The identification did not confirm that the soil and manure isolates belonged to *Acinetobacter* species associated with human infections, such as *A. baumannii*, *A. pittii*, *A. ursingii*, *A. junii*, *A. bereziniae*, *A. colistiniresistence*, *A. courvalinii*, *A. lwoffii*, *A. parvus*, *A. radioresistence*, *A. schindleri* or *A. nosocomialis* [1]. Two *Acinetobacter* isolates from manure-affected soil belonged to *A. amyesii*, a taxon described by Nemec

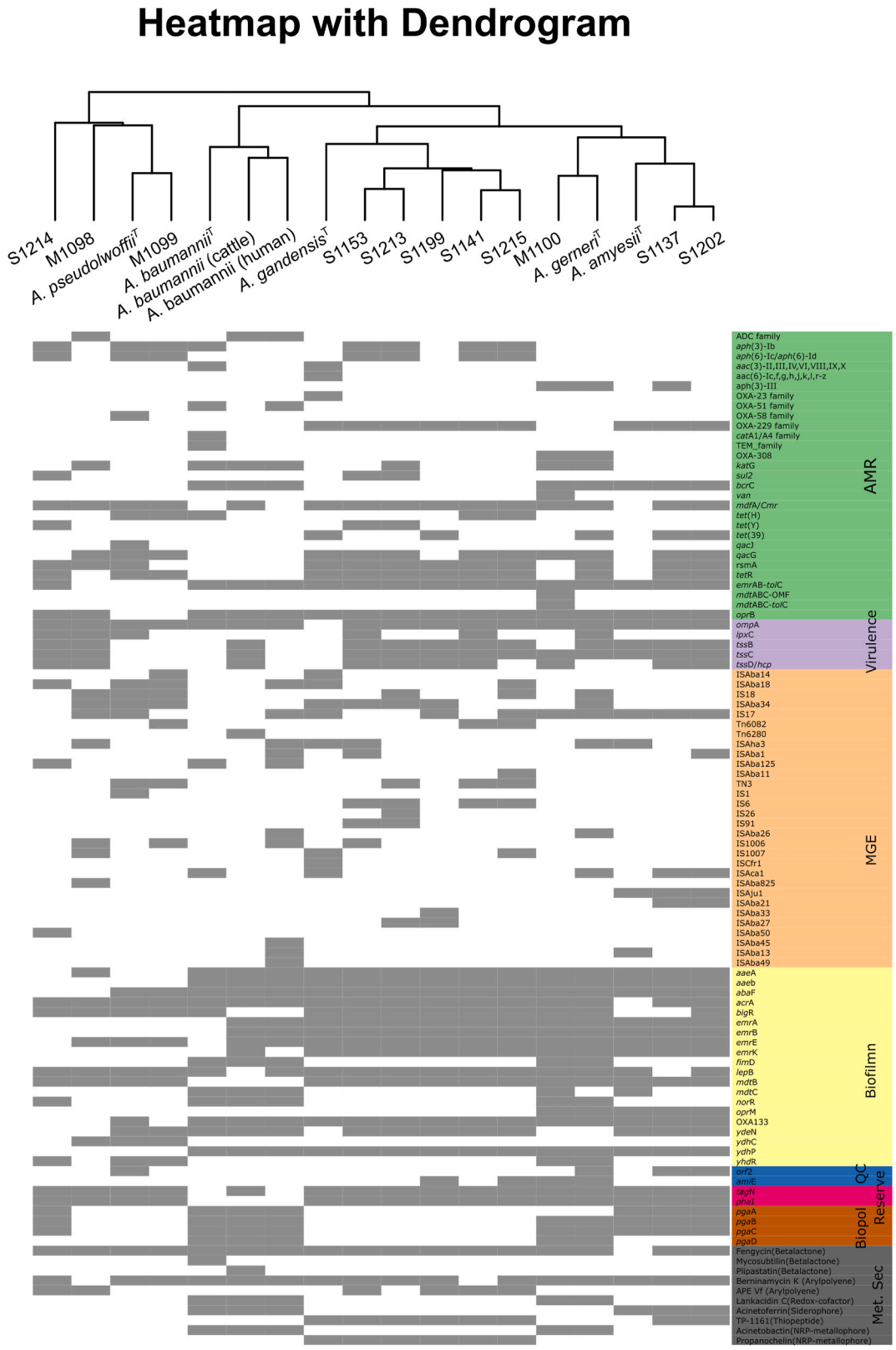
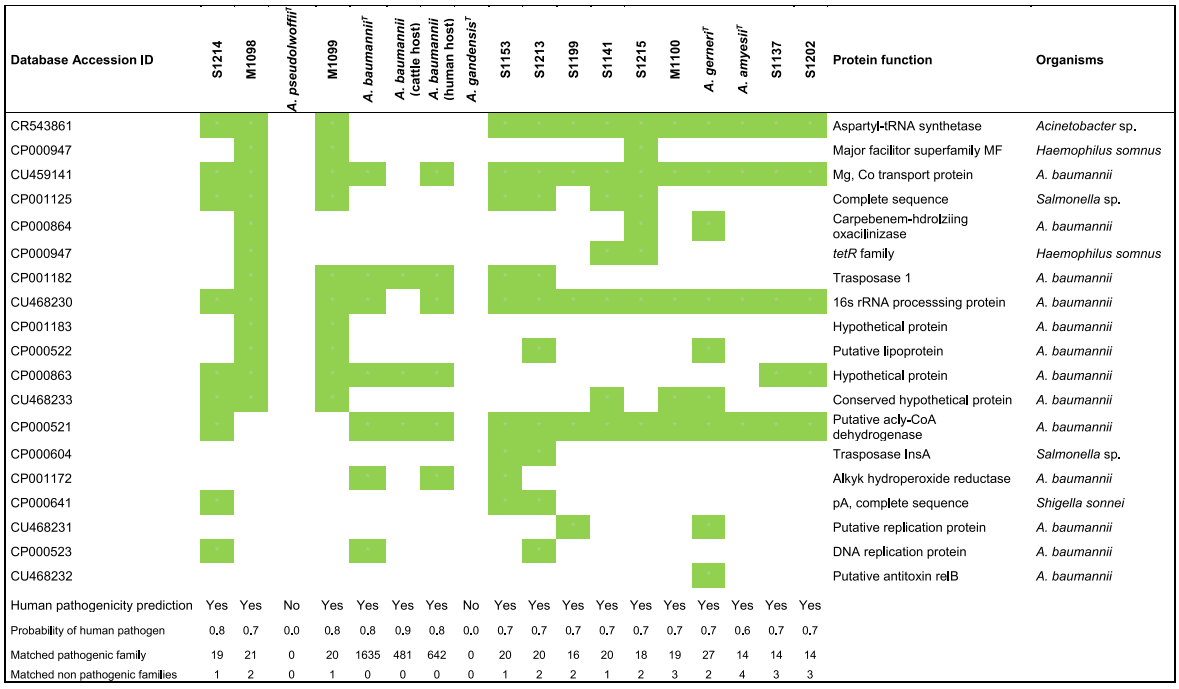


Fig. 3. Hierarchical clustering based on the presence and absence of potentially risky genes (associated with AMR, virulence, MGE, biofilm, motility-chemotaxis, quorum quenching, quorum sensing, reserve compounds, synthesis of biopolymers, and secondary metabolites), and heatmap showing the most relevant genes. Genes that show no variation across the isolates (i.e., those present in all isolates) were removed from the plot and the analysis.

Table 2
Presence of genes associated with pathogenicity and predicted probability of the isolates as human pathogens.



Database Accession ID	S1214	M1098	*A. pseudolwoffii*^{T}	M1099	*A. baumannii*^{T}	*A. baumannii* (cattle host)	*A. baumannii* (human host)	*A. gandensis*^{T}	S1153	S1213	S1199	S1141	S1215	M1100	*A. gerneri*^{T}	*A. amyesii*^{T}	S1137	S1202	Protein function	Organisms
CR543861																			Asparyl-tRNA synthetase	*Acinetobacter* sp.
CP000947																			Major facilitor superfamily MF	*Haemophilus somnus*
CU459141																			Mg, Co transport protein	*A. baumannii*
CP001125																			Complete sequence	*Salmonella* sp.
CP000864																			Carpebenem-hdrolziing oxacilinizase	*A. baumannii*
CP000947																			*tetR* family	*Haemophilus somnus*
CP001182																			Trasposase 1	*A. baumannii*
CU468230																			16s rRNA processsing protein	*A. baumannii*
CP001183																			Hypothetical protein	*A. baumannii*
CP000522																			Putative lipoprotein	*A. baumannii*
CP000863																			Hypothetical protein	*A. baumannii*
CU468233																			Conserved hypothetical protein	*A. baumannii*
CP000521																			Putative acly-CoA dehydrogenase	*A. baumannii*
CP000604																			Trasposase InsA	*Salmonella* sp.
CP001172																			Alkyk hydroperoxide reductase	*A. baumannii*
CP000641																			pA, complete sequence	*Shigella sonnei*
CU468231																			Putative replication protein	*A. baumannii*
CP000523																			DNA replication protein	*A. baumannii*
CU468232																			Putative antitoxin relB	*A. baumannii*
Human pathogenicity prediction	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		
Probability of human pathogen	0.8	0.7	0.0	0.8	0.8	0.9	0.8	0.0	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.7	0.7		
Matched pathogenic family	19	21	0	20	1635	481	642	0	20	20	16	20	18	19	27	14	14	14		
Matched non pathogenic families	1	2	0	1	0	0	0	0	1	2	2	1	2	3	2	4	3	3		

Table 3
Growth of *Acinetobacter* isolates at 37 °C and 40 °C on tryptic soya agar (TSA, 24 h) and at 41 °C and 44 °C on brain-heart infusion broth (BHIB, 48h).

ISOLATE	Growth at temperature			
	TSA		BHIB	
	37 °C	40 °C	41 °C	44 °C
M1098	+/+	−/−	nd	nd
M1099	+/+	−/−	nd	nd
M1100	+/+	−/−	nd	nd
S1137	+/+	weak/weak	weak/weak	−/−
S1202	+/+	+/+	weak/+	−/−
S1141	+/+	−/−	nd	nd
S1153	+/+	−/−	nd	nd
S1199	+/+	−/−	nd	nd
S1213	+/+	−/−	nd	nd
S1215	+/+	−/−	nd	nd
S1214	+/+	+/+	+/+	−/−

Results of two independent growth experiments: +, positive growth.
weak, weak growth; -, no growth; nd, not done.

et al. [69] as geographically widespread in soil, water environments, and livestock feces. Additionally, five soil isolates were identified as *A. gandensis,* which has previously been reported in the dung of hospitalized horses [70] and in domestic ruminants, including their milk [71]. Polluted riverine environments [12,72] and raw, unwashed vegetables [73], are also sources of *A. gandensis*. In contrast, the isolates from the cow manure were identified as *A. gerneri* and *A. pseudolwoffii*. *A. gerneri* was isolated from manure for the first time in our study. Its occurrence had been previously recorded in activated sludge [74], untreated hospital sewage [75], influent wastewater [76], tannery effluents and solid wastes [77], in swine carcass [78] and in human mouth sampled postmortem [79]. In contrast, *A. pseudolwoffii* has been previously found in a wide range of domestic animals as the predominant *Acinetobacter* species [71], and is a ubiquitous taxon also found in humans, soil, and water [1]. It is noteworthy that *A. pseudolwoffii* has been investigated as a cause of foodborne illness and food spoilage from the bullfrog, produced as a gourmet delicacy in Mexico [80]. Nevertheless, *A. pseudolwoffii* has not yet been reported to cause disease.

Antimicrobial resistance in the genus *Acinetobacter* is a key feature that makes this versatile bacterial genus a threat to critically ill, injured and debilitated humans and animals. We were primarily interested in resistance to beta-lactam and tetracycline antibiotics administered on farms where we sampled the cow manure. Phenotypic resistance or reduced susceptibility to beta-lactam antibiotics as well as the presence of genes coding for resistance to beta-lactamases characterized the majority of isolates. The gene OXA-58, detected in the manure isolate M1098 (*A. pseudolwoffii*), has been found to be a stable part of the multidrug-resistant *A. baumannii* strain of the European epidemic clone II, which is widespread in Czech hospitals [81,82]. Another gene representing the carbapenem-hydrolyzing class D beta-lactamase OXA-229, detected in most of our soil isolates, deserves attention and surveillance. The OXA-229 gene has been reported in the pathogen *A. bereziniae* originating from infected humans and animals, highlighting the need for its monitoring as a risk factor in circulation and transmission at the human-animal-environment interface [83,84]. Phenotypic resistance to tetracycline was found only in the multidrug-resistant soil isolate S1153 (*A. gandensis*), which carried the gene *tet*(Y) encoding an efflux pump, located on contigs predicted to be plasmid DNA. Additionally, two other genes, i.e., *tet*(H) and *tet*(39), that are unique to *A. baumannii* within the genus *Acinetobacter* [85], were also located in plasmid DNA in four other isolates, further increasing their potential risk. The *tet*(39) gene was predominant in oxytetracycline-resistant *Acinetobacter* spp. isolates from integrated fish farms in Thailand as results of antimicrobials administration in animal production [86]. The manure isolates M1098 and M1099, identified as *A. pseudolwoffii,* also carried a high-risk variant of the gene *aph*(6)-Id, which is a top risk human-associated gene related to aminoglycoside resistance. This gene was found in contigs predicted to be plasmid DNA in the manure isolate M1098, similar to the soil S1153 isolate. The S1153 contained other aminoglycoside resistance genes categorized in the lowest risk rank IV, non-human associated genes [57]. The S1153, identified as *A. gandensis,* was the only isolate that manifested multi-resistance to clinically important antimicrobial categories

Table 4
Susceptibility of the *Acinetobacter* isolates to antibiotics (Antibiotic disk diffusion test).

ISOLATE	ATB AGENTS - antimicrobial category[a] AV±SD, inhibition zone of antibiotics in mm[b]								
	FEP - cephalo-sporins	AKN -aminogly-cosides	GME -Aminogly-cosides	DOX -tetra-cyclines	MNO -tetra-cyclines	CIP -fluoro-quinolones	SXT -folate pathway inhibitors	COL -polymyxines	PTZ -penicillin + β-lactamase inhibitors
M1098	25 ± 0.0	25 ± 0.6	29 ± 1.2	14 ± 1.0	25 ± 2.1	27 ± 2.5	18 ± 1.5	15 ± 1.0	**11 ± 0.6**
M1099	20 ± 0.6	21 ± 0.0	24 ± 0.0	18 ± 1.0	22 ± 1.0	21 ± 1.5	22 ± 1.2	14 ± 1.0	**18 ± 0.0**
M1100	**18 ± 0.0**	24 ± 1.0	26 ± 0.6	17 ± 1.2	24 ± 1.2	34 ± 1.0	21 ± 1.5	15 ± 1.0	**15 ± 0.0**
S1137	22 ± 1.0	21 ± 0.0	19 ± 1.0	18 ± 3.5	25 ± 1.5	26 ± 1.0	24 ± 1.2	14 ± 1.2	**18 ± 1.7**
S1202	20 ± 0.0	20 ± 0.0	22 ± 2.5	17 ± 0.0	26 ± 0.6	27 ± 1.0	23 ± 0.6	14 ± 1.0	**18 ± 1.7**
S1141	24 ± 0.6	20 ± 0.6	23 ± 0.0	15 ± 0.0	21 ± 0.6	26 ± 0.6	25 ± 0.0	13 ± 0.6	**19 ± 1.2**
S1153	19 ± 1.7	22 ± 0.6	19 ± 1.5	**7 ± 0.0**	24 ± 1.2	**20 ± 0.6**	**7 ± 0.0**	**7 ± 0.0**	**16 ± 3.5**
S1199	20 ± 0.6	22 ± 0.6	24 ± 1.2	15 ± 0.0	24 ± 1.2	26 ± 0.0	23 ± 0.0	14 ± 0.6	**19 ± 0.0**
S1213	27 ± 0.0	21 ± 1.0	23 ± 1.2	15 ± 0.0	24 ± 0.6	26 ± 0.6	**7 ± 0.0**	13 ± 0.6	**16 ± 1.2**
S1215	30 ± 0.0	21 ± 0.6	24 ± 1.2	21 ± 1.2	27 ± 0.6	28 ± 0.0	25 ± 1.2	13 ± 1.0	23 ± 1.7
S1214	**16 ± 0.0**	**15 ± 1.5**	20 ± 0.6	14 ± 0.6	21 ± 1.2	**16 ± 2.0**	20 ± 0.0	14 ± 0.0	**17 ± 0.6**

[a] ATB agents (FEP – Cefepime 30 μg, AKN – Amikacin 30 μg, GME – Gentamicin 30 μg, DOX – Doxycycline 30 μg, MNO – Minocycline 30 μg, CIP – Ciprofloxacin 5 μg, SXT – Trimethoprim-sulfamethoxazole 1.25–23.75 μg, COL – Colistin 10 μg, PTZ – Piperacillin + Tazobactam 30-6 μg) were divided to antimicrobial categories to define type of multiresistance of Acinetobacter spp [68].

[b] Criteria specified by the NCCLS (https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf) were used to interpret the zones of inhibition for antibiotics. Interpretative categories: resistant (bolt type and underlined value), intermediate = reduced susceptibility (bolt type value) and susceptible (unhighlited values).

[68]. In our previous study [36], *A. gandensis* was identified as the dominant species in the mixed culture of dairy cow manure. The study by Pulami et al. also reports the isolation of *A. gandensis* in raw cattle manure [32]. The potential risks associated with antibiotic-resistant *Acinetobacter* strains is closely related to their ability to spread in the environment and the colonization of hosts, which is facilitated by a number of properties of this highly adaptable bacteria [12].

While *Acinetobacter* colonization is more common than infection, infections can become life-threatening if they manifest in the host [69]. Genes related to adherence mechanisms, and in particular to the first steps of host colonization, such as *pilC*, *G*, *H*, *T*, were found in all isolates, as well as the *csrA* gene. The *csrA* encodes a carbon storage regulator known to control stress response and virulence in many bacterial pathogens [87]. This gene plays an important control role in desiccation and osmotic tolerance, essential for *A. baumannii* growth on host-derived substrates such as urine or blood, as well as for survival and transmission in hospital environments [88,89]. Based on the current knowledge about the genetic background of *Acinetobacter* spp. for host infection reviewed by Wong et al. [90], we found the genes *ompA* and *IpxC*, important for immune modulation, in all and in four isolates (M1098, S1214, S1153, and S1141), respectively.

Siderophore production is an important virulence factor of *A. baumannii* [5]. During infection, *A. baumannii* needs siderophores such as acinetobactin to acquire iron from the host environment, and at the same time it competitively inhibits the growth of members of the commensal microbiota [91]. All *A. baumannii* genomes included in our study, as well as the M1100, S1137 and S1202 isolates, and the *A. gerneri* and *A. amyesii* reference genomes, had genes related to synthesis of acinetobactin and acinetoferrin. Other genes encoding the synthesis of various important types of secondary metabolites, such as NRP-metallophores, arylpolyene, and betalactone, which are considered as a means of competitive advantage in acquiring micronutrients and evading the host immune system and thus as virulence factors, were found in all our soil and manure isolates. The strong metabolic capacity and the utilization of a wide range of substrates as carbon and energy sources make organisms of the genus *Acinetobacter* very adaptable to the environment [12]. All the genomes studied showed genetic potential for the synthesis of reserve material. Since the genomes of environmental isolates had a higher number of genes related to the synthesis of reserve material than the genomes of *A. baumannii* isolated from human or animal hosts, we assume that reserve substances may be more important for *Acinetobacter* strains in the environment. In contrast, the consistent presence in all *A. baumannii* genomes of the *pgaA*, *pgaB*, *pgaC*, and *pgaD* genes, which are essential for the biosynthesis of poly--β-(1–6)-N-acetylglucosamine (PNAG), as opposed to their incomplete or entirely absent occurrence in environmental strains, suggests that PNAG production plays a more critical role under host-associated conditions than in external environments. The PNAG has been well described as a major component of biofilms and may play an important role in biofilm-mediated pathogenesis of *A. baumannii* [92]. Many genes linked with biofilm formation and regulation were found in all studied isolates, indicating that biofilm also belongs to the life strategy of the studied environmental strains. The genes linked to quorum sensing are important for *A. baumannii* virulence, including biofilm development capability [93], and were found both in all *A. baumannii* as well as all environmental strains. The presence of various MGEs in all genomes is consistent with the importance that MGE might have for the genus *Acinetobacter* in acquiring important genes for adaptability and possibly evolution [94], including the spread of resistance among different hosts and environments [95].

We evaluated the potential pathogenicity of the isolates and the reference strains using PathogenFinder, which provides predictions based on the analysis of whole genomes [61]. PathogenFinder predicted the manure M1099 (*A. pseudolwoffii*) and soil S1214 (*Acinetobacter* spp.) isolates to be human pathogens with a high probability (0.8), comparable to that of *A. baumannii* isolated from human hosts. However, it must be noted that this is only a deductive and experimentally unsupported prediction based solely on sequence homology of factors whose pathogenic potential has been demonstrated in different allelic forms, in other organisms or in other biological contexts. Nevertheless, these results provide valuable insights for mapping the pathogenicity potential of environmental *Acinetobacter* species. The ability to grow at 37 °C for all isolates and even at 41 °C for the S1214 (*Acinetobacter* spp.), S1137 and S1202 (*A. amyesii*) isolates is significant from a clinical perspective. Indeed, bacterial growth at 37 °C and higher temperatures is associated with strains of clinical relevance [1] and is related to resistance to host macrophage uptake [90].

5. Conclusion

Numerous genotypic and phenotypic human-health-risk features related to virulence and antibiotic resistance were found in all *Acinetobacter* isolates from livestock manure under antibiotic administration and from the soil affected by the manure. All *Acinetobacter* isolates were predicted to be human pathogens, with the highest probability for the manure isolate identified as *A. pseudolwoffii*, which carried several high-

risk mobile antibiotic resistance genes, among other features. When considering the potential health risks associated with environmental *Acinetobacter* strains, we must also consider the health of the hosts and the environment in which they live.

CRediT authorship contribution statement

Alper Dede: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eduardo Pérez-Valera:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dana Elhottová:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.107610.

Data availability

The identified bacterial cultures were deposited in the Biology Centre Collections of Organisms (BCCO 40_1098/40_1215, https://soilbacteria.bcco.cz/index.php?page=records_list). DNA reads from the Illumina sequencing and assembled genomes were deposited at the NCBI SRA database under BioProject accession **PRJNA905257**.

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