Final Report JRP

JRP15-AMR2.1-FED-AMR

Responsible Partner: 2-AGES

Contributing partners: 7-SZU, 9-BfR, 10-FLI, 13-SSI, 14-UT, 20-IP, 23-UoS, 25-NUIG, 33-NVI, 34-PIWET, 36-INSA

GENERAL INFORMATION

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Dissemination *Author's suggestion to inform the following possible interested parties.*	OHEJP WP 1 ☐ OHEJP WP 2 ☐ OHEJP WP 3 ☒ OHEJP WP 4 ☒ OHEJP WP 5 ☐ OHEJP WP 6 ☒ OHEJP WP 7 ☐ Project Management Team ☒ Communication Team ☐ Scientific Steering Board ☐ National Stakeholders / Program Owners Committee ☐ ECDC ☐ EFSA ☒ EEA ☐ EMA ☐ FAO ☐ OIE ☐ WHO-EURO Other international stakeholder(s): …… Social Media: …… **Other recipient(s):** ……

Final Report FED-AMR

1. Consortium composition

Project Lead: Werner Ruppitsch,
Deputy Project lead and
Scientific Manager: Manuela Caniça
Administrative Manager: Karin Rainer

JRP15-	Title	Leader	deputy
WP1	Project Management and Communication	Ruppitsch	Cabal Rosel
WP1 T1	Scientific Management		
WP1-T1.1	Coordination of sampling, laboratory experiments and building a database, QM		
WP1-T1.2	Webinars (WP1.4 in Annex Deliverables) bimonthly in proposal (start proposal M25, Annexes M30)		
WP1-T1.3	Project Meetings		
WP1-T2	Administrative Management Consortium agreement, Monitoring of scientific progress, risk mitigation measures		
WP1-T3	Data and protocol management plan (T3) - not as pressing (email from OHJEP)		
WP2	Field experiments: Determination of the naturally occurring ARG background load and microbial biodiversity in the tested environmental compartments	Caniça	Cabal Rosel
WP2-T1	Assemble list of sampling compartments and points. Determination of test areas representative for the European regions (North, West, East, South).		
WP2-T2	Establish common protocol for sampling and data analyses to facilitate comparability of the results between European test areas (North, West, East, South) and local sampling locations		
WP2-T3	Assess microbial and ARG diversity with NGS in selected test enviroments, compare between ecostystems, characterization of cultivable environmental bacteria on complete nutrient and minimal media		
WP2-T3.1	Shotgun sequencing and bioinformatic analyses of AMR genes and MGEs		
WP2-T3.2	Gene enrichment with gene capture probes		
WP2-T4	Quantification of clinically relevant ARG tested by qPCR and or qPCR arrays		
WP2-T5	Identify naturally transformable bacterial species in tested compartments (NGS)		
WP2-T6	Asses clonal/lineage diversity in selected ARB species		
WP2-T7	Isolation and assessment of the quantity, diversity and stability of free extracellular ARG encoding DNA in tested environments, seq. Comparison		

WP3	Elucidating the role of Clostridium difficile as an ARG transfer platform over ecosystems boundaries and its linkage between human and non-human (zoonotic) reservoirs	Oleastro	Persson
WP3-T1	Epidemiological survey of zoonotic ribotypes across participant countries		
WP3-T2	WGS and AMR characterization of human and non-human *C. difficile* isolates		
WP3-T3	Evaluation of the extent of genetic overlap between human and non-human C. difficile lineages		
WP3-T4	*C. difficile* / AMR dissemination between the human, animal and the environment: pig farm as a proof of concept		
WP4	Determination of the selection pressures in the tested compartements of human, animal and environmental ecosystems	Brandtner	Gajda/Gbylik-Sikorska
WP4-T1	Selection of essential antimicrobials to be quantified in the tested compartments (published antibiotic consumption data, farmers' questionnaire, personal experience, expert interviews (veterinarians)		
WP4-T2	Water: Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in aqueous matrices (water)		
WP4-T3	Manure: Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in manure		
WP4-T4	Faeces: Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in faeces		
WP4-T5	Soil: Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in soil		
WP4-T6	Quantification of herbicides in agricultural soil		
WP4-T7	Measurement of the concentration of trace elements in environmental samples gathered across participants countries		
WP 5	Identification of environmental conditions modulating transformation frequencies in soil microcosms and an in vitro porcine gut model (poGutMo) (laboratory studies	Chambers	La Ragione
WP5-T1	Establish basement levels of HGT in the model organism (*E. coli*) arising from transformation in the poGutMo		
WP5-T1.1	Ability of *E. coli* to acquire AMR to serve as a donor DNA		
WP5-T1.2	Determine the optimal growth parameter for cultivating *E. coli* strains within the gut model		
WP5-T1.3	Rates of transformation calculated by taking samples from the gut model and plating on agar plates supplemented with the appropriate antibiotics		
WP5-T1.4	DNA transfer rates via bacterial conjugation will be calculated using the endpoint method		

Kommentiert [RK2R1]: @Racha/Hein please confirm, that we should keep the names of tasks the same

Kommentiert [CMP(oVM1]: Krista - do we have to keep these tasks exactly as they were in the original proposal, as we gave up the possibility of doing any conjugation experiments in this project?

WP5-T2	Evaluate conditions that drive HGT and the emergence of AMR via transformation (WP 4 & 6 will suggest drivers)		
WP5-T2.1	Iterative evaluation of candidate drivers (antibiotic, herbicide, cation) of HGT evaluated in the gut model - round 1		
WP5-T2.2	Iterative evaluation of candidate drivers (antibiotic, herbicide, cation) of HGT evaluated in the gut model – round 2		
WP5-T2.3	Conjugation-mediated HGT between the clostridial donor and recipient strains within the gut model determined		
WP5-T3	Effect of different environmental conditions on the expression of competence genes in *E. coli* determined using soil microcosms		
WP 6	Probabilistic and mechanistic models of the links between antimicrobial usage in animals, AMR in the environment, and the risks for public health.	Lo Iacono	Chambers
WP6-T1	Build a probabilistic mathematical model of the emergence of AMR in target bacteria and the relative contribution of transformation and conjugation to ARG acquistion (afterwards: Factors influencing the prevalence of antibiotic resistance in the environment)		
WP6-T1.1	Data integration, Annotation and Association Analysis (afterwards: Systematic review: environmental factors associated with AMR)		
WP6-T1.2	Data integration, annotation and association analysis Y4		
WP6-T1.3	Data integration, annotation and association analysis Y5		
WP6-T2	Develop mechanistic models to adress key questions regarding the spatio-temporal changes observed in microbiological communities		
WP6-T2.1	Modelling microbial communities		
WP6-T2.2	Modelling microbial communities II		

Kommentiert [CMP(oVM3]: Similarly, these tasks no longer reflect the work we've actually done.

2. Summary of the work carried out in the Project

FED-AMR project aimed to understand the role of free extracellular DNA (exDNA) in dissemination of antimicrobial resistance (AMR) over ecosystem boundaries along the food/feed chain in one-year crop growing season. As transformation is an important driver for genetic plasticity of bacterial genes and genomes and natural transformation does not require physical contact between donor and recipient bacteria we hypothesized that this may facilitate antibiotic resistance genes (ARG) crossing ecosystem barriers and invading new habitats compared to HGT by conjugation.

The WP1 aims were met and were mainly based on project coordination, including scientific and administrative management, organization of regular activities, facilitation of cooperation between the WPs and integration of their outputs and results.

WP2 aimed to quantify and assess microbial and AMR diversity with next generation sequencing (metagenomics) in different compartments of food production chain, during one-year-crop growing

season. In particular we was interested in studying the role of extracellular DNA in the dissemination of AMR genes. We had 476 samples from six European countries (Austria, Czech Republic, Estonia, Ireland, Portugal, United Kingdom), distributed in four EU regions from up to eleven compartments along the food/feed chain. Both extracellular free DNA (exDNA) and total DNA were subjected to 16S rDNA sequence-based microbial profiling to measure the bacterial diversity. The Phyla in exDNA are dominated by *Proteobacteria* and *Firmicutes*. The principal component analysis suggests a separation between three groups of compartments: 1) soil 2) crops, feeds and drinking water 3) farmers, pigs, wild animals, manure and wastewater, which may show e.g. a smaller shifting between the microorganisms found globally in the compartments of these three groups. No significant differences were detected between soil amended with manure and without it, which may be due to an history of exposure to manure in the preceding years. ExDNA and total DNA were also subjected to target enrichment (equivalent to gene capture) to characterize and quantify ARGs in the different compartments. The analysis started with samples obtained from the wild animal compartment where we detected ARG conferring resistance to about 30 antibiotic classes, where β-lactams, tetracyclines, aminoglycosides and fluoroquinolones were the most prevalent. In the same compartment, we identified e.g. 11 ARG-types conferring resistance to fluoroquinolones. As for the competent bacteria the highest loads were observed in feed (exDNA), groundwater (total DNA) and field drainage (total DNA). We can conclude that bacteria known to be able to take up extracellular DNA are present in all environmental compartments and may serve as receptors for ARGs that are harboured on free exDNA. We also characterized genetically (WGS-based typing) and phenotypically (antimicrobial susceptibility testing) more than 500 cultivable bacteria. In all countries, isolates retrieved from wastewater, pig manure and pigs were those carrying more ARGs, including extended-spectrum beta-lactamases, but also ARGs associated to antimicrobials used usually in animal husbandry such as aminoglycosides, tetracyclines or sulfonamides. However, AMR data in our study (including those from metagenomics) are not thought to be a direct result of antimicrobial drug use, because 'globally' there is no direct linkage of AMR (associated with the antibiotics from the classes identified in WP4) across the various compartments and countries, therefore, there will be other risk factors (to be identified yet, if achievable).

WP3 aimed at elucidating the role of *Clostridioides difficile* as a pathogen/ARGs transfer platform over ecosystems boundaries and the genetic overlap between human and non-human zoonotic reservoirs. At the farm level, dominant clones in environmental compartments associated with pig production were identified, suggesting a transmission chain between compartments involving these animals. The results contributed to unveil the role played by animal and environmental reservoirs within the *C. difficile* epidemiology. The studies assessing genetic overlap between human and non-human *C. difficile* lineages at different One Health settings support the zoonotic relevance of *C. difficile* and its presence in novel reservoirs.

In WP4 we aimed at determining the selection pressures (antimicrobials, elements and herbicides) for antimicrobial resistance in environmental ecosystems (soil, water, faeces, manure, plants, feeds). We analysed samples collected by WP2. The highest concentrations of antimicrobials were detected in manure and faeces and are likely to select bacteria resistant to these compounds. The results of WP4 will be used for an impact evaluation of the analysed substances on the prevalence and quantities of ARGs encoded on extracellular DNA in exposed bacterial populations residing in the tested environmental compartments.

In WP5 we successfully established and optimised (pH, temperature, flow rates and volumes) an *in vitro* model of the pig large intestine using pig faeces and conditions to represent the natural complex microbial community of the gut. We demonstrated sufficient growth and maintenance of an *E. coli* J53 strain to serve as a recipient of exDNA to study the impact of antibiotics and/or trace elements on the efficiency of natural transformation within the gut. Also, a soil microcosm setting was created using non-manured agricultural soil and *Acinetobacter baylyi* ADP1 as receptor of extracellular DNA. The results showed a repression of the mRNA levels of two competence genes (*comA* and *dprA*) during the

first 6 hours after spiking if the microcosm was incubated at 35°C compared to 20°C. These results indicate that elevated environmental temperatures may diminish the ability of naturally competent soil bacteria to take up extracellular DNA and may reduce AMR spread by bacterial transformation.

WP6 focused on generating a protocol for a systematic review on factors influencing the prevalence of antibiotic resistance in the environment and on formulating and validating a mathematical model for the dynamics of microbial communities. The protocol was registered in a public repository and accepted in Dec/2022 and published in Jan/2023 in an international journal (*Environment International*: https://doi.org/10.1016/j.envint.2022.107707). With respect to the task on modelling microbial communities, we have formulated a new model and validated it with *in-silico* data purposely developed.

FED-AMR has a societal, policy and economic impact, as well as scientific impact through cross-sector communication of data contributing to the advancement of science. This project used the true concept of One Health, namely with an important environmental component (farmers, pigs, wild animals, manure, air of pig barns, feeds, crops, soil, water).

3. Work carried out in the JRP/JIP, scientific results and integrative outcomes

WP1: Project Management and Communication

WP1-T1 Scientific Management (M25-M60)

The scientific manager (SM) Manuela Caniça and the project leader (PL) Werner Ruppitsch as well as their deputy Adriana Cabal Rosel have worked continuously to ensure the scientific soundness of the project. Budget and strategy changes (i.e. Ares genetics involvement) were discussed and voted by the FED-AMR Scientific Supervisory Board (SSB) and Advisory Board (AB) members in 2021 (Y4). The scientific management has worked closely with the administrative management team consisting of Karin Rainer (project manager, PM), Krista Rathammer (deputy PM) and Nadine Peischl (project administrator).

WP1-T1-ST1.2 Webinar forum and Skype meetings for instant scientific interactions (M29-M60)

Regular scientific and administrative exchange between the project partners was facilitated and encouraged through consistently scheduled monthly online meetings. These TCs included a short update of all WPs and plenary discussions as well as administrative input. Minutes of these meetings were shared with the project partners for approval. A definitive version incorporating all suggestions received was distributed via email and published on the internal workspace on the website (https://fed-amr.ages.at) hosted by AGES. In addition, regular online meetings were done between members of one or more WPs, in order to discuss strategies, overarching tasks and to exchange knowledge. Such meetings last the whole duration of the project. Furthermore, webinar forums were established for which recognized experts were invited to give talks on scientific topics of relevance to the project consortium.

WP1-T1-ST1.3: Project Meetings (M25-M52)

The kick-off meeting of this project was held face-to-face at the AGES facilities in Vienna in Y3. The second consortium meeting had to be postponed to April 2022 (Y5) due to the travel restrictions. This meeting took place at INSA for two full days, on the 21st and 22nd of April 2022, in a hybrid format (24 in-person participants) and with the option of joining online (12 participants). All six WPs made an update on the data already achieved, followed by discussion, namely with the aim of clarifying questions about several scientific issues, reorienting the analysis of these data, and talking about respective publications. Each WP leader was responsible for organizing and coordinating the work

during the time allocated to the respective WP, ensuring the involvement of all participants. At the end, the next steps were discussed both in plenary and WP-dedicated sessions. Particular attention was given to the outputs and the importance of leaving a scientific legacy available and useful to the scientific community, stakeholders and policy makers in the field of the project: AMR spread. Between the 1st and 2nd meeting there was an interim meeting online, in December 2021, where an overview of all work already performed was presented by each WP leader and colleagues. The project closing meeting was held online in December (Y5) in order to honour the projects budget requirements.

WP1-T2: Administrative Management (M25-M60)

The coordination of joint activities in the frame of the FED-AMR project mainly coordinated by AGES. Additionally, each partner appointed an Administrative Representative who was in direct contact with the AGES Administrative Manager (AM), Karin Rainer. The administrative team at AGES and the Scientific Manager (SM) defined a risk management strategy for the project. The overarching risk management strategy initiated in year 3 of the project and was put in place by the AM and the SM, in consultation with the FED-AMR Scientific Supervisory Board (SSB) to ensure that adverse situations were properly handled along the course of the project, which was being highlighted in the Data Management Plan.

WP1-T3: Data and Protocol Management (M25-M58)

A first version of the Data and Protocol Management Plan was already delivered on M34. In year 4, the DMP was updated in the new OHEJP data management platform CDP, regarding details of FED-AMR data throughout the project, with information provided to the leader and deputy leader, by task leaders on their datasets. In addition, a metadata file was generated for all samples collected in WP2. This file facilitated the introduction of metadata in the CPD platform and data analysis. The final version of the DMP will be delivered by end February 2023 upon agreement with the OHEJP coordination team and will be uploaded to Zenodo.

WP2: Field experiments: Determination of the naturally occurring ARG background load and microbial biodiversity in the tested environmental compartments (M25-M60)

Overall, WP2 aimed to answer four main questions: which ARGs and how many of them are present in each compartment, and which and how many bacteria are present in the compartments. The tasks below have been answering these questions through a large amount of results obtained derived from the sequencing (16S, gene enrichment, WGS).

WP2-T1: Assemble list of sampling compartments and points. Determination of test areas representative for the European regions (North, West, East, South) (M25-M30)

In this task, the countries involved in the WP2 sampling elaborated first a list of sampling compartments and points. Sample collectors integrated four EU regions: East (Czech Republic, Poland), West (Austria, Ireland and Great Britain), North (Estonia and Norway), and South (Portugal). In addition, a sample timeline by collectors, a sample distribution list, and the transportation and conservation requirements for the samples was generated by WP2. Unique identifiers by compartment and time point were given to all samples. The outcome of this task is summarized in D-JRP15- FED-AMR–WP2.1 and the corresponding annexes.

WP2-T2: Establish common protocol for sampling and data analyses to facilitate comparability of the results between European test areas (North, West, East, South) and local sampling locations (M25-M33)

In this task, common protocols for the collection of different sample types, their processing and cultivation, DNA extraction (extracellular and total), antimicrobial susceptibility testing, whole genome sequencing and metagenomics sequence analysis, were generated by WP2. They supported the harmonization of testing procedures and enhanced comparability of the results obtained from different regions of Europe. The outcome of this task is also summarized in D-JRP15- FED-AMR–WP2.1 and the corresponding annexes.

WP2-T3- Assess microbial and ARG diversity with NGS in the selected test environments (metagenomics). Compare microbial and ARG diversity between ecosystems and over ecosystem boundaries. Characterization of cultivable environmental bacteria on complete nutrient and minimal media (M27-M54)

The WP2 « analysis team » finalised the statistical analysis on the 16S rDNA microbial profiling data. The whole genome sequencing-based analysis of all consortium isolates is also finished, as well as the susceptibility testing. Results derived from these analysis were presented at several international conferences. At the moment, the group continues to distribute the analysed data in different possible manuscripts in accordance with the objectives. This task is finished.

Regarding the characterization of cultivable bacteria by WGS-based typing, more than 300 animal and environmental bacterial isolates belonging to clinically-relevant species, such as *E. coli*, *K. pneumoniae*, *S. enterica*, *S. aureus*, *E. faecium* and *E. faecalis* were obtained after sample cultivation by the six participating countries. Also, nearly 200 strains belonging to other bacterial species, mostly environmental, were gathered by the partners. The first 300 strains were tested for antimicrobial susceptibility and paired-end sequenced was carried out on Illumina devices at each institute. Afterwards, raw genome sequences (FASTQ) were forwarded to 2-AGES for genome assembly, species re-identification, species-specific assessment of the genetic relatedness by core genome MLST using publicly available schemes, extraction of the sequence types of the classical MLSTs, and extraction of ARGs and virulence genes using CARD and VFDB databases, respectively. Afterwards, the assemblies (FASTA) were shared with 33-NVI for further analyses. The Mobile Element Finder was used to identify mobile genetic elements (MGE). Also, an in-house pipeline (Ellipsis (https://github.com/NorwegianVeterinaryInstitute/Ellipsis) was used for detection of plasmid replicons and for re-extraction of ARGs and VGs in all FASTA files; ARGs were determined using ResFinder, virulence genes using VirulenceFinder, and plasmid replicons using PlasmidFinder and mob-suite. The analysis of associations between ARGs and the compartments and countries in which the respective strains was isolated was performed. The identified MGE will provide more information about the possible dissemination via horizontal gene transfer of ARGs and VGs between clones of the same or different species, countries and compartments.

cgMLST-based analysis showed intra-country isolate clusters of the same species, but no multi-country cluster was detected. However, for several strains, the same ST was detected in more than one country, especially among the *E. coli* isolates. In all countries, isolates retrieved from wastewater, pig manure and pigs were those carrying more ARGs, including extended-spectrum beta-lactamases (ESBL), but also ARGs associated to antimicrobials usually used in animal husbandry such as aminoglycosides, tetracyclines or sulfonamides.

Two manuscripts have been already published on phenotypical and WGS-based characterization of 89 environmental bacteria from Austria belonging to clinically-relevant species. As for Portugal, ARG in isolates from the *Enterobacter cloacae* complex related with non-susceptibility to β-lactams, quinolones, fosfomycin and macrolides as well as several MGEs were identified. Phylogenetic analysis

identified two main clusters with closely related strains from different compartments. Almost all isolates were non-susceptible to colistin and resistant to at least one carbapenem due to carbapenemase production, and were obtained from three compartments (river water, sludge, and effluent from a stabilization pond). These results were presented at ECCMID 2022 and were part of an MSc thesis defended and published in 2022. Results from the WGS-based characterization and the phenotypical tests carried out by Czech Republic, UK, Estonia, Ireland and Portugal (other species different from *E. cloacae*) are also ready and we have given potential titles with the future manuscripts that will be drafted with those results.

WP2-T3-ST1- Shotgun sequencing and bioinformatic analyses of AMR genes and MGEs (M37-M58)

The FED-AMR partners agreed on the evaluation and comparison of ARGs using a novel methodology based on gene capture probes (equivalent to gene enrichment, see subtask WP2-T3.2 for more information). This methodology allowed us to analyse the ARGs and MGEs, and dispense with both conventional shotgun metagenomics (this subtask) and qPCR (task WP2-T4) for the collected samples. So, this subtask does no longer exist in a methodological point of view, but the aim persisted, and results came from WP2-T3.ST2.

WP2-T3-ST2- Gene enrichment with gene capture probes (M31-M48)

To ensure that target enrichment could be used instead of PCR and conventional shotgun metagenomics to analyze all the exDNA and/or total DNA obtained from the WP2 samples, we conducted a small pilot experiment comparing the results obtained for a pair of samples with shotgun metagenomics and target enrichment. As target enrichment showed superiority in detecting more ARGs, especially in the exDNA, the FED-AMR Supervisory Board agreed on the increased sensitivity and novelty of the use of target enrichment exclusively as a better approach to detect and quantify ARGs in the different compartments and EU countries.

Afterwards we performed the DNA extraction (exDNA and/or total DNA) and quality control of all samples within WP2 that were further enriched and sequenced. Results involved the generation of two large datasets grouping all the ARGs obtained in each of the samples by marker class and mechanism of resistance. These two large datasets included two different approaches to analyse gene enrichment data: 1) the one offered by the company ARES Genetics and 2) the one described by Lanza *et al.* (2015). WP2 considered the second approach as the best option, since it had been previously published and showing the benefit. Intensive data cleaning had been carried out in both cases, for both 16S and AMR data. Regarding AMR, we encountered some irregularities in the results provided by Ares Genetics (that did metagenomics for all the consortium) as did not contain the annotation of the information in the final format for a direct analysis, so it took more time (e.g. the ARG names, particularly regarding their classification into different AMR groups, which necessitated a manual revision of the ARGs names and classification). Statistical analyses have been completed using several R packages by the "analysis team". These include evaluation of the ARGs diversity, among others, in each of the samples, DNA types (extracellular vs. total), compartments and countries. The correlation between all WP2 and other WPs results needs to be completed. In parallel, we are drafting a first paper including the wildlife results for both exDNA and total DNA from samples collected in Austria, Ireland and Portugal, which can also be considered a martyr document from where we will be able to correct and fine-tune the analysis and writing of the papers on the other compartments, related to metagenomics.

WP2-T4 Quantification of clinically relevant ARG tested by qPCR and or qPCR arrays (M34 – 43)

As explained in the 12M report of Year 3, the detection of ARG through qPCRs was eliminated from the project. The Scientific Supervisory Board (SSB) contributed to this decision-making process.

However, the quantification of relevant ARGs in the analysed compartments was performed, inferring from the number or reads that cover each detected ARG.

WP2-T5- Identify naturally transformable bacterial species in the tested compartments (NGS) (M42-M56)

To identify naturally transformable bacteria in the compartments tested, a query database containing bacterial species proven to be able to take up extracellular DNA under naturally occurring environmental conditions was generated. For this purpose, a literature search for naturally competent bacteria covering the years from 1994 to 2021 was performed using defined search strings, with PubMed and SCOPUS as reference databases. Only hits describing the development of competence in bacteria under physiological conditions were eligible for entering the query database. The bacterial species names retrieved from literature were annotated and the appropriate phylogenetic levels were allocated according to the taxonomic classification system as laid down in SILVA 138.1. For the annotation and classification of 16S NGS data generated in the frame of the project the same version of SILVA database was employed. The resulting collection of observed bacterial species in FED-AMR samples was used as the searched database. This search database was checked for the presence of bacteria sampled in the query database using the level "genus" as qualifier. The research questions were: 1. Are naturally transformable bacteria present in FED-AMR isolates? 2. If yes, in which compartments? 3. Which competent bacteria are to be found in these compartments?

The query database contained 171 entries (= bacterial species) with empirically obtained evidence for their natural transformability. The searched database contained the 16S sequence information of 488 samples and 5763 different bacterial species. Naturally transformable bacteria were identified in almost all samples (except for a sample from soil and from a farmer – both of exDNA as sample type), in all sample types and environmental compartments. The overall most prevalent taxons encountered in FED-AMR samples were *Pseudomonads a*nd *Sphingomonads*. *Lactobacilli* were found in a significantly lower number of samples, but showed highly abundant genus-specific sequence reads. Samples from wildlife (exDNA), farmers (ex/total DNA) and crops (ex/total DNA) carried the lowest abundance of competent bacteria. The highest loads with competent bacteria could be observed in feed (exDNA), groundwater (total DNA) and field drainage (total DNA). We can conclude that bacteria known to be able to take up extracellular DNA are present in all environmental compartments and may serve as receptor for ARG encoded on free exDNA.

The detailed statistical evaluation of the obtained results focus on country-specific comparisons and the comparison with *in silico* alignment studies based upon comEC homologies.

WP2-T6- Assess clonal/lineage diversity in selected ARB species (M43-M52)

In the original task WP2-T6, phylogenetic trees using 16S amplicon sequencing and the shotgun sequencing data were intended. However, since the number of samples that the consortium analysed was significantly higher than the initially planned, the methodological approach was modified and no phylogenetic analysis was done using the raw data (FASTQ). See deliverable D-JRP15-FED-AMR-WP2.6 for additional information.

Really, as well as the resistome referred above, the microbial biodiversity in the tested environmental compartments from all partners was evaluated by characterising all 16S V1-V9 regions through 16S rRNA metagenomics and target gene enrichment. Thus, after cleaning up the data, all OTUs (Operational Taxonomic Units) with no reads, and correcting some errors we ended up with 573646 OTUs in 476 samples. We then proceeded to create a Phyloseq (R package) object using the available data in the literature (McMurdie & Holmes, 2013).

We divided the samples into 11 compartments: Crop – crops harvested in the agricultural fields; Feed – proceed feeds for animal consumption; Farmers – faeces samples from farmers; Pigs_Barn – faeces

from farm pigs; Manure – faeces of pigs and other organic material used to fertilize the soil; Wild_animals – faeces samples of wild animals like deal, hares, but also sheep and goat; Soil_Fs_Ms_Ctrl_Bline – soil samples from forests, meadows and agricultural fields before being cultivated or fertilized; Soil_field_noMan – soil samples from agricultural fields fertilized without the use of manure; Soil_field_Man – soil samples from agricultural fields fertilized with of manure; water_DRG – water samples from drainage, river and ground water; water_WTP – water samples from waste treatment plants.
Overall, water_DRG is the only compartment exclusively with samples that did not pass the quality control. If we separate the data sample type we will have 232 samples and 1725 genera for extracellular DNA samples and 223 samples and 1810 genera in total DNA samples. Phyla in extracellular and total DNA samples, are dominated by *Proteobacteria* and *Firmicutes*. Most of the Phyla are present in both extracellular and total DNA samples. *Acidobacteriota, Choloflexi* and *Gemmatimonadota* have higher reads in soil samples while *Bacteroidota* and *Firmicutes* have higher reads in farmers, pigs, manure and wild animals samples. Concerning alpha diversity, we explored the data with and without rarefaction (a procedure commonly used in microbial diversity analysis, but which may lead to loss of data). Our samples present high variability in the number of reads: 16 to the sample with least reads and 939820 for the sample with most reads. 72449 and 90698 for median and mean respectfully. Pielou's evenness index does not seem to be greatly affected by the total number of reads in each sample, both Chao1 and Shannon are. According to the rarefaction curves we identified that most of the samples approach the asymptote, which signalled that sampling depth was enough to capture the diversity in the sampled populations. The compartments that are significantly different from each other in terms of alpha diversity were determined both in the extracellular and total DNA samples. Most of the compartments were significantly different. From a total of 55 possible comparisons between compartments, we have 42 agreements extracellular and total DNA samples when rarefication was used and 45 agreements when rarefication was not used. This suggest that there are some punctual differences concerning alpha diversity between the extracellular and total DNA. Note that soil samples from forests, meadows, controls and baselines are significantly different than the other soil samples in extracellular DNA, but not in total DNA. In both types of samples, soil with and without manure did not present significant differences, which may be due to a history of exposure to manure in the preceding years (ARG "memory effect").

To perform ordination analysis we used CLR (centered log ratio) transformation that allowed us to use Aitchison distance. In both extracellular and total DNA samples PC1 separate soil samples from everything else, PC2 and PC3 separate the other compartments. The first three principal components explain 41.6%, 8.9% and 4.0% of the data variability in extracellular DNA samples and 45.2%, 7.3% and 5.3% in total DNA samples. PC2 and PC3 will separate crops, feeds and water samples from farmers, pigs and manure, with wild animals samples bridging these two clusters. We used multiple differential abundance methods to help ensure robust biological interpretations and we choose ALCOM-BC, ALDEx2 and the Wilcoxson test with CLR transformation. For the remaining compartments, the conclusions are not yet clear.

Regarding OUT's in both extracellular and total DNA samples, the compartments are divided into two main groups that separate the soil samples from everything else, as in the ordination analysis. The remaining samples are further divided into two groups: one with the mammals, another with the crops, feeds and water_DRG. Water_WTP and manure seem not to cluster with any other compartment. Concerning the separation for the OUT's we see that there are separations, which are common to the extracellular and total DNA and others that are specific. In both types of samples, we note large OUT's clusters exclusive from soil, others that are exclusive from manure and others exclusive for water_WTP. Following the group order in the dendrogram for the OUT's in the total DNA samples, we have a first cluster shared by mammals and manure, another that is shared by mammals, water_WTP, manure and soil samples. These are followed by a cluster shared by every compartment. For other clusters it is difficult to assign a characteristic. The total DNA samples seem to have a cluster that is

shared by wild_animals, crops, feeds and soil samples that is not as evident in the extracellular DNA heatmap.

Overall, the data suggest: A) Separation between three groups: 1) soil; 2) crops, feeds and drinking water; 3) farmers, pigs, wild animals, manure and waste water. B) No significant difference was detected between soil in fields with and without manure. C) Differences were detected between extracellular and total DNA samples in: farmers, feeds and soil. D) Very few samples of water that also did not pass quality control. This preliminary analysis may show a smaller shifting between the microorganisms found globally in the compartments of these three groups, suggesting, however, a greater (or more complex?: need to deepen the analysis further) interplay between the microorganisms of the compartments that correspond to farmers, pigs, wild animals , manure and wastewater.

WP2-T7- Isolate and assess quantity, diversity and stability of free extracellular ARG encoding DNA in the tested environments. Sequence comparisons (M34-M52)

The extracted extracellular free DNA from all the collected samples was used according to the generated protocols based on previous literature available. Gene enrichment was performed as described in WP2-T3.

We first evaluated the quality of the extracted total and extracellular free DNA. Qubit measurements of double stranded DNA were done for all the samples. Further quality assessments of the samples were done as explained in D-JRP15-FED-AMR-WP2.6. In addition, we removed from the analysis all those genes with a coverage below 0.5. For a total of 475 samples 7252 different ARGs were detected.

Most samples presented good amount of Transcripts Per Kilobase Million (TPMs). Only 21 out of 475 had an insufficient number of reads. TPMs in the samples by country were analysed. Overall, the number of TPMs for the exDNA was similar to that of the total DNA and the ARGs with the highest TPM sums corresponded to the tetracycline ARG *tetW*, which was present in 343 samples, and mutations in ribosomal subunits. As for the number of ARGs in each compartment, there was variations among compartments, DNA type and countries.

After this overview on ARGs quantity and diversity the more complex statistical analyses is clarifying the differences between compartments, countries and DNA type regarding ARGs, their antibiotic class and resistance mechanism.

All ARG results provided by Ares Genetics were analyzed and grouped according to AMR mechanisms, as follows: antibiotic inactivation, target alteration (replacement and alteration), target protection and efflux impermeability; in the group called 'others' we have mainly resistance genes to heavy metals and biocides, as well as virulence genes (adherence, motility, chemotaxis, etc). The first specific analysis on ARG concerns the wildlife compartment; we found that the ARGs obtained in samples from this compartment confer resistance to about 30 antibiotic classes, where the most prevalent were mainly the following classes (in descending order): β-lactams, tetracyclines, aminoglycosides and fluoroquinolones. Among β-lactams the prevalent resistance mechanism was the β-lactamase production [mostly class A β-lactamases, including ESBLs (e.g. encoded by bla_{CfxA}), class C, namely PMAβ (plasmid-mediated AmpC β-lactamases; e.g. encoded by bla_{CMY}, bla_{ACT}, bla_{ACC}, bla_{MIR}, bla_{DHA}, bla_{ADC}), and class D β-lactamases, such as carbapenemases (e.g. encoded by $bla_{OXA\text{-}types}$)]. In the same compartment, we identified genes encoding resistance to aminoglycosides through the action of aminoglycoside phosphotransferase (e.g. *aph(2'')-IIa*, *aph(3')-Ia*, *aph(3'')-Ib*), and 11 ARG-types

conferring resistance to fluoroquinolones, which included episomal (such as *qnrB, qnrD, qnrE*) and chromosomal (*oqxA*, *gyrA, gyrB*) mutated gene variants. We also identified resistance to heavy metals, such as (more prevalent in descending order) to copper, mercury, tellurium, arsenic, nickel-cobalt, chromium and silver.

AMR data in our study (including those from metagenomics) are not thought to be a direct result of antimicrobial drug use, because 'globally' there is no direct linkage of AMR across the various compartments and countries associated with the antibiotics from the classes identified in WP4, therefore, there will be other risk factors.

Comparison of microbial and antimicrobial resistance gene diversity across ecosystems and along ecosystem boundaries, as well as discussion of results are included in the manuscripts under preparation (see Chapter 7).

WP3 Elucidating the role of Clostridioides difficile as an ARG transfer platform over ecosystems boundaries and its linkage between human and non-human zoonotic reservoirs

WP3- T1 Epidemiological survey of zoonotic ribotypes across participant countries

WP3 participants aimed at investigating the epidemiology of zoonotic *C. difficile* through the identification of zoonotic types of *C. difficile* across the WP3 partner countries by generating an epidemiological survey of zoonotic ribotypes (RTs). Based on this survey, a database was constructed comprising of genomic data (WGS reads) and associated metadata on potential zoonotic toxigenic *C. difficile* isolates from various sources (human, animal and environment). Metadata included demographic and epidemiological data, as well as strain type (namely RT), toxin profile and AMR profile, when available. Discrimination between zoonotic and non-zoonotic types was based on *C. difficile* RTs and other genetic markers described in the literature. In addition, participating partners started sampling campaigns in order to enrich the collection of *C. difficile* isolates available from different sources, more specifically from diverse animal, environmental and food sources (FED-AMR isolates). Harmonized protocols for *C. difficile* isolation from samples of animal, environment and food origin were developed and used by WP3 partners, for the several sampling campaigns.

WP3-T2 WGS and AMR characterization of human and non-human C. difficile isolates

A dataset of *C. difficile* isolates from zoonotic RTs (RT002, RT033, RT078 and RT049) covering different origins was selected from existing strain collections of WP3 and external partners as wells as the FED-AMR isolates. The phenotypic (AMR) and genomic characterization (toxin profile, RT, WGS) was performed for the collection isolates not previously characterized and for all the FED-AMR isolates.

WP3-T3 Evaluation of the extent of genetic overlap between human and non-human C. difficile lineages

The changing epidemiology of *C. difficile* reflects a well-established and intricate intercommunity transmission network. With rising numbers of reported community-acquired infections, research should focus on the role of alternative reservoirs. Within WP3-T3, several studies were conducted with that purpose. 1) A study aiming to evaluate the role of companion animals as possible reservoirs for

human cases of infection was carried out, involving 335 faecal samples from dogs and 140 samples from cats in Portugal (INSA), collected during 2021 and 2022. The phylogenetic analysis revealed close genetic overlap between animal and human isolates involving main RTs present in both species (RT106 and RT014/RT020), supporting the possibility of clonal interspecies transmission or a shared environmental contamination source. Pets' isolates presented high rates of AMR to antibiotics that are important for human therapy: clindamycin (27.9%), metronidazole (17.1%) and moxifloxacin (12.4%). The study showed that companion animals could be reservoirs of toxigenic and antimicrobial resistant human-associated *C. difficile* isolates or vice-versa. Additionally, this study contributed important data on the genetic proximity between *C. difficile* isolates from humans and companion animals. This could help to clarify the role of animal reservoirs in the establishment of community associated transmission networks and alert for potential public health risk (one poster in the OHEJP Annual Scientific Meeting 2022; one manuscript submitted to Frontiers in Public Health, in review). 2) Another study targeted pig isolates vs human isolates in Denmark (SSI): the phylogenetic analysis revealed close genetic overlap between pig and human isolates within two clusters (RT078, RT066), indicating either direct transmission or a shared intermediate reservoir. The antimicrobial resistance gene (ARG) profile within each human/veterinary cluster confirmed similarity and relevant AMR carriage. This study showed that food chain animals could serve as reservoirs of toxigenic and antimicrobial resistant human-associated *C. difficile* isolates (one poster at ECCMID (Lisbon 2022); one manuscript in preparation). 3) Similarly, a study on *C. difficile* in New World Camelids, performed on 43 alpaca-/llama-husbandries in Saxony, Saxony-Anhalt and Thuringia in 2019 (Germany, FLI) showed a positivity rate of 16% (7/43) at farm-level; different RTs and toxin profiles were found, including the hypervirulent, zoonotic ribotype RT078. This study showed that New World Camelids are potential reservoirs for *C. difficile* and represent a previously unknown factor for zoonotic transmission in the One Health (OH) context (manuscript in preparation). From this study, a genomic analysis to assess genetic overlap between isolates from RT002 from different sources (human, food/environment, animals, including New Camelids) is being undertaken (one manuscript is scheduled). 4) Lastly, a study focused on food was performed (Germany, BfR), whose goal was to investigate strawberries from self-picking fields, since these often lead to the consumption of unwashed fruit directly on the field, posing a potential risk for *C. difficile* infection. The ratio of samples with *C. difficile* was low (2%). The only detected isolate from a self-picking field belonged to non-toxigenic RT051 that is not commonly associated with human CDI. Overall, the risk of *C difficile* infection via consumption of unwashed strawberries appears to be low but not impossible (1 poster at the One Conference 2022).

While performing this task, a MGE database for *C. difficile* (MGE-Database for *C. difficile*) was constructed for the identification of transposons, plasmids and phages (FLI) in WGS data; this MGE database was tested and validated by WP3 partners.

Overall, these studies supported the high zoonotic importance of *C. difficile* (RT078, RT106, RT014/020, RT002) and its presence in novel reservoirs, and showed genetic overlap between human and non-human *C. difficile* lineages at different OH settings.

WP3-T4. C. difficile / AMR dissemination between the human, animal and the environment: pig farm as a proof of concept

In order to assess *C. difficile*/AMR dissemination between humans, animals and the environment, studies were conducted on samples from four countries (two HOALs from Portugal and Austria, one agricultural facility from Estonia, single compartments from Ireland). The results obtained allowed to

perform a classification of pig farm compartments according to their role in the epidemiology of *C. difficile*, which was identified in all the following ecosystems: pig barn (faeces), manure (pig), wild animals, soil (agricultural fields), river water, ground water, wastewater treatment plant and air. In addition, in each HOAL, a different *C. difficile* RT (RT033, RT078 and RT049, respectively) was dominant, reflecting well-established transmission chains and a resilient source. The presence of those clones in compartments associated with the pig production unit suggests a transmission chain involving these animals and contributes to unveil the role played by animal and environmental reservoirs in *C. difficile* epidemiology.

The results obtained from the HOAL in Portugal (INSA) are a good example of these events. The study aimed to establish a *C. difficile* transmission network involving all the OH components using a pig farm as a proof-of-concept, as well as to assess the diversity of potential zoonotic types and to unveil genomic features of dominant clones. From 188 studied samples, a predominant clone of RT033 was found in samples from all compartments connected to the pig production unit, with core-genome SNV-based analysis supporting a clonal transmission between them (mean distance of 0.1 ± 0.1 core-SNVs). The phylogenetic positioning of this clone was clearly distinct from the classical RT033 cluster, being the first report of a toxigenic RT033 clone. This clone displayed a unique combination of genetic elements that may contribute to host tropism, environmental dissemination and maintenance. We are currently performing a study focused on this novel RT033 clone with zoonotic potential, involving genomic diversity assessment and relevant phenotype assessment (germination, sporulation, biofilms and toxins production) (one manuscript in preparation). Lastly, two large-scale genomic studies are ongoing to assess genetic overlap between isolates from different sources (human, food/environment, and animals), targeting the dominant clones RT078 (HOAL from Austria) and RT049 (agricultural facility from Estonia) (two manuscripts are scheduled).

Overall, the results obtained from the three HOALs/agricultural facility showed the presence of dominant clones in compartments associated with the pig production unit and suggest a transmission chain involving these animals. They contributed to unveil the role played by animal and environmental reservoirs within the *C. difficile* epidemiology. Scientific outputs from this task: two scientific papers with peer-review (doi: 10.3389/fmicb.2022.858310, doi: 10.1016/j.anaerobe.2022.102651) and two posters (OHEJP Annual Scientific Meeting 2021).

WP4 Determination of the selection pressures in the tested compartments of human, animal and environmental ecosystems (M25-M50)

WP4-T1- Selection of essential antimicrobials to be quantified in the tested compartments (published antibiotic consumption data, farmers' questionnaire, personal experience, expert interviews (veterinarians) (M25-M30)

This task was finished in M30 and the corresponding deliverable (D-JRP15-FED-AMR-WP4.1) was uploaded into the members area of the OHEJP website; this deliverable contains three protocols as annexes on the quantification of antibiotics, elements and herbicides in the different compartments.

Four antimicrobial classes to be tested in the compartments were selected: tetracyclines, macrolides, sulphonamides, trimethoprim and fluoroquinolones (task JRP15-WP4-T1) in accordance with the ARGs [*tet*(M), *tet*(W), *tet*(Z), *sul*1, *sul*2, *sul*3, *erm*-like genes, PMQR-encoding genes] that were investigated in the environment (faeces, manure, agricultural soil, drainage, surface and ground-water; see WP2). We focused on antibiotics from these groups that were marked as the most important according to published antibiotic consumption data and EFSA report on antibiotic residues in live animals and food. They were included in the analytical method by liquid chromatography-tandem mass spectrometry (LC/MSMS) performed by 34-PIWET (WP4-T2 to WP4-T5). The chosen herbicides glufosinate and glyphosate, as well as its degradation product aminomethylphosphonic acid (AMPA), 2,4-

Dichlorophenoxyacetic acid (2,4-D) are among the most often used herbicides in agriculture. Quantification of these substances (Task WP4-T6) was performed by an AGES associated sister company (UBA Vienna). Heavy metals and trace elements were chosen among those that are triggering co-selection and that have been used in co-selection studies: Cd, Cr, Cu, Ni, Hg, Co, Pb, Zn. The samples were analysed by inductively coupled plasma mass spectrometer (ICP/MS) carried out by 23-UoS (Task WP4-T7).

WP4-T2-Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in aqueous matrices (water) (M31-M50)

29 antibiotics are in the analytical scope of the LC-MSMS method used in WP4-T2, T3, T4 and T5: 4 tetracyclines, 7 sulphonamides, 10 fluoroquinolones, 7 macrolides and trimethoprim. 72 samples were analysed and in 10 samples antibiotics from 4 antimicrobial classes (macrolides, tetracyclines, fluoroquinolones and sulphonamides) were detected: azithromycin, oxytetracycline, enrofloxacin and sulfadimidine).

WP4-T3- Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in manure (M31-M50)

Results of 22 manure samples are available. Antibiotics from 2 antimicrobial classes (fluoroquinolones and tetracyclines) were detected in 12 samples (maximum values are 41 µg/kg marbofloxacin, 69 µg/kg enrofloxacin and ciprofloxacin; 124 µg/kg oxytetracycline; 768 µg/kg doxycycline). The concentrations of all five antibiotics exceeded the minimum selective concentrations. This indicated that under the given circumstances the detected antimicrobials were likely to select bacteria resistant to these compounds.

WP4-T4- Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in faeces (M35-M50)

24 samples were analysed. Antibiotics from 2 antimicrobial classes (fluoroquinolones and tetracyclines) were detected in 2 samples (maximum values are 265 µg/kg enrofloxacin; 1250 µg/kg oxytetracycline; 3440 µg/kg doxycycline). The concentrations of all 3 antibiotics exceeded the minimum selective concentrations. This indicated that under the given circumstances the detected antimicrobials were likely to select bacteria resistant to these compounds.

WP4-T5- Quantification of four antimicrobial classes (tetracyclines, macrolides, sulphonamides and fluoroquinolones) in soil (M31-M50)

In this task, 118 samples were analysed. The tetracycline doxycycline was detected in 8 samples (6.8 %) in the range between 9 µg/kg and 20 µg/kg. The highest concentration could be detected at the minimum selective concentration.

WP4-T6- Quantification of herbicides in agricultural soil (M31-M50)

The following substances were in the analytical scope of the LC-MSMS method: Glyphosate and its more stable degradation product AMPA (Aminomethylphosphonic acid), Glufosinate, 2,4-D (2,4-Dichlorophenoxyacetic acid), Metolachlor ESA the degradation product of Metolachlor, Bentazon and the degradation products of Metazachlor (Metazachlor ESA and Metazachlor OA).

Analytical results: 114 soil samples: herbicides were detected in 66 samples (58 %) (maximum values are 200 µg/kg AMPA and 74 µg/kg glyphosate); 30 water samples: herbicides were detected in 26 samples (86.7 %) (maximum values are 4.3 µg/L AMPA and 0.7 µg/L Metolachlor ESA); 7 manure samples: herbicides were detected in 6 samples (maximum values are 91 µg/kg AMPA and 41 µg/kg glyphosate).

The observed glyphosate levels in the tested compartments were below the MICs and sub-inhibitory concentrations as analysed for *Salmonella* sp strains. It is likely that the observed glyphosate concentrations (wet weight) do not select for resistant bacteria or perturb bacterial populations.

WP4-T7 Measurement of the concentration of trace elements in environmental samples gathered across participant countries

Samples of water (77), soils (98), crops and animal feeds (56), manure and faeces from wild animals and farm pigs (43) were received at the University of Surrey, Department of Chemistry from members of the consortium (namely Austria, Estonia, Czech Republic, Republic of Ireland, United Kingdom and Portugal), for the subsequent determination of trace elements. The key elements considered for analysis were chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), mercury (Hg) and lead (Pb). These analytes were selected on the basis of their toxicity or their nutritional/physiological importance. In addition to these key elements, analyses were also completed and data are available for vanadium (V), manganese (Mn), iron (Fe), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), cadmium (Cd), antimony (Sb) and barium (Ba).

The analysis of water samples was completed by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7800) after filtration and acidification. The concentration profiles for all partners showed similar trends, with Zn showing the highest concentration in all areas when comparing the 8 key elements. Only samples from Czech Republic, Republic of Ireland and Portugal presented measurable concentrations of Hg (limit of detection 0.13 ng/mL). Samples from wastewater plants showed the highest concentrations for all elements, presenting as well as a clear seasonal trend over the period of sampling.

The bioavailable fraction of elements was determined from the soils samples. For this purpose, the soils were homogenised with 0.43 mol/L acetic acids and the extracts were analysed by ICP-MS after filtration. The concentration profiles varied largely amongst the consortium partners, due to the differences in the geochemical makeup of the soil. In general terms, Zn presented the highest concentrations amongst the 8 key elements and the highest variability (ca. 2 to 9 µg/g), followed by Ni (approx. 1.5 µg/g) and Cu (0.5 µg/g). The concentrations of Cd and Hg were below the limit of detection (0.002 µg/g Cd and 0.005 µg/g Hg), but the presence of Pb and Cr was measurable in all samples.

The elemental composition of crops and animal feed ranged in very wide intervals and no clear geographical differences were observed when considering the concentration of the 8 key elements. Iron (Fe) presented the highest concentration of all elements analysed (max concentration 600 mg/kg), but when considering the 8 key elements, Zn again presented the highest concentration values, ranging from 150 to 10 mg/kg, followed by Cu from 2 to 50 mg/kg.

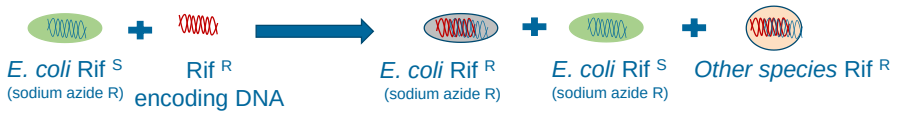
The profiles of all the elements in the manure and faeces were almost identical for all samples, indicating that the concentrations of these considered elements may be controlled by homeostatic processes. The highest concentrations observed were for Fe, followed by Mn and Zn. Measurable concentration of Cd and Pb were recorded for all samples, and the lowest measured values were for Cd.

WP5: Identification of environmental conditions modulating transformation frequencies in soil microcosms and an in vitro porcine gut model (poGutMo) (laboratory studies) (M32-M60)

WP5-T1 Establish basement levels of HGT in the model organism (E. coli) arising from transformation in the poGutMo

We first determined suitable *E. coli* strains with which to demonstrate ARG transmission arising from transformation. For this we used an *E. coli* J53 strain that was sensitive to rifampicin, but resistant to sodium azide. This served as the recipient (reporter strain) of transformation with exDNA encoding Rif,

whilst the DNA amplicons encoding rifampicin resistance and bacterial DNA lysates from other *E. coli* strains served as sources of AMR encoding exDNA.



An important aspect of the poGutMo was that it would represent faithfully the bacterial abundance and diversity of the natural host. To determine this, DNA has been extracted from independent pig gut model experiments and submitted for 16S rRNA sequencing. Analysis is on-going to confirm whether bacterial abundance and diversity is being maintained within the model.

Optimal growth parameters for cultivating *E. coli* in the gut model were established in terms of primary bacterial inoculum concentration, time of inoculation, pH and flow rate. Once these conditions were established, we conducted experiments to establish the baseline efficiency of transformation using Rif^R-encoding DNA. We demonstrated successful transformation from 24 hours in the gut model. Further experiments are currently undergoing to demonstrate transformation with bacterial DNA lysate and plasmids as an additional source of exDNA.

Practical challenges were encountered in maintaining sufficient *E. coli* J53 recipient bacteria to be able to detect the low numbers of transformation events that may be occurring. We addressed this through co-incubation of the recipient strain with bacterial faeces before using this material to seed the gut model.

WP5-T2 Evaluate conditions that drive HGT and the emergence of AMR via transformation

As described later in the project self-assessment section, we encountered substantial technical difficulties in delivering T1 that meant it was no longer possible to achieve our ambition in T2 of evaluating environmental conditions that drive HGT and the emergence of AMR via transformation (WP4 & WP6 were intended to suggest such drivers, such as antibiotic, herbicide, cation). The hope was that we would evaluate these iteratively in the model. However, it took too long to establish a baseline of natural transformation in the model under T1 and there was insufficient time left to introduce antibiotics or different cation concentrations in the gut model and evaluate their effect on transformation efficiency. However, we did conduct trace element analysis on the faecal content of the gut model in anticipation that we might manipulate the concentration of cations in the model to evaluate their impact upon transformation efficiency. The analysis demonstrated the lack of detectable antibiotics residues in the collected pig faeces and manure samples while variable concentrations of trace elements were detected.

In a similar way, the challenges we faced in T1 meant that we were unable to generate data in sufficient time to feed into the iterative *in silico* modelling we had envisaged in WP6.

WP5-T3 Effect of different environmental conditions on the expression of competence genes in A. baylyi determined using soil microcosms

A soil microcosm setting was created by utilizing non-manured agricultural soil. Heat exposure was chosen as the environmental parameter to be analysed, thus, soil samples were incubated at 35°C and at 20°C. The soil samples were spiked with the naturally transformable bacterium *Acinetobacter baylyi* ADP1 in order to track the expression of two competence genes: *dprA* and *comA*. DprA is an exclusively transformation-specific ssDNA binding protein which is essential for recruiting the recombinase *recA* to bind intruding ssDNA during transformation. ComA is a competence factor forming a transmembrane tunnel, which is required for DNA uptake. RNA was isolated from samples collected at

4 different sampling timepoints: Prior to incubation and spiking with *A. baylyi* (tinitial), immediately after spiking (tspike), six hours and 24 hours after spiking. The obtained RNA was analysed using RT-qPCR to quantify the expression of the 16S rRNA reference gene and the two transformation/competence-specific targets: *dprA* and *comA*. The delta delta Ct method was utilized for relative quantification. A 30 (*comA*)- to 40 *(dprA*)-fold decrease in target gene expression was noticeable in the *A. baylyi* spiked soils incubated up to 6 hours at 35°C in comparison to the control. However, this competence gene repression vanished after 24h and the expression-levels of the 35°C and 20°C soil samples converged. Preliminary results show that within a time window of 6 hours, expression of *A. baylyi* specific competence genes can be repressed due to heat exposure. This effect is time restrained as the mRNA-levels of *dprA* and *comA* are similar after 24h at either temperature. This observation indicates that uptake of free extracellular DNA by naturally competent soil bacteria might be reduced at elevated environmental temperatures. This experiment is currently fine-tuned and additional reference genes are tested. This experiment will be repeated under improved conditions and an additional timepoint (3 hours after heat incubation) will be added to obtain more information about competence gene expression levels.

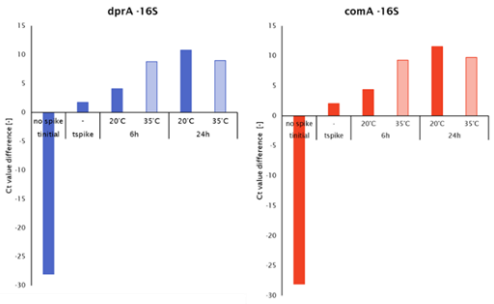


Figure 1 Temperature-dependent expression of competence-specific genes dprA and comA in relation to 16S rRNA using the Delta Delta Ct method. Sampling timepoints were tinital (before spiking with *A. baylyi*), tspike (immediately after spiking with *A. baylyi*), 6h and 24h after spiking with *A. baylyi* and at two different temperatures (35°C and 20°C).

WP6- Probabilistic and mechanistic models of the links between antimicrobial usage in animals, AMR in the environment, and the risks for public health.

WP6-T1 Factors influencing the prevalence of antibiotic resistance in the environment (PRIOR name: Build a probabilistic mathematical model of the emergence of AMR in target bacteria and the relative contribution of transformation and conjugation to ARG acquisition) (M32-M60)

This task refers to the systematic evidence map "Factors associated with the prevalence of antibiotic resistance in the environment from a One Health perspective: Protocol for a systematic evidence map" (doi: 10.17605/OSF.IO/A8GV6). The protocol successfully passed a very stringent editorial review, requiring a resubmission of the manuscript based on editors' feedback. The manuscript was sent out for review. Following a request of major modifications, a revised manuscript was re-submitted to the

journal. The manuscript was accepted in Dec/2022, published Jan/2023. As requested by the journal, the protocol is extremely detailed; a Qualtrics online form has already been prepared for data extraction. Most of the questions are in closed form reducing the risk of ambiguity. This will render the process more efficient and analysis simpler. Thus most of the work is already done.

WP6-T1-ST1 Data Integration, Annotation and Association Analysis (afterwards: Systematic review: environmental factors associated with AMR) (M32-M60)

Because of personnel departure at UoS two tasks (WP6-T2.1 "Modelling microbial communities" and WP6-T2.2 "Modelling microbial communities II") have been merged to result in one combined paper.

WP6-T2 Develop mechanistic models to address key questions regarding the spatio-temporal changes observed in microbiological communities (M34-M60)

This task refers to the development and validation of a model that dynamically tracks changes in microbial communities to address specific questions. The plan is to focus on the impact of external fluctuations and/or impact of rare species on the dynamics of the community. The final decision on the specific research question will be made once the model is fully validated. At the moment we are continuing working on the model to improve the numerical code. The model can successfully infer the parameters (growth rate, interaction, mortality) of an independent agent based model which mimics microbial five different taxonomic units and the rest of the communities is treated as whole. We are aiming to increase the number of taxonomic units that the model can discriminate to make the model more impactful.

WP6-T2. ST1 - Modelling microbial communities (M34-M60)

This task refers to the development and validation of a model that dynamically tracks changes in microbial communities to address specific questions. The plan is to focus on the impact of external fluctuations and/or impact of rare species on the dynamics of the community. The final decision on the specific research question will be made once the model is fully validated. At the moment, we are continuing working on the model to improve the numerical code. The model can successfully infer the parameters (growth rate, interaction, mortality) of an independent agent based model which mimics microbial five different taxonomic units and the rest of the communities is treated as whole. We are aiming to increase the number of taxonomic units that the model can discriminate to make the model more impactful.

4. Project self-assessment

In general, the project objectives as laid out in the project proposal have been met. However, as the very ambitious goals we set at the beginning of this project were not sufficiently reflected in the working plan and the budget of the project proposal, adaptations on the methodological side and regarding the time frame were necessary, being all the sole responsibility of the respective WP leaders (except for the decision to eliminate the qPCR method and its replacement in WP2, which was discussed with the partners within WP2and with the consortium, and approved by them and by the SSB). Additional costs arose partially due to increased administrative difficulties during the pandemic, as well as from enhanced analytic pathways. Most of the delays in the FED-AMR project were either a direct (closed laboratories, reduced staff availability, ...) or indirect (i.e. supply chain issues, late sampling, ...) consequence of the COVID-19 pandemic, as our project started in early 2020 (year3).

In WP2, the prior limited knowledge on free exDNA posed consequently diverse methodological

Kommentiert [AC4]: This task hs been modified. It refers now to the Systematic evidence map.

Kommentiert [HI5R4]: Please explain clearly for the reviewers. Thank you.

Kommentiert [RK6R4]: @Nadine: there was an email from Gianni I think, I cant seem to find it

Kommentiert [PN7R4]: Because Brian's departure the two different tasks (WP6-T2.1 "Modelling microbial communities" and WP6-T2.2 "Modelling microbial communities II") have been merged with the plan to get one paper. So, the information below apply to both *WP6-T2.1 and WP6-T2.2 (From Gianni-Mail 29.11.)*

challenges, such as the generation and validation of novel protocols for extraction of this DNA fraction. Despite the low exDNA concentrations obtained for some of the samples, the quality of the sequencing data exceeded our expectation. There is nevertheless room for improvement in the future with respect to the exDNA extraction protocols. Indeed, the time planned in the project proposal for the development of these protocols, specific to each of the compartments, was not realistic enough. Regarding target enrichment, this new approach has undoubtedly been the method that has allowed us to obtain harmonized AMR data for all participating countries in shorter time and with better results than with the initially chosen qPCR approach. Although there are still questions to be answered, we consider that all the initial objectives for this WP have been met. We have generated a unique dataset containing comprehensive information, from exDNA and tDNA, on microbial biodiversity (16S rDNA) and ARGs present in different compartments along the food/feed chain and in one-year crop growing season. Up to our knowledge, there are no studies integrating so many compartments that use target enrichment to detect ARGs in exDNA. The large datasets obtained here together with the associated metadata make our results a unique resource that will be available to other researchers and from which further studies can be performed.

In WP3, zoonotic vs anthropogenic antimicrobial resistance (AMR) transmission was studied with *Clostridioides difficile* as a model organism in WP3. At farm level, dominant clones in environmental compartments associated with the pig production unit were identified, suggesting a transmission chain involving these animals. These results contributed to unveil the role played by animal and environmental reservoirs within the *C. difficile* epidemiology. The studies assessing genetic overlap between human and non-human *C. difficile* lineages at different OH settings support the high zoonotic importance of *C. difficile* and its presence in novel reservoirs.

In WP4 we aimed at determining the selection pressures (antimicrobials, elements and herbicides) for antimicrobial resistance in environmental ecosystems. The highest concentrations of antimicrobials were detected in manure and faeces and are likely to select bacteria resistant to these compounds. The results of WP4 will be used for an impact evaluation of the analysed substances on the prevalence and quantities of ARGs encoded on extracellular DNA in exposed bacterial populations residing in the tested environmental compartments.

WP5 encountered substantial technical difficulties that could not have been anticipated at the outset of the project. These were exacerbated by delays with the laboratory work due to COVID-19. The financial resources available proved inadequate to conduct the full range and scope of objectives originally envisaged. As such, in Y5 the whole WP was re-focussed on demonstrating that the pig gut model could serve as a representative model of the in vivo large intestine in terms of the bacterial microbiota. The model conditions were optimized to establish whether natural transformation could be detected with sufficient sensitivity to likely detect what was expected to be a low-efficiency event. This refocus meant it was no longer possible to consider conjugation using *C. difficile* in the model. This was not a major consideration since the focus of this project was on natural transformation, and conjugation was only included in this WP as a comparator of the efficiency of conjugation versus transformation. More disappointingly, the technical challenges we faced meant that we could not achieve our ambition of evaluating environmental conditions that drive HGT and the emergence of AMR via transformation. The hope was that we would evaluate these iteratively in the model. However, without first being able to establish a reliable baseline of natural transformation in the model we had nothing against which to compare. This was always going to be an ambitious program of work. Lessons learnt include the need for more time and resource to establish the model. However, we have been successful in establishing an experimental in vitro pig gut model that can form the basis of future studies. The soil microcosm suffered similar issues due to COVID-related delays of laboratory reagents, changes in and lack of personnel, establishing a new laboratory method and resources to carry out this work package. Nonetheless, a soil microcosm pilot study was successfully conducted and functioned as a basis to identify heat as an environmental parameter that could repress the expression of competence genes in the naturally transformable bacterium *A. baylyi*. Temperature-levels that were

non-lethal for the soil bacterium could temporarily repress the expression of certain competence factors, and hence, the ability to take-up free extracellular DNA and recombine it with the bacterial genomic DNA. Due to time- and staff-limitations we could not test the effect of additional parameters (e.g. antibiotics, herbicides, heavy metals, etc.) on competence gene expression. However, continuation of the work is secured and will build up on the already obtained results and experience. An RNA-Seq platform will be implemented to gain more insight on competence gene expression on *A. baylyi* and bacterial population levels.

In WP6, the main tasks were: 1) completion of the protocol for a systematic evidence map; and 2) completion of a model to dynamically track changes in microbial communities to address specific questions (e.g. impact of external fluctuations and/or impact of rare species on the dynamics of the community). The work on the systematic evidence map has required a longer time than anticipated. This is perhaps not surprising considering the scientific quality standards of the journal 'Environment International', to which we submitted our manuscript. Following a first pre-submission enquire, we have had multiple meetings with the editors of Environment International. Although they have been supportive, they have provided an in-depth and rigorous critique which required extensive revisions. As expected, the departure of Dr Gardner (he is working on the project only part-time, for a few hours a week) has had a large impact on the project. To mitigate this, we asked Dr Deza Cruz to step in and he has largely contributed to developing and finalising the protocol. For the modelling part, we had to revisit our objectives because of Dr Gardner's departure. Initially our plan was to assess the impact of external perturbation on microbial communities and the impact of rare species. We are still interested in these two questions, but at the moment it is difficult to anticipate and fully assess the performance and computational cost (e.g. the required time to complete the task) of the numerical code. Thus, we reserve to make a final decision after the model is validated and the computational cost estimated.

5. Progress of the project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
15	D-JRP15-FED-AMR - WP1.1	Scientific Supervisory Board (SSB) installed. Local administrative representatives nominated (T1, T2)	M25	M25	Confidential (contains e-mail addresses of the members of the consortium) OHEJP: available Public Zenodo: https://doi.org/10.5281/zenodo.5081685	10
15	D-JRP15-FED-AMR - WP1.2	Unified sampling and experimental protocols (T1.1.)	M33	M33	Public OHEJP : available Zenodo: https://doi.org/10.5281/zenodo.5081689	2
15	D-JRP15-FED-AMR - WP1.3	Data and protocol management plan (T3)	M34	M34	Public OHEJP : available Zenodo: https://doi.org/10.5281/zenodo.5078099	8
15	D-JRP15-FED-AMR - WP1.4	Webinars (T1.2.)	M31	M31	Public OHEJP: available Zenodo: https://doi.org/10.5281/zenodo.5081710	5

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
15	D-JRP15-FED-AMR - WP1.5	Annual project report	M38	M38	Public Zenodo: https://doi.org/10.5281/zenodo.5078024	8
15	D-JRP15-FED-AMR - WP1.6	Interim project report (T1.3.)	M52	M52	Public Zenodo: https://doi.org/10.5281/zenodo.7057286	
15	D-JRP15-FED-AMR-WP1.7	Final project report (T1.3.)	M59	M60	This document. Zenodo Link will follow after this document is approved.	
15	D-JRP15-FED-AMR-WP2.1	List of sampling compartments, points and European test areas and harmonized protocols in alignment with EFFORT project protocols available in data repository (T2.1, T2.2)	M33	M33	Public Zenodo: https://doi.org/10.5281/zenodo.5081756	
15	D-JRP15-FED-AMR-WP2.2	Preliminary data collection on ARG prevalence and ARG background load in the compartments analysed	M37	M37	Public OHEJP: available Zenodo: https://doi.org/10.5281/zenodo.5078064	8

Kommentiert [CRA8]: We will uploaded to the OHEJP Website when you give us the green light for the final version.

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
		so far (T2.4)				
15	D-JRP15-FED-AMR-WP2.3	Annual report Y3 (WP2)	M39	M38	Public Zenodo: https://doi.org/10.5281/zenodo.5078149	8
15	D-JRP15-FED-AMR-WP2.4	Determination of naturally transformable bacteria in tested environmental compartments (T2.5)	M56	M60	Public Zenodo: https://doi.org/10.5281/zenodo.7446361	
15	D-JRP15-FED-AMR-WP2.5	Shotgun sequencing: ARG diversity in tested environmental compartments (T2.3.1)	M46	M46	Public Zenodo: https://doi.org/10.5281/zenodo.5726732	10
15	D-JRP15-FED-AMR-WP2.6	16S metagenomics results: Microbial biodiversity and phylogenetic relationships of ARBs over ecosystem boundaries (T2.3, T2.6)	M54	M56	Public Zenodo: https://doi.org/10.5281/zenodo.7057188	

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
15	D-JRP15-FED-AMR-WP2.7	Quantity and stability of free extracellular DNA observed in environmental compartments tested so far (T2.7)	M56	M60	Public Zenodo: https://doi.org/10.5281/zenodo.7547822	
15	D-JRP15-FED-AMR-WP2.8	Annual report for Y4 (WP2)	M49	M49	Public Zenodo: https://doi.org/10.5281/zenodo.7057332	
15	D-JRP15-FED-AMR-WP2.9	ARG dynamics in an agricultural testing area: Response of ARG concentrations according to different fertilisation techniques and crops over an annual growth period (WP2)	M58	M60	This deliverable is finished. The document will be uploaded to Zenodo soon.	
15	D-JRP15-FED-AMRWP2.10	Final report on WP2 and draft version of peer-reviewed publication (WP2)	M58	M60	This deliverable is finished. The document will be uploaded to Zenodo soon.	
15	D-JRP15–FED-AMR-WP3.1	Database of zoonotic *Clostridium difficile* isolates across participant countries (task 3.1)	M36	M36	Public OHEJP: available Zenodo: https://doi.org/10.5281/zenodo.5078164	3

Kommentiert [CRA9]: Will be uploaded next week

Kommentiert [CRA10]: Will be uploaded next week

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
15	D-JRP15–FED-AMR-WP3.2	Overview of genetic overlap between human and non-human *C. difficile* isolates (task 3.2 and task 3.3)	M60	M60	Public OHEJP: available Zenodo: https://doi.org/10.5281/zenodo.7472663	
15	D-JRP15–FED-AMR-WP3.3	Classification of pig farm compartments according to their role in the epidemiology of *C. difficile* (task 3.4).	M60		Public OHEJP: available Zenodo: https://doi.org/10.5281/zenodo.7472732	
15	D-JRP15-FED-AMR-WP4.1	Standardize protocols for sampling and testing of environmental samples	M30	M30	Public OHEJP: available Zenodo: https://doi.org/10.5281/zenodo.5482759	2
15	D-JRP15-FEDAMR-WP4.2	Quantitative results of antibiotics in water	M50	M50	Public. Zenodo: https://doi.org/10.5281/zenodo.6396025	
15	D-JRP15-FEDAMR-WP4.3	Quantitative results of antibiotics in manure	M50	M50	Public. Zenodo: https://doi.org/10.5281/zenodo.6396070	
15	D-JRP15-FEDAMR-WP4.4	Quantitative results of antibiotics in faeces	M50	M50	Public. Zenodo: https://doi.org/10.5281/zenodo.6396112	
15	D-JRP15-FEDAMR-WP4.5	Quantitative results of antibiotics in soil	M50	M50	Public. Zenodo: https://doi.org/10.5281/zenodo.6396145	

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
15	D-JRP15-FEDAMR-WP4.6	Quantitative results of herbicides in environmental samples	M50	M50	Public. Zenodo: https://doi.org/10.5281/zenodo.6396160	
15	D-JRP15-FEDAMR-WP4.7	Quantitative results of trace elements in environmental samples	M53	M53	Public Zenodo: https://doi.org/10.5281/zenodo.7057243	
15	D-JRP15-FED-AMR-WP5.1	*E. coli* strains demonstrated to be suitable for transformation	M38	M38	Deliverable will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines. Zenodo: deliverable description https://doi.org/10.5281/zenodo.5121159	10
15	D-JRP15-FED-AMR-WP5.2	Optimal growth parameters for cultivating *E. coli* and transformation pilot experiments using AMR-encoding DNA are tested	M57	M60	This deliverable is mostly finished but we are delaying it till the end of the year as we recruited additional staff support to finish the WP.	10
15	D-JRP15-FED-AMR-WP5.11	First results from soil microcosm experiments on effects of environmental conditions on transformation	M60		Pilot experiments have been performed. RNA isolation from soil samples has been established. RT Taqman qPCR systems for competence and reference genes have been designed and are available. Delays were due to bottlenecks in availability of laboratory staff. First results were delivered along with final	

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
					summary of results and combined with D-JRP15-FEDAMR- WP5.13 Since competence genes and their expression are essential for transformation, we used the results for both deliverables, since WP5.13 is only an in-depth topic for WP5.11.	
15	D-JRP15-FEDAMR-WP5.13	Results of the soil microcosm experiments regarding environmental effects on competence gene expression	M58	M59	Please see "D-JRP15-FED-AMR-WP5.11" Public Zenodo: https://doi.org/10.5281/zenodo.7547851	
15	D-JRP15-FED-AMR-WP6.1	Main code for the mathematical modelling made available in public repository (e.g. GitHub) with associated documentation (which can be used as "Material and Method" section of the forthcoming publications).	M30	M37	This is now a protocol for systematic review (not a code for mathematical modelling). Confidential until publication or registration of the protocol for the systematic review, except for FED-AMR or other One-Health EJP members. Zenodo: deliverable description https://doi.org/10.5281/zenodo.5139307	3

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
					Following a request of major modifications, a revised manuscript was submitted to the journal. We are now waiting for the decision.	
15	D-JRP15-FEDAMR-WP6.2	Findings presented at one international conference and one national conference.	M54	M57	We presented our results at two international conferences (ASM 2021 and One Conference 2022). See output section for more information.Public Zenodo: https://doi.org/10.5281/zenodo.7341753	
15	D-JRP15-FED-AMR-WP6.3	Update of codes and documentations in public repository (e.g. GitHub).	M36	M43	Confidential until full validation of the code or publication (except for FED-AMR or other One Health EJP members). Public Zenodo: https://doi.org/10.5281/zenodo.7341773	3
15	D-JRP15-FED-AMR-WP6.4	Submission/publication of 1/2 paper(s) on how resilience of microbial communities depends on external environmental drivers and richness and diversity of the	M60	M60	Zenodo: https://zenodo.org/record/7547834#.Y8pfdnbMLEY DOI of paper: https://doi.org/10.1016/j.envint.2022.107707	

JRP/JIP code	Project deliverable number *(Original number, if different from the actual one)*	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered on Project Group (month)	Comments *Please mention:* ***public or confidential, the Zenodo reference****, other comments*	Proposed categories* *(1 to 8) (several categories may be applicable)*
		community.				
15	D-JRP15-FED-AMR-WP6.5	Main code for the mathematical modelling made available in public repository (e.g. GitHub) with associated documentation (which can be used as "Material and Method" section of the forthcoming publications).	M57	M60	In the previous document this deliverable appeared as D-JRP15-FED-AMR-WP6.3, not D-JRP15-FED-AMR-WP6.5. Both deliverables are similar, 6.5 now is just an update on the code but the deliverable titles were switched. This deliverable relates to WP6 task 2, 'Modelling microbial communities', and describes prototype code written in Python, along with associated documentation, uploaded to the University of Surrey's online repository GitLab for version control purposes. https://doi.org/10.5281/zenodo.7456214 We are continuing with this task, specifically to improve the Python code to make it more efficient. https://doi.org/10.5281/zenodo.7456214 https://osf.io/a8gv6/	

** Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities); 7. Prevention: aligned use of facilities and models; 8. Other (please specify);*

Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
15	M-JRP15-FED-AMR-01	Kick off meeting	M26	M26	
15	M-JRP15-FED-AMR-02	Database repository active	M27	M27	
15	M-JRP15-FED-AMR-03	Webinar forums started	M30	M30	The consortium initiated the scientific exchange via online teleconferences and formally installed regular webinars by M31.
15	M-JRP15-FED-AMR-04	Interim meeting	M48	M52	Had to be moved online and scheduled for convenience, date was decided in the TCs and through an online meeting on Zoom.
15	M-JRP15-FED-AMR-05	Final meeting (T1.3.)	M60	M60	Online meeting
15	M-JRP15-FED-AMR-07	List of sampling compartments, points and European test areas available. Harmonized protocols for sample collection + transportation, DNA extraction, qPCR,	M26	M33	The list of sampling compartments, points and European test areas have been defined. Harmonized protocols for sample collection and transportation and DNA extraction protocols are already available for all project members. Protocols for WGS, metagenomics, gene capture and bioinformatics were developed.

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
		metagenomics, shotgun sequencing, gene capture and bioinformatics and statistical analysis of sequence data available. Alignment with EFFORT project protocols (T2.1, T2.2)			
15	M-JRP15-FED-AMR-08	Start of annual field study (i.e. sampling campaign, sample collection) (T2.3)	M27	M30	
15	M-JRP15-FED-AMR-09	Start of DNA isolations and cultivation of ARB strains (immediately after reception of the first environmental samples) (T2.3, T2.7)	M27	M28	

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
15	M-JRP15-FED-AMR-10	Start of performing qPCRs (T2.4)	M28	Not applicable	qPCR were replaced by Ares genetics services
15	M-JRP15-FED-AMR-11	Start collecting data for annual report (WP2)	M34	M34	
15	M-JRP15-FED-AMR-12	Starting preparations for gene capture probe approach (T2.3.2)	M35	M38	
15	M-JRP15-FED-AMR-13	Finishing annual report (WP2)	M36	M37	
15	M-JRP15-FED-AMR-14	Starting preparations for shotgun sequencing (T2.3.1)	M37	Not applicable	Shotgun Metagenomics was replaced for target enrichment (see Task 2.3.1).
15	M-JRP15-FED-AMR-15	Stop: field sampling campaign (T2.3), DNA isolations (T2.7)	M40	M43	
15	M-JRP15-FED-AMR-16	Starting 16S metagenome analysis of obtained DNA	M40	M43	

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
		isolates. Batch format (T2.3)			
15	M-JRP15-FED-AMR-17	Start: Determination of naturally transformable bacteria (T2.5)	M41	M43	
15	M-JRP15-FED-AMR-18	Finalizing ARG qPCRs (T2.4)	M42	Not applicable	qPCRs were replaced by target enrichment
15	M-JRP15-FED-AMR-19	Finalizing determination of transformable bacteria (T2.5)	M56	M60	
15	M-JRP15-FED-AMR-20	Start: Assessment of clonal/lineage diversity of ARB (T2.6)	M43	M45	
15	M-JRP15-FED-AMR-21	Finalizing 16S metagenome analysis of obtained DNA isolates (T2.3)	M52	M60	
15	M-JRP15-FED-AMR-22	Finalizing assessment of clonal/lineage diversity of ARB (T2.6)	M52	M60	

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
15	M-JRP15-FED-AMR-23	Finalizing shot gun sequencing and bioinformatics and statistical analysis of NGS data (T2.3.1, T3.2.2, T2.3)	M53	M60	
15	M-JRP15-FED-AMR-24	Delivery of final annual report on WP2 + draft version of peer reviewed paper (WP2)	M54	M60	
15	M-JRP15–FED-AMR-25	Completed database with zoonotic types	M36	M36	
15	M-JRP15–FED-AMR-26	Complete WGS on all included isolates	M46	M60	
15	M-JRP15–FED-AMR-27	Complete AMR profiles on all included isolates	M59	M60	
15	M-JRP15–	Genetic overlap-analysis human vs. non-human	M60	M60	

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
	FED-AMR-28				
15	M-JRP15–FED-AMR-29	Identification of transmission network as described above	M60	M60	
15	M-JRP15-FED-AMR-30	Starting the selection of essential antimicrobials to be quantified in the tested compartments	M25	M27	
15	M-JRP15-FED-AMR-31	Starting the analysis of antimicrobials in aqueous matrices	M27	M31	
15	M-JRP15-FED-AMR-32	Starting the analysis of antimicrobials in manure	M31	M31	
15	M-JRP15-FED-AMR-33	Starting the analysis of antimicrobials in faeces	M29	M35	
15	M-JRP15-FED-AMR-34	Starting the analysis of	M31	M35	

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
		antimicrobials in soil			
15	M-JRP15-FED-AMR-35	Starting the quantification of herbicides in agricultural soil	M31	M40	
15	M-JRP15-FED-AMR-36	Starting the measurement of the concentration of trace elements in environmental samples	M27	M48	
15	M-JRP15-FED-AMR-37	Bacterial strains supplied to UoS	M25	M34	Bacterial strains have been supplied to UoS and used successfully.
15	M-JRP15-FED-AMR-38	Porcine gut model set up using faecal samples obtained through WP2, samples stored for trace element analysis (WP4) – experiments can start	M44	M45	

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
15	M-JRP15-FED-AMR-39	Samples from gut model experiments stored for trace element analysis (WP4)	M41	M45	
15	M-JRP15-FED-AMR-40	Samples from gut model experiments stored for trace element analysis (WP4)	M52	M52	
15	M-JRP15-FED-AMR-41	Antibiotics, herbicides and heavy metals most likely to drive HGT through transformation supplied to UoS.	M57	M57	Antibiotics and transposable elements analysis results has been supplied to WP5.
15	M-JRP15-FED-AMR-42	DNA sent for whole-genome sequencing	M55	M55	Bacterial isolates have been sent to WP2 for WGS and results have been received.
15	M-JRP15-FED-AMR-43	Sub-strains of *E. coli* with appropriate antibiotic resistances made and chromosomal	M55	M55	Rifampicin resistant *E. coli* strain has been generated and appropriate nucleic acid preparations have been made.

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
		DNA and lysates prepared			
15	M-JRP15-FED-AMR-44	Porcine gut model set up using faecal samples obtained through WP2, samples stored for trace element analysis (WP4) – experiments can start	M51	M51	All samples are stored for trace element analysis.
15	M-JRP15-FED-AMR-47	Samples from gut model experiments stored for trace element analysis (WP4)	M50	M50	All samples are stored for trace element analysis
15	M-JRP15-FEDAMR-52	Deliver results from soil microcosm experiments to WP6 leader for final modelling	M52	M60	This will depend on results from soil microcosm experiments. In addition, due to the departure of Dr Gardner we need to reassess feasibility of this milestone.
15	M-JRP15-FED-AMR-53	Literature review on concept of	M27	M28	Mainly done at the beginning of the project, which led to the type of modelling proposed here (Lotka-Volterra system and agent-based models).

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
		resilience and modelling in microbial communities.			We keep constantly monitoring the literature to be updated with the state-of-the-art.
15	M-JRP15-FED-AMR-54	Identification of relevant available data. Formulation and implementation of the model for the microbiological community within-host. Conditioned to data availability, potential extension of the model to natural environment (e.g. soil).	M30	M30	We found that a dataset particularly suitable to our type of model is the one provided by Stein *et al.* which used cecal content data in (https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1003388). We also developed our own agent-based models to validate the model.
15	M-JRP15-FED-AMR-56	Literature review on fluctuating ecological systems and gut model approach.	M45	M45	The review of the literature is an ongoing task to ensure we are updated with the state of the art.
15	M-JRP15-FED-AMR-59	Application of the model to address specific	M50	60	Partially achieved: the mathematical model has been presented in the OHEJP conference and Brian in continuing work on this milestone.

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWPs	Date of achievement	Comments
		questions and dissemination of findings in peer-reviewed publications and conferences.			
15	M-JRP15-FEDAMR-62	Finding presented at one international conference and one national conference	M54	M54	The mathematical model has been presented in OHEJP conferences.
15	M-JRP15-FED-AMR-64	Finishing annual report (WP2)	M48	(M60)	Since the FED-AMR project was extended till December 2022, the WP2 annual report for the Y5 be delivered at the end of the project.

6. Follow-up of the recommendations and comments by the Ethics Advisors

None is remaining. All were timely addressed.

7. Publications and additional outputs

Publication title, DOI reference and Zenodo reference	Manuscript status	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing charges (in €)
Draft Genome Sequence of a Multidrug-Resistant *Escherichia coli* Sequence Type 1193 Pandemic Clone Isolated from Wastewater in Austria. Microbiology Resource Announcements DOI: 10.1128/MRA.00762-21 https://zenodo.org/record/5770120#.YeZ1rd8xl4F	published	Yes	No	1,050.00 $ 929.38 €
Assessment of the Transmission Dynamics of Clostridioides difficile in a Farm Environment Reveals the Presence of a New Toxigenic Strain Connected to Swine Production. Frontiers in Microbiology DOI: 10.3389/fmicb.2022.858310 https://zenodo.org/record/6857162#.Yyh-SXZByUk	published	Yes	No	2,950.00 $ 2,750.00 €
Characterizing Antimicrobial Resistance in Clinically Relevant Bacteria Isolated at the Human/Animal/Environment Interface Using Whole-Genome Sequencing in Austria. Journal International Journal of Molecular Sciences. Doi: 10.3390/ijms231911276 https://zenodo.org/record/7446455#.Y5xQ-FGZND8	published	Yes	No	2,173.86 €

Airborne spores' dissemination of a swine associated *Clostridioides difficile* clone. *Anaerobe.* DOI: 10.1016/j.anaerobe.2022.102651 https://zenodo.org/record/7446535#.Y5xSj1GZND8	published	Yes	No	free of charge
Gardner, Brian; Betson, Martha; Cabal Rosel, Adriana; Caniça, Manuela; Chambers, Mark; Contadini, Francesca M.; Gonzales Villeta, Laura C.; Hassan, Marwa M.; La Ragione, Roberto; De Menezes, Alexandre; Messina, Davde; Nichols, Gordon; Olivenca, Daniel V.; Phalkey, Revati; Prada, Joaquin M.; Ruppitsch, Werner; Santorelli, Lorenzo A.; Selemetas, Nick; Tharmakulasingam, Mukunthan; van Vliet, Arnoud H. M.; Wögerbauer, Markus; Deza-Cruz, Iñaki; Lo Iacono, Gianni (2022) Factors associated with the prevalence of antibiotic resistance in the environment from a One Health perspective: Protocol for a systematic evidence map. Environment International. DOI: 10.1016/j.envint.2022.107707 https://zenodo.org/record/7446911#.Y6G50nbMJD8	published	Yes	No	
Alves F, Castro R, Pinto M, Nunes A, Pomba C, Moreira O, Silveira L, Gomes JP and Oleastro M (2022) Molecular epidemiology of *Clostridioides difficile* in companion animals: genetic overlap with human strains and public health concerns. Frontiers in Public Health. DOI: 10.3389/fpubh.2022.1070258 https://zenodo.org/record/7528151#.Y7-_bHbMJD8	published	Yes	No	3,100 €
Coelho, Rodrigo. Characterization of Antibiotic Resistance in Strains Isolated from Different Environmental Reservoirs	published	Yes	No	Free of charge. Zenodo link will follow soon.

[Caracterização da Resistência aos Antibióticos em estirpes isoladas de diferentes reservatórios ambientais]. Master thesis, 2022. http://hdl.handle.net/10451/53801				
Ines Dost, Mostafa Abdel-Glil, Gernot Schmoock, Christian Menge, Christian Berens, Belén González Santamarina, Elisabeth Wiegand, Heinrich Neubauer, Stefan Schwarz, Christian Seyboldt. *Clostridioides difficile* in South American camelids in Germany: First insights into molecular and genetic characteristics and antimicrobial resistances. Antibiotics. DOI: 10.3390/antibiotics12010086 https://zenodo.org/record/7550849#.Y9Dmvv6ZND8	published	Yes	No	
Microbial composition and ARGs diversity in wild mammals of Portugal, Austria and Ireland (WP2).	under preparation			
Assessing the microbial composition and ARGs diversity in Europe in different compartments along the feed/food chain (WP2).	under preparation			
Tackling the dissemination of ARGs between compartments of the feed/food chain with target enrichment (WP2).	under preparation			
Prevalence of naturally competent bacteria in European Agroecosystems (and their relevance in AMR dissemination) (WP2).	under preparation			
Correlation analysis reveals strong association of free extracellular ARGs with certain trace elements in environmental compartments of the food/feed chain (WP2)	under preparation			
Alves F, Nunes A, Castro R, Serrano M, Bolt C, Persson S, Maurischat S, Gomes JP, Henriques AO and Oleastro M. Genomic diversity, sporulation, germination and biofilm phenotypes of *Clostridioides difficile* ribotype 033 from different One Health settings (WP3).	under preparation			

Semeh Bejaoui, Jesper Nielsen, Monica Oleastro, Christian Seyboldt, Sven Maurischat, Adriana Cabal Rosel, Christelle Mazuet, Dorte Frees and Søren Persson (+NN to be determined later). *Clostridioides difficile* (ST11). Time resolved phylogeny and genetic overlap between human and non-human isolates across Europe (WP3).	under preparation			
Clostridioides difficile RT002 epidemiology and zoonotic potential". Authors involved would be FLI staff and WP3 Partners (WP3).	under preparation			
Maurischat, S., Seyboldt, C., Scholtzek, A., Tenson, T. Transmission and Persistence of *Clostridioides difficile* within agricultural settings and the environment (WP3).	under preparation			
Changes in the metal bioavailability and content of antimicrobial and herbicide agents as a result of application of manure to agricultural soils (WP4)	under preparation			
The development of a chemostat pig gut model for maintaining the microbiome composition and investigating the transfer of AMR (WP5).	under preparation			

Additional output

WP1

Oral presentations

Werner Ruppitsch. "FED-AMR: The role of free extracellular DNA in dissemination of antimicrobial resistance over ecosystem boundaries along the food/feed chain (One Health EJP)", One Conference, 21st-24th June, 2022, Brussels, Belgium.

- Werner Ruppitsch. Dissemination workshop on preparedness 2022 organized by OHEJP-WP5 on the topic AMR movement over ecosystem boundaries. 25th March 2022 (online).

- Manuela Caniça. FED-AMR: The role of free extracellular DNA in dissemination of antimicrobial resistance over ecosystem boundaries along the food/feed chain. 22th November, 2022, POC Meeting, Stockholm, Sweden.

WP2

Poster presentations

- Abstract in ECCMID 2021 (e-poster): Antimicrobial resistance and genetic relatedness of environmental bacteria across the animal-human-wildlife interface in Austria (WP2, 2-AGES) https://zenodo.org/record/5499916#.YTs2z-dCR4E

- Abstract presented in Annual Scientific Meeting 2021 (e-poster): Diversity of bacterial communities and genes encoding AMR in different environmental compartments along the food/feed chain (WP2, 36-INSA) https://zenodo.org/record/4916135#.YTsqrudCR4E

- D. Olivença; A. Cabal; T. Tenson; M. Hassan; V. Manageiro; E. Dias; T. Rosado; V. Kõiv; V. Kisand; G. Rab; J. Jeremejeva; K. Telling; K. Arbo; M. Chambers; R. Laragione; E. Voit; Z. Drahošová; M. Kořínková; M. Woegerbauer; W. Ruppitsch; M. Caniça; A. de Menezes 2022. "Microbial and antimicrobial resistance gene diversity in extracellular and total DNA across rural ecosystem barriers in Europe"; OHEJP ASM 2022, Orvieto, Italy. https://doi.org/10.5281/zenodo.6857015

- A Cabal Rosel; N. Peischl, B. Daza, A Stöger, G Rab; K Rathammer; F Allerberger; M Wögerbauer, W Ruppitsch. 2022. "Antimicrobial resistance and genetic relatedness of environmental bacteria across the animal-human-wildlife interface in Austria"; ECCMID 2022, Lisbon, Portugal. https://doi.org/10.5281/zenodo.6641985

- V Manageiro, R Coelho, T Rosado, P Vieira, L Reis, R Matias, J Rodrigues, C Menezes, E Ferreira, A Sequeira, O Moreira, E Dias, M Caniça. 2022. Resistome, mobilome and virulome analysis of Enterobacter cloacae complex strains isolated from the water environment. ECCMID 2022, Lisbon, Portugal

- Olivença D; Cabal A; Tenson T; Hassan M; Manageiro V; Dias E; Rosado T; Kõiv V; Kisand V; Rab G; Jeremejeva J; Telling K; Arbo K; Chambers M; Laragione R; Voit E; Drahošová Z; Kořínková M; Woegerbauer M; Ruppitsch W; Caniça M; de Menezes 2022. Microbial and antimicrobial resistance gene diversity in extracellular and total dna across rural ecosystem barriers in Europe, ECCMID 2022, Lisbon, Portugal, https://zenodo.org/record/6857015#.Y6G9SXbMJD8

- A de Menezes, M Caniça, S Ciutti, L Griffin, A Haigh, AC Rosel, W Ruppitsch. 2022. Antibiotic resistance and AMR gene seasonality patterns in fallow deer (Dama dama) with different levels of human interaction: a case study of Ireland. Annual Conference of the UK-Ireland Microbiological Society, Belfast, UK (Oral communication).

WP3

Poster presentations

- Abstract presented in Annual Scientific Meeting 2021 (e-poster): Dissemination of antimicrobial resistant Clostridium difficile RT078/ST11 in Austria across the human-animal-wildlife interface (WP3, 2-AGES) https://zenodo.org/record/4915872#.YTsrmOdCR4E

- Abstract presented in Annual Scientific Meeting 2021 (e-poster): Zoonotic spread of multi-resistant *C. difficile* (WP3, 13-SSI) https://zenodo.org/record/4915901#.YTsqsOdCR4E

- Abstract presented in Annual Scientific Meeting 2021 (e-poster): Diversity and Dynamics of *Clostridioides difficile* in a farm environment PT (WP3, 36-INSA). https://zenodo.org/record/4916135#.YTs1O-dCR4EF. Alves; R. Castro; M. Pinto; M. Oliveira; C. Pomba; M. Oleastro 2022. "*Clostridioides difficile* in companion animals, a One Health concern?" One Health EJP Annual Scientific Meeting 2022, Orvieto, Italy.2022. https://doi.org/10.5281/zenodo.6641860

- "Potential for the zoonotic spread of multi-resistant Clostridioides difficile " ECCMID 2022 https://zenodo.org/badge/DOI/10.5281/zenodo.6641860.svg) resulted in the following article in the Guardian: "Pigs can pass deadly superbugs to people, study reveals". https://www.theguardian.com/society/2022/apr/24/pigs-can-pass-deadly-superbugs-to-people-study-reveals

- Scholtzek; P. Witt; G. Raatz; A. Bhatte; S. Maurischat 2022. "Prevalence of *Clostridioides difficile* in strawberries from Germany". One Conference 2022, Brussels, Belgium. https://doi.org/10.5281/zenodo.6856987

WP4

Abstracts

- Abstract presented in Annual Scientific Meeting 2021 (e-poster): FED-AMR: Determination of selection pressures for AMR in environmental samples (WP4) https://zenodo.org/record/4916158#.YTs1CudCR4E

- Presentation by SZU in the Ekomonitor Seminar on Waste Analytics in 2021: English title: International project to map the potential for horizontal transfer of antibiotic resistance genes across different ecosystems, original language: Czech. https://doi.org/10.5281/zenodo.5770033

- WP4- poster presentation entitled "Analysis of antimicrobials in environmental samples as a part of determination of selection pressures for antimicrobial resistance" accepted at Euroresidue IX conference. OHEJP Annual Scientific Meeting 2021, https://doi.org/10.5281/zenodo.4916158

WP5

Poster presentations

- M. M Hassan; M. Getino; J. Leng; M. Chambers; R. M. La Ragione. 2022. "Development of an in vitro pig gut model for evaluating factors driving antimicrobial resistance" ; OHEJP ASM 2022, Orvieto, Italy. https://doi.org/10.5281/zenodo.6642824

WP6

Abstracts

- Abstract accepted to ONE – Health, Environment, Society – Conference , 21-24 June 2022. "Modelling the dynamics and long-term stability of perturbed gut microbiota" (WP6, 23-UoS)https://zenodo.org/record/6860460#.Y6HAFXbMJD8
- Abstract presented in Annual Scientific Meeting 2021 (e-poster): Ecological modelling of microbial communities subject to perturbation (WP6, 23-UoS) https://zenodo.org/record/4915941#.YTsqs-dCR4E
- B Gardner, M M Hassan, M Chambers, R M La Ragoine, G Lo Iacono, 2021. "Ecological modelling of microbial communities subject to perturbation", ASM 2021, https://zenodo.org/record/4915941#.YTsqs-dCR4E https://doi.org/10.5281/zenodo.4915941

Poster presentations

- B Gardner, L C Gonzalez Villeta;J Leng; M M. Hassan; M Chambers; R M La Ragione; G Lo Iacono. 2022. "Mechanistic modelling of microbial communities with insights from an in vitro pig gut model", ASM 2022, https://doi.org/10.5281/zenodo.6856915
- B. Gardner; M. M. Hassan; M. Chambers; R. M. La Ragione; G. Lo Iancono 2022. "Modelling the dynamics and long-term stability of perturbed gut microbiota", One Conference 2022, Brussels, Belgium. https://doi.org/10.5281/zenodo.6860460

Cooperation other organizations and dissemination

- On April 6 2021 partner 2-AGES presented our project to MediPIET (Mediterranean and Black Sea Field Epidemiology Training Programme Network), at the Training of Trainers on One Health.
- In the context of WP2, we have established a small collaboration with the University of Delft for comparison of our different extracellular DNA extraction methodologies and further analysis with target enrichment were performed for two water samples that University of Delft.
- Presentation of the FED-AMR project at the One Health Session, Project Review Module 2021 and facilitation at the Introductory course and Phylogeny Sessions 2021, as part of the ECDC Fellowship Program.
- EFSA was updated (in)directly on the progress of the FED-AMR project through a stakeholder that belongs to the FED-AMR Advisory Board (Beatriz Guerra).
- The protocol for the systematic evidence map has attract some media attention (Altmetric score 64). More details at https://www.altmetric.com/details/140395101?src=bookmarklet

Outcomes (deliverable, publication, folder, tool, etc.) of the project that might be suited for communication purposes to various audiences, for instance food safety or AMR scientists, national and international stakeholders, specific professionals, the general public, etc.

- Main code for the mathematical modelling is available in GitLab as private and can be shared upon request. Once the paper is finished, it will be publicly available in GitHub.
- FASTQ files from the 16S sequencing and the ARG profiling will be available on ENA for other researchers once the WP2 results are published. Due to the limited amount of studies on gene capture for AMR, our project provides valuable datasets that can be used by other researchers

interested in using similar methodologies for deep characterisation of AMR gene diversity.

- New documents produced during FED-AMR project, e.g. Protocols, are available for the scientific community.
- Risk Management Plan has been very well designed and is publicly available.

Outcomes of this project that are already discussed or even implemented and in use at any institute of the project consortium, at stakeholders' organisations (ECDC, EFSA, EMA, EEA, FAO, OIE, WHO), or at the level of national authorities?

Some of the outcomes of the project may already be in use or planned to be implemented in one or more partner institutes, or in external organisation (including stakeholders' organisations), or at the level of national authorities. Please describe.

The HOALs, which correspond to real experimental laboratories, will be disseminated as an important model for in-depth studies that intend to continue to evaluate the spread of AMR.

In WP2 we have implemented the laboratory protocol related to the collection and phenotypic characterisation of clinically-relevant bacteria present in different environmental compartments. These were identified by the WHO for their relevance in terms of their resistance to last line antibiotics.

WP2 has developed important strategies for 16S and AMR metagenomic analysis, which will be disseminated to the scientific community through scientific publications.

Regarding WP3, we highlights the harmonization and dissemination of protocols for *C. difficile* collection and isolation in animal, food and environmental samples, and the development of a pipeline for identification of MGEs in *C. difficile* (ClosTyper). All these tools are already in use by WP3 partners and were or will be disseminated externally through scientific publications.

In WP4 selection pressures (antimicrobials, elements and herbicides) for antimicrobial resistance in environmental ecosystems (soil, water, faeces, manure, plants, feeds) were determined. Most of the samples showed low or usual concentrations of the analysed substances, but in some samples (soil, faeces and manure) antibiotics from 2 antimicrobial classes (fluoroquinolones and tetracyclines) were detected at or above the minimum selective concentrations for bacteria. The results will be used for an impact evaluation of antimicrobials, elements and herbicides on the prevalence and quantities of ARGs encoded on extracellular DNA in the tested environmental compartments.

There is a close liaison of AGES, and other institutes participating in the FED-AMR project, with ministry policy makers; they are informed and sensitized to the issue of AMR in a One Health context, namely through the this project.

8. One Health impact

Antimicrobial resistance gene (ARG) pollution is a global threat to the environment and a public health issue. Risk evaluation of pathogens, ARGs as well as mobile genetic elements (MGEs) in diverse environments and matrixes are mainly based on total DNA (tDNA) data. The role of extracellular (ex) DNA is still neglected in most environmental studies. In general, the FED-AMR project contributed:

- to improve the methods and knowledge in environmental exDNA research and its role in AMR transmission.

- to collect and analyse large amounts of data and metadata from an agricultural environment, which outcomes have been presented to stakeholders.

- to underline the importance of the role of exDNA as a source of diverse antimicrobial resistances in the environment, trough the results of the FED-AMR project, which, in combination with the extremely

long persistence and its important role as a structural component of biofilms, might represent a serious future public health threat that is currently neglected.

- to still perform data analysis with the metadata produced and to combine all data i.e. exDNA, tDNA, pesticides, antibiotics, heavy metals, microbiomes, as well as to identify clinically relevant bacterial species, which may help to elucidate the transfer and development of resistances in microbial communities in an agricultural environment.

- to have identified new potential sources of emerging resistant microorganisms along the feed/food chain with a focus on the environment and wildlife as a typically neglected component of One Health.

- to confirm that *C. difficile* can spread clonally across the different compartments (animal and environment); the compartments soil and wastewater showed greater diversity in terms of *C. difficile* ribotypes, while fecal and manure samples indicating the dominant strain; this suggests a unidirectional spreading out of the animal compartments into the environment, while the soil and wastewater compartments seem to act as a reservoir for different input pathways beyond the animal compartments.Of particular interest is the possibility that *C. difficile* is transferred back to animal compartments via groundwater and surface water. The introduction of antibiotics or other substances that promote the development of antimicrobial resistance in *C. difficile* into any of the compartments can have an impact on the other compartments. - to demonstrate the genetic overlap between human and non-human *C. difficile* lineages at different One Health settings, supporting the high zoonotic importance of this human pathogen.

- to develop new ongoing projects such as HERA NGS II, funded by the EU Commission, which will incorporate and strengthen the knowledge acquired in this project.

- to understand the need to further improve any new method and new technologies, dynamically, despite the promising results we may have, as in the case of the FED-AMR.; therefore, with FED-AMR further research is essential before implementation into regional/ national surveillance systems.

- to a private company working on machine learning for detection of AMR from WGS data and a university institute working with exDNA, with whom a collaboration was established and further scientific exchanges in regards exDNA can occur in the future.

- to networking and communication between partners and reference laboratories from different backgrounds, sectors and countries, leading to new knowledge (such as at the level of the design of new protocols, or the implementation of new pipelines, or still for analyzing data in the interface of genomics and informatics).

Overall, we can still underline:

The Scientific impact of FED-AMR:

Cross-sector communication of data contributing to the advancement of science, namely e.g.:

- ✓ By a study capitalized on advancements in **high-throughput sequencing methods** and **analytical tools**, as it provides the large scalability necessary to investigate bacterial communities, in a way to explore and mapping AMR in exDNA, either in human, animal, and environmental settings, and in several sample matrices.
- ✓ By sampling campaigns that provided basic information for **establishing ARG monitoring in environmental compartments**, which is recommended by EFSA and has the potential to become compulsory for EU MS.
- ✓ By using the true concept of One Health, namely with an **important environmental component** (farmers, pigs, wild animals, manure, air of pig barns, feeds, crops, soil, water).

The societal, policy-making and economic impact of FED-AMR:

- ✓ Impactful research that **adds value** to a European, national and international level.
- ✓ Decisive **for assessing** the potential of exDNA to serve as **a high-risk source** of resistance determinants in agricultural soils and along the food/feed chain.
- ✓ Impact on **strategies to improve and/or upgrade** wastewater treatment plants, as it is decisive for WWTP engineers to know if they have to design devices that only kill bacteria or if strategies to eliminate bacterial DNA from the waste streams would have to be applied.
- ✓ As reliable and accurate surveillance is fundamental to characterize the risk of AMR in a given region, the results obtained **show how essential it is to track the spread of specific ARGs** geographically and over time, identify new ARGs and support preventive measures and interventions against AMR pathogens.
- ✓ Through a systematic evidence map, we will gather and collate data to inform future research, policy-relevant systematic review questions, and future funding strategies, concerning risk mitigation for antibiotic resistance emerging in the environment. As such, we expect that the evidence map will be relevant to e.g. WHO, ECDC and other EU agency, and national public health agencies. We expect that the evidence will contribute to the current debate about the role of environmental fluctuations always occurring in nature on the emergence and dynamics of antimicrobial resistance.

9. Data Management Plan

The project DMP should be finalized on the CDP tool, all the data should be made public and the DMP should be extracted from the CDP Tool and uploaded to Zenodo.

Zenodo link to be provided here.

A first version of the Data and Protocol Management Plan was already delivered on M34. In year 4, the DMP was updated in the new OHEJP data management platform CDP, with details of FED-AMR data throughout the project, with information provided to the leader and deputy leader by task leaders on their datasets. In addition, the task leader and deputy leader generated a metadata file for all samples collected in WP2. This file facilitated the introduction of metadata in the CPD platform and data analysis. The final version of the DMP will be delivered by end February 2023 upon agreement with the OHEJP coordination team and will be uploaded to Zenodo.

10. Future dissemination activities

The main stakeholders we identified are policy makers, the scientific community (e.g. through publications), as well as students and the general public (e.g. farmers); dissemination to the wider public was not in the foreground or scope of this project, so it is a world to explore.

The presentation of scientific findings for specific target groups would be an added-value to help combat the problem of antibiotic resistance was promoted.

11. List of dissemination and communication activities

Name of the activity:			
Date:			
Place:			
Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories. Include hyperlink, if relevant. Zenodo grants the open access, it could be used as a repository for the presentations, posters, and other dissemination materials.			
	Yes / No		Yes / No
Organisation of a Conference		Participation to a Conference	
Organisation of a Workshop		Participation to a Workshop	
Press release		Participation to an Event other than a Conference or a Workshop	
Non-scientific and non-peer-reviewed publication (popularised publication)		Video/Film	
Exhibition		Brokerage Event	
Flyer		Pitch Event	
Training		Trade Fair	
Social Media		Participation in activities organized jointly with other H2020 projects	
Website		Other	
Communication Campaign (e.g. Radio, TV)			
Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categories			
	Number		Number
Scientific Community (Higher Education, Research)		Media	
Industry		Investors	
Civil Society		Customers	
General Public		Other	
Policy Makers			

APPENDIX 1

This information is from the original Full Proposal of the project, submitted in 2018. It will help the evaluators when assessing the final report.

Please do not modify this part.

Objectives description

O1: Origins and transmission dynamics of resistant bacteria (O1.1: Import vs. local selection; O1.2: Zoonotic vs. anthropogenic vs. environmental transmission). Objective 1 will be met in WP2 by analyzing microbial biodiversity and antimicrobial resistance genes (ARGs) along the food/feed chain in a one year, longitudinal study utilizing an open air testing catchment comprising of agricultural fields, an associated pig farm, meadows, forests, surface and ground water. ARG transmission relating to import vs local selection will be addressed by analyzing cultivated crops (feed) and commercially available feed diets. Zoonotic vs anthropogenic antimicrobial resistance (AMR) transmission will be exemplarily studied with Clostridioides (former Clostridium) difficile as a model organism in WP3. Probabilistic and mechanistic modeling in WP6 will be used to model transmission dynamics of resistant bacteria and associated risks to public health. Selection pressures in environmental

compartments are determined in WP4.

O2: Clonal spread of resistant bacteria vs. horizontal gene transfer (HGT) in the dissemination of resistance

(O2.1: AMR dissemination over different ecosystem boundaries (Human – Animal – Environment)). Objective 2 will be addressed in WP2 by comparison of metagenomic data from DNA samples collected from different compartments along the catchment testing area, including human ecosystems. In laboratory settings (soil microcosms, pig gut model) the effect of environmental conditions on clonal spread and conjugation and transformation frequencies will be investigated using *Acinetobacter* spp. (transformation) and *C. difficile* (conjugation) as prototype gene-exchange platforms (WP5).

O3: Geographical differences and trends in AMR and antimicrobials in the natural environment (O3.1: Influence of animal husbandry on the occurrence and spread of AMR and antimicrobials in the environment; O3.2: Influence of animal husbandry on the occurrence and spread of AMR and antimicrobials in people living in proximity to farms). The influence of animal husbandry on the occurrence and spread of AMR and antimicrobials in the environment and in people living in proximity to farms will be analyzed in WP2 as described under O1. Geographical differences will be determined by sampling compartments similar to the environmental compartments tested in the catchment area from Northern (EE, NO) and Southern European areas (PT).

O4: Focus on multidrug and emerging resistances (O4.1: Resistance to critically important antimicrobials for human and animal treatment). Critically important antimicrobials will be quantified in environmental samples (manure, faeces, soil, water) in WP4. The corresponding resistance determinants will be identified in WP2 via metagenomics (NGS) and qPCR.

O5: Multidisciplinary approach (O5.1: Microbiology, O5.2: Genomics (i.e. WGS), O5.3: Epidemiology).

The consortium makes use of a broad range of conventional microbiological techniques (cultivation, MIC determination etc. (WP2)), but also has a strong focus on modern techniques like metagenomics and NGS, gene capture and enrichment (WP2), real time qPCR (WP2), chemostat pig gut models (WP5), soil microcosms (WP5), epidemiological analyses (WP3) and sophisticated bioinformatics and probabilistic and mechanistic modelling (WP6).

Project abstract

The relevance of horizontal antimicrobial resistance gene (ARG) transfer on free extracellular DNA (exDNA) over ecosystem boundaries relative to bacterial conjugation will be evaluated. ExDNA is omnipresent in natural environments and sufficiently stable to constitute an important reservoir for ARGs. The dissemination of AMR on exDNA over ecosystem boundaries will be monitored under controlled but naturally occurring environmental conditions in an open-air agricultural research area: The Hydrology Open Air Laboratory (HOAL) in Petzenkirchen, Austria. ARG concentrations, diversity, dynamic variability, mobility and bacterial biodiversity will be determined in an annual longitudinal study covering a crop growing period, different fertilization and land management techniques and various different – but interlinked – environmental compartments along the route: pig farm --> manure --> soil --> crop/food/feed --> ground/surface water --> pig farm (and other associated human compartments). The results obtained from HOAL will be compared with data retrieved from equivalent compartments in Northern, Eastern and Southern Europe. Movement of ARGs over ecosystem barriers will be inferred by sequence comparisons and construction of phylogenetic trees of ARGs and ARBs.

The linkage between human and non-human reservoirs of AMR will be investigated exemplarily with *C. difficile* as ARG transfer platform over ecosystem boundaries and conjugation as means for HGT.

The prevailing selection pressure in each tested habitat during the longitudinal study will be determined by quantifying antimicrobials, herbicides and trace elements in the tested compartments. Environmental conditions which may induce or inhibit the expression of competence genes that are necessary to enable the uptake of free extracellular DNA by bacteria will be identified in soil microcosms and in a pig gut model. The impact of transformation relative to conjugation will be evaluated using *Acinetobacter* sp. (transformation) and *C. difficile* (conjugation) as model organisms in these experiments.

Experimental data obtained during the project will be used to feed and tune probabilistic modelling of the emergence of AMR in target bacteria and to delineate the relative contribution of transformation and conjugation to ARG acquisition in soil environments. Mechanistic models will address key questions regarding the spatio-temporal changes observed in microbial communities and shall support the identification of critical control points for intervention to reduce the spread of AMR from environmental sources.

Background

The contribution of bacterial transformation to horizontal gene transfer (HGT) of antimicrobial resistance genes (ARGs) in environments crucial for animal and human health, such as agricultural soils, is unclear and empirical data on the impact of natural transformation in these ecosystems are lacking (1).

Free extracellular DNA (exDNA) is omnipresent and persisting in natural environments like soil (2-5), sediment (6-8) and water (9-14). Free exDNA has been identified as a source of (ARGs) in wastewater (15-17), manure (18-25), food (26-31) and feed (32), and is an important matrix component of bacterial biofilms (33-35). It is detectable in the mammalian gastrointestinal tract (36, 37) , faeces (38) , cattle rumen (39) and body fluids like saliva (40), urine (41) and blood plasma (42). ARGs encoded by free exDNA are disseminated by natural genetic transformation of environmental bacteria and human and animal pathogens competent for the uptake of free DNA (43, 44). Natural bacterial transformation is a HGT process characterized by the uptake of exDNA of plasmid or chromosomal origin by a competent bacterium, its chromosomal integration or extrachromosomal stabilization, and its expression, which leads to a new phenotype of the recipient (45, 46). Free exDNA is assumed to be a major contributor to the environmental resistome (47) considering the fact that up to 60% of the total DNA extracted from soil may originate from extracellular sources (48, 49). It has been shown that even MGEs are efficiently disseminated via transformation (43). Transformation is an important driver for genetic plasticity of bacterial genes and genomes and plays a decisive role during the formation of mosaic

ARGs like the genes for mosaic penicillin binding proteins (50). Natural transformation does not require physical contact between donor and recipient bacteria but allows large tempo-spatial separation between source and recipient of genetic information and is exclusively regulated by the recipient cell (51). This may facilitate ARG crossing ecosystem barriers and invading new habitats as growth inhibition of nonautochthonous species in new environments (i.e. the "founder effect") is alleviated.

To achieve our aims we shall use a state of the art, dedicated open-air agricultural research facility: the Hydrology Open Air Laboratory (HOAL) in Petzenkirchen, Austria. The HOAL consists of a model pig farm and agricultural fields of approx. 660 000 m2 with different crops (= food/feed) and various forms of fertilization. In contrast to point prevalence studies that only provide a snapshot view of the situation, the use of HOAL will allow us access to the dynamics of the environmental resistome and variation in the microbial biodiversity in several compartments (e.g. pigs, soil, crops (feed), surface water, farmers) in real-time.

The results of the project are decisive for assessing the potential of extracellular DNA to serve as a high-risk source of resistance determinants in agricultural soils and along the food/feed chain. The conclusions drawn from these exercises will also have an impact on strategies to improve and/or upgrade wastewater treatment plants, as it is decisive for WWTP engineers to know if they have to design devices that only kill bacteria or if strategies to eliminate bacterial DNA from the waste streams would have to be applied.

The project involves 13 laboratories from 12 partner institutions and covers a broad range of molecular, microbiological, epidemiological, biostatistical, analytical chemistry and probabilistic modelling expertise. The planned endeavours will strengthen inter-institutional co-operation, knowledge exchange and harmonisation of AMR testing approaches, which is of key importance for reliable results and conclusions for evidence-based decision-making in risk management. In particular, the project incorporates decisive elements developed by the EFFORT project. As such, the project will support harmonization of AMR testing procedures throughout Europe by building upon experience gained by the EFFORT project and similar activities and by an open access data and protocol repository.

Our project is in line with the "One Health" approach as set up by WHO, EMA, EFSA and various national action plans on reducing the spread of AMR from environmental sources and tackles knowledge gaps as identified by several high-level stakeholder conferences in 2017 – 2019.

Progress beyond state of the art

- Our approach relies on an open air agricultural testing ground comprised of all environmental compartments relevant for ARG dissemination from environmental/animal sources to human pathogens and vice versa.

  Our approach allows a comprehensive view on the research questions posed and access to process-relevant metadata collected routinely already for decades. We offer a methodological transition from point prevalence to longitudinal studies, which mirrors the highly dynamic AMR dissemination processes in and between natural habitats over the course of a year much more adequately in a controlled but still natural testing area.

- The hazards of ARGs encoded on free extracellular DNA for adverse effects on human and animal health will be determined: the focus of research is shifted here from conjugation to the impact evaluation of bacterial transformation as mediator of ARG dissemination in natural environments like agricultural soils under naturally occurring conditions. According to experts' interviews this area shows currently a substantial lack of empirical data and knowledge.

- Empirical data will be acquired on the potential of free exDNA to more easily overcome ecosystem boundaries compared to cross-boundary transfer and establishment of ARG carrying bacteria. This exercise will identify currently unknown hot spots of resistance and critical points of control which may help set measures to efficiently mitigate AMR

dissemination from environmental sources.

- We provide access to a large pool of metadata (climatic conditions, water flow, soil characteristics etc.) which can be correlated to ARG prevalence and concentrations in the tested habitats.
- NGS sequencing data will be used to identify naturally transformable bacteria capable to take up ARG encoding exDNA and expand the list of known of bacterial species capable to take up DNA (by identification competence gene homologs).
- qPCRs will be established in the way to facilitate and cheapen routine monitoring of clinically relevant ARGs also from non-human sources.
- Validated and harmonized sampling, analysis and evaluation protocols.
- Establishment of economical routine ARG monitoring systems may offer the opportunity of acquiring a return of invest for the participating partner institutions by offering testing capacities for stakeholders (e.g. competent authorities, NGOs etc.).
- Understand zoonotic potential and transmission networks of *C. difficile.*

Project aims

Evaluate the role of natural bacterial transformation relative to bacterial conjugation in the dissemination of AMR over environmental ecosystem boundaries.

We shall conduct a longitudinal study over a one-year crop-growing period (WP2, see Figure 1). DNA sequence comparisons will allow us to identify transmission routes over ecosystem boundaries and identify points for intervention to reduce the spread of AMR via exDNA. The data obtained will be compared with data collected from similar sample retrieval locations in Northern, Eastern and Southern regions of Europe to allow a comparative assessment of the risk of AMR dissemination via exDNA over ecosystem boundaries and to identify feasible ARG-monitoring and intervention strategies. Epidemiological analyses and the linkage between human and non-human (zoonotic) reservoirs of ARGs will be explored in WP3 using *C. difficile* as a paradigmatic gene exchange platform. Field studies will be complemented by data from lab experiments intended to identify drivers of AMR in the tested compartments: selection pressure (WP4); and identification of environmental conditions modulating transformation frequencies (gut model, soil microcosms; WP5). Risk management options will be derived and assessed through probabilistic and deterministic models that explain the links between antimicrobial usage in animals, AMR in the environment, and the risks for public health (WP6). The following hypotheses will be tested:

1. **Hypothesis 1 (H1):** Horizontal transfer of ARG-encoding free exDNA overcomes ecosystem-specific barriers and bottlenecks for AMR spread by relieving the requirement for direct cell-to-cell contacts and growth of non-autochthonous bacteria.
2. **Hypothesis 2 (H2):** A high microbial biodiversity constitutes a barrier for the spread of AMR.

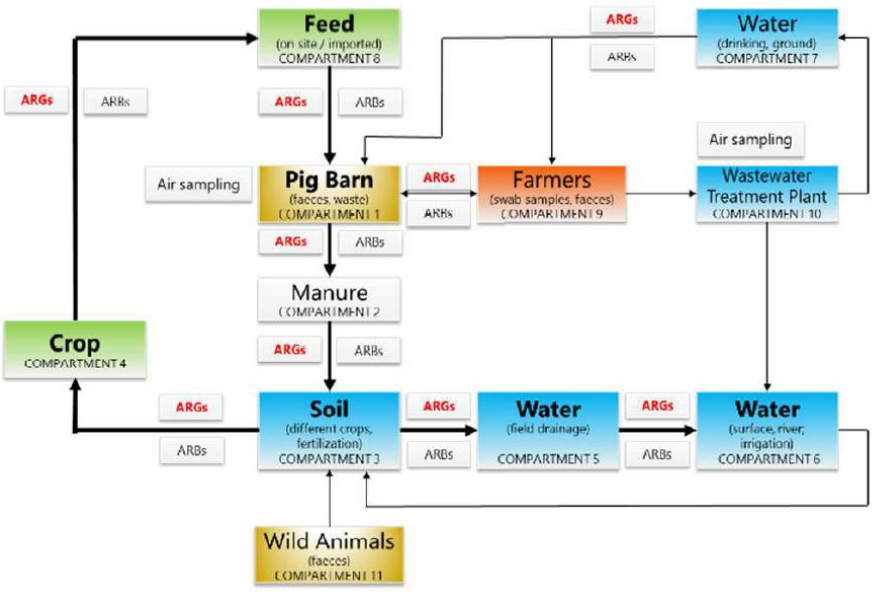


Figure 1. Potential pathways for ARG dissemination over environmental ecosystem barriers

Bold letters/arrows = compartments and pathways for ARG movement that are monitored on the HOAL testing range

ARGs: antimicrobial resistance genes, ARBs: antimicrobial resistant bacteria

Animal compartments (pigs, stable; wild animals in HOAL catchment area): gold

Human compartments (farmer, workers exposed to animal husbandry in HOAL): red

Compartments associated to plants (crops, animal feed): green;

Genuine environmental compartments (soil, water): blue

The design of the experiments was led by the following research questions:

2. **Question Q1:** What are the most prevalent ARGs in the tested environmental compartments?

3. **Question Q2:** Which environmental compartments show an especially high contamination with ARGs of anthropogenic origin?

4. **Question Q3:** Does ARG-encoding free exDNA overcome ecosystem boundaries and bottlenecks more easily compared to the transfer of non-autochthonous bacteria in newly invaded ecosystems?

5. **Question Q4:** What are the main drivers of AMR dissemination via free exDNA in the tested environmental compartments?

6. **Question Q5:** Which strategies can be applied to reduce the spread of AMR caused by the transfer of free exDNA between ecosystems highly contaminated with ARGs of anthropogenic origin?

The following aims are targeted by the respective work packages:

Objective 1 (WP2): *Determination of the resistome and microbial biodiversity in the tested environmental compartments – longitudinal study over a crop-growing season (1 year; field studies). Identification of the role of exDNA for HGT in the tested compartments.*

Objective 2 (WP3): *Elucidating the role of* C. difficile *as an ARG transfer platform over ecosystems boundaries and its linkage between human and non-human (zoonotic) reservoirs.*

Objective 3 (WP4): *Determination of the selection pressures in the tested compartments of human, animal and environmental ecosystems*

Objective 4 (WP5): *Identification of environmental conditions modulating transformation frequencies in soil microcosms and porcine chemostat gut models (laboratory studies)*

Objective 5 (WP6): *Developing probabilistic and mechanistic models explaining the links between antimicrobial usage in animals, AMR in the environment, and the risks for public health*