Deliverable 2.9
ARG dynamics in an agricultural testing area:
Response of ARG concentrations according to different fertilisation techniques and crops over an annual growth period

Workpackage 2

Responsible Partner: 36-INSA, 2-AGES
Contributing partners: 7-SZU, 14-UT, 23-UoS, 25-NUIG, 33-NVI

GENERAL INFORMATION

European Joint Programme full title	**Promoting One Health in Europe through joint actions on foodborne zoonoses, antimicrobial resistance and emerging microbiological hazards**
European Joint Programme acronym	**One Health EJP**
Funding	**This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830.**
Grant Agreement	**Grant agreement n° 773830**
Start Date	**01/01/2018**
Duration	**60 Months**

DOCUMENT MANAGEMENT

Title OHEJP deliverable	ARG dynamics in an agricultural testing area: Response of ARG concentrations according to different fertilisation techniques and crops over an annual growth period
WP and task	WP2
Leader	M. Caniça (36-INSA)
Other contributors	A. Cabal (2-AGES)
Due month of the deliverable	M60
Actual submission month	M60
Type *R: Document, report* *DEC: Websites, patent filings, videos, etc.; OTHER*	R **Save date**: 16-Mar-23
Dissemination level *PU: Public (default)* *CO: confidential, only for members of the consortium (including the Commission Services).*	PU *See updated Grant Agreement*
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 ☐ EFSA ☐ ECDC ☐ EEA ☐ EMA ☐ FAO ☐ WHO ☐ OIE ☐ Other international stakeholder(s): …… Social Media: ..... **Other recipient(s):** .....

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)

Introduction:

WP2 aims at determining the naturally occurring Antimicrobial Resistance (AMR) genes (ARG) background load and the microbial biodiversity in the tested environmental compartments.

The deliverable WP2.9 belongs to the WP2 and is associated to task JRP17-R2-WP2-T7: *"Isolate and assess quantity, diversity and stability of free extracellular ARG encoding DNA in the tested environments. Sequence comparisons."*

Due to the number of samples, and the short time and the computation resources needed for the analysis, as well as because the participating institutes could not pre-analyse and preformat their own samples/sequences, we opted for a harmonized analysis that was mostly carried out by a statistician and mathematician and the remaining "WP2 analysis team", which as a whole comprised 4 countries.

Aim:

The main aim of this deliverable was to answer to the following question: Which and how many ARG exist in the different countries and compartments?

Strategy for analysis:

The strategy for the statistical analysis was the following:

- Prepare the data for analysis.
- Separate analysis was performed for 16S and ARG data. For the ARG data, we used a clustering methodology proposed by Lanza et al. (Lanza et al., 2018) to improve the sensitivity and specificity of the metagenomics analysis.
- All analysis was performed for extracellular and total DNA separately.
- Separate analysis was performed for differences between countries and compartments.
- Organize the data into a Phyloseq object (McMurdie & Holmes, 2013). Because Ares Genetics did not provide the sequence alinement we could not include a phylogenetic tree in the Phyloseq object and preform analysis that depend in it.
- Characterize of the data retrieving information like number of samples per country and compartment, bacterial species and ARG detected in the different samples, compartments and countries, reads per sample and basic statistics, among others.
- The analysis of alpha-diversity was performed with and without rarefication to try to account for library sizes differences. We studied the richness using Chao1, ACE and Fisher indexes; evenness using the Pielou index; and diversity using Shannon and Simpson indexes. We used Anova, Kruskal-Wallis and the Wilcoxon test to access differences.
- For ordination and differential abundance analysis we first performed a centred log ratios (CLR) transformation to the data, which removes the compositional constraints to make the standard multivariate techniques suitable for analysis (Quinn et al., 2019).
- For ordination analysis we used Aitchison distance and principal component analysis. We used Permanova, Permadist and Tukey's honest significant differences test to infer the significance of the differences of the observed clusters.

- For differential abundance analysis, as recommended by Nearing et al. (Nearing et al., 2022), we used multiple differential abundance methods to help ensure robust biological interpretations. We used ALCOM-BC, DEseq2, ALDEx2 and the Wilcoxon test with CLR transformation.
- In correlation analysis we used three methods: Pearson correlation, Spearman correlation and sparCC (Friedman & Alm, 2012).
- We used Benjamini-Hochberg and Bonferroni p-value corrections for multiple testing when necessary throw-out the study.

Here we present some data:

ARG were searched in all samples. We start with 435646 rows, 5070 genes and variants in 535 samples in the AMR data. The 471 samples in 16S and AMR sets after cleanup are shown in Table 1.

Table 1. Samples in 16S and AMR sets after cleanup.

ID2541	ID2542	ID2543	ID2544	ID2955	ID2956	ID2957	ID2958
ID2959	ID2965	ID2966	ID2967	ID2968	ID2969	ID2970	ID2971
ID2972	ID2973	ID2975	ID2976	ID2977	ID2978	ID2979	ID2980
ID2981	ID2982	ID2983	ID2984	ID2986	ID2987	ID2988	ID2989
ID2990	ID2991	ID2992	ID2997	ID3000	ID3001	ID3002	ID3003
ID3004	ID3005	ID3006	ID3007	ID3008	ID3009	ID3010	ID3011
ID3012	ID3013	ID3014	ID3015	ID3017	ID3018	ID3019	ID3020
ID3021	ID3022	ID3023	ID3024	ID3025	ID3027	ID3028	ID3029
ID3030	ID3032	ID3033	ID3041	ID3042	ID3043	ID3044	ID3200
ID3201	ID3202	ID3203	ID3204	ID3205	ID3206	ID3207	ID3208
ID3209	ID3210	ID3211	ID3212	ID3213	ID3214	ID3215	ID3216
ID3217	ID3218	ID3219	ID3220	ID3221	ID3222	ID3223	ID3224
ID3225	ID3226	ID3227	ID3228	ID3229	ID3231	ID3232	ID3233
ID3234	ID3235	ID3236	ID3237	ID3238	ID3239	ID3240	ID3241
ID3242	ID3243	ID3244	ID3245	ID3246	ID3247	ID3248	ID3249
ID3250	ID3251	ID3252	ID3253	ID3254	ID3255	ID3256	ID3257
ID3258	ID3259	ID3260	ID3261	ID3262	ID3263	ID3264	ID3265
ID3266	ID3267	ID3268	ID3269	ID3270	ID3271	ID3272	ID3273
ID3274	ID3275	ID3276	ID3277	ID3278	ID3279	ID3280	ID3281
ID3282	ID3283	ID3284	ID3285	ID3286	ID3287	ID3288	ID3289
ID3290	ID3291	ID3292	ID3293	ID3294	ID3295	ID3296	ID3297
ID3298	ID3299	ID3300	ID3301	ID3302	ID3303	ID3304	ID3305
ID3306	ID3307	ID3308	ID3309	ID3310	ID3311	ID3312	ID3313
ID3314	ID3315	ID3316	ID3317	ID3318	ID3319	ID3320	ID3321
ID3322	ID3323	ID3324	ID3325	ID3326	ID3327	ID3328	ID3329
ID3330	ID3331	ID3332	ID3333	ID3334	ID3335	ID3336	ID3337
ID3338	ID3339	ID3340	ID3341	ID3342	ID3345	ID3346	ID3347
ID3348	ID3349	ID3350	ID3351	ID3352	ID3353	ID3354	ID3355
ID3356	ID3357	ID3358	ID3360	ID3362	ID3363	ID3364	ID3365
ID3366	ID3367	ID3368	ID3369	ID3370	ID3371	ID3372	ID3373
ID3375	ID3376	ID3377	ID3378	ID3379	ID3380	ID3381	ID3382
ID3383	ID3384	ID3385	ID3386	ID3387	ID3388	ID3389	ID3391
ID3392	ID3393	ID3394	ID3395	ID3396	ID3397	ID3398	ID3399
ID3400	ID3401	ID3402	ID3403	ID3404	ID3405	ID3406	ID3407
ID3408	ID3412	ID3413	ID3414	ID3415	ID3417	ID3425	ID3426
ID3427	ID3428	ID3429	ID3445	ID3446	ID3447	ID3448	ID3454
ID3455	ID3456	ID3457	ID3458	ID3459	ID3460	ID3461	ID3462
ID3463	ID3464	ID3465	ID3466	ID3467	ID3468	ID3469	ID3470
ID3471	ID3472	ID3473	ID3474	ID3475	ID3476	ID3477	ID3478
ID3479	ID3480	ID3481	ID3482	ID3483	ID3484	ID3485	ID3486
ID3487	ID3488	ID3489	ID3490	ID3491	ID3492	ID3493	ID3494
ID3495	ID3496	ID3497	ID3498	ID3499	ID3500	ID3501	ID3502
ID3503	ID3504	ID3505	ID3506	ID3507	ID3508	ID3509	ID3714
ID3715	ID3716	ID3717	ID3718	ID3719	ID3720	ID3721	ID3722
ID3723	ID3724	ID3725	ID3726	ID3727	ID3728	ID3729	ID3730
ID3731	ID3732	ID3733	ID3734	ID3735	ID3736	ID3737	ID3738
ID3739	ID3740	ID3741	ID3742	ID3743	ID3744	ID3745	ID3746
ID3747	ID3748	ID3749	ID3750	ID3751	ID3752	ID3753	ID3754
ID3755	ID3756	ID3757	ID3758	ID3759	ID3760	ID3761	ID3762
ID3763	ID3764	ID3765	ID3766	ID3767	ID3768	ID3769	ID3770
ID3771	ID3772	ID3773	ID3774	ID3775	ID3776	ID3777	ID3778
ID3779	ID3780	ID3781	ID3782	ID3783	ID3784	ID3785	ID3786
ID3787	ID3788	ID3789	ID3790	ID3791	ID3792	ID3793	ID3794
ID3795	ID3796	ID3859	ID3860	ID3861	ID3862	ID3863	ID3864
ID3865	ID3866	ID3867	ID3868	ID3869	ID3870	ID3871	ID3872
ID3873	ID3874	ID3875	ID3876	ID3877	ID3878	ID3879	ID3880
ID3881	ID3882	ID3883	ID3884	ID3885	ID3886	ID3887	ID3888
ID3889	ID3890	ID3891	ID3892	ID3893	ID3894	ID3895	ID3896
ID3897	ID3898	ID3902	ID3903	ID3904	ID3905	ID3906	

Regarding the number of reads per samples, most samples have less than 1000000 reads (Fig. 1). AMR clusters in the different compartments and countries, before Lanza clustering are represented in Fig. 2.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830.

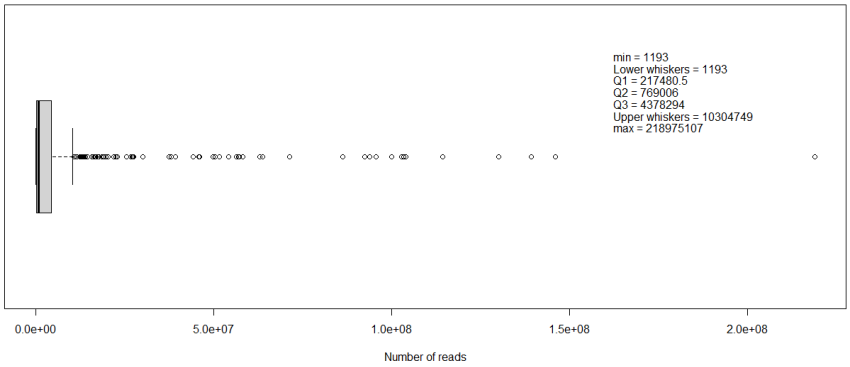

Fig 1. – Distribution of the number of reads per sample.

Indeed, 1044 of the ARG are common to all countries. United Kingdom and Portugal share 496 genes. 405 genes are excusive of one of the 6 countries, which suggests that most genes are shared by two or more countries.
Among all, 249 ARG are exclusive from waste treatment plant water. The highest number of ARG were 445 that are common to manure, feeds and soil from forests, meadows, controls and baselines. Waste treatment plant water and feeds presented the highest ARG counts, reinforcing the diversity of ARG in these compartments.

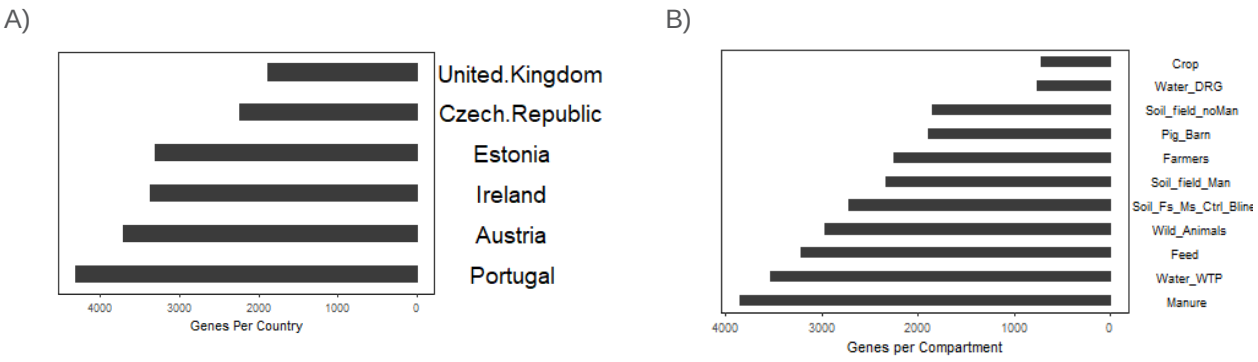

Fig. 2. – Number of ARG, A) by country and B) by compartment.

The ARG identified conferred resistance through different resistance mechanism as represented in Fig. 3 (analysis RPG by Country).

Fig. 3 - Resistance mechanisms in which the identified ARG were involved, by country.

Globally, we notice that feeds present high richness. Except for tDNA in Estonia, feeds have a richness comparable to manure, farmers and pigs. Concerning farmers they show high richness. Pairwise Wilcoxon reinforce these conclusions, we did not found significant differences between feeds, wild animals and waste water, both in eDNA and tDNA. Also, no significant differences in richness between waste water and farmers. There are significant differences between the forest and fields without manure fertilization and the fields with manure. Much more significant differences in richness in tDNA. Both DNA types show significant differences in richness between countries in crops and pigs. There is much more variation on the ARG comparing to 16S analysis.

Publication:

These results and all related ones will be part, in 'detail', of a manuscript to be submitted and that will also be available to the international scientific community.

References:

Friedman, J., & Alm, E. J. (2012). Inferring Correlation Networks from Genomic Survey Data. PLoS Computational Biology, 8(9), e1002687. https://doi.org/10.1371/journal.pcbi.1002687

Lanza, V. F., Baquero, F., Martínez, J. L., Ramos-Ruíz, R., González-Zorn, B., Andremont, A., Sánchez-Valenzuela, A., Ehrlich, S. D., Kennedy, S., Ruppé, E., van Schaik, W., Willems, R. J., de la Cruz, F., & Coque, T. M. (2018). In-depth resistome analysis by targeted metagenomics. Microbiome, 6(1), 11. https://doi.org/10.1186/s40168-017-0387-y

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE, 8(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Nearing, J. T., Douglas, G. M., Hayes, M. G., MacDonald, J., Desai, D. K., Allward, N., Jones, C. M. A., Wright, R. J., Dhanani, A. S., Comeau, A. M., & Langille, M. G. I. (2022). Microbiome differential abundance methods produce different results across 38 datasets. Nature Communications, 13(1), 342. https://doi.org/10.1038/s41467-022-28034-z

Quinn, T. P., Erb, I., Gloor, G., Notredame, C., Richardson, M. F., & Crowley, T. M. (2019). A field guide for the compositional analysis of any-omics data. GigaScience, 8(9), 1–14. https://doi.org/10.1093/gigascience/giz107