PhD DAY (III)

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Title: Potential Role of Treated Wastewater and Heavy Rainfall Events on Environmental Spreading of AMR into the Lambro River (Lombardy Region, Italy)

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Background: Antibiotic-resistance indicators (ARB, ARGs, and MGEs) are frequently detected in environmental matrices, including Wastewater Treatment Plant (WWTPs) effluents. Wastewater Bypass could also contribute to their dissemination, especially during stormwater events. This project aimed to assess the potential impact of heavy rainfall on the release of ARGs and ARB into the receiving river.

Method: Water samples were collected along the Lambro River (Northern Italy), at five sampling points, in the absence and in the presence of high-intensity rainfall events. ARB were quantified using culture-based methods. DNA was then extracted for the quantification of *16S rRNA*, *intI1*, and seven ARGs. Bacterial community composition was investigated via *16S rRNA* gene Amplicon Sequencing on MiSeq – Illumina platform.

Results: In absence of rainfall, several ARGs showed significantly higher concentrations downstream of the WWTP compared to upstream, without a corresponding increase in culturable ARB. During rainfall events, both ARGs and ARB significantly increased along all the river sites, suggesting a potential contribution of wastewater bypass, stormwater runoff, and potentially additional upstream sewer overflows. PCA analysis revealed that the riverine bacterial communities collected during rainfall clustered closely with wastewater influent samples, highlighting a strong wastewater-related signature in the river during high-intensity rainfall conditions.

Conclusions: WWTP effluents may contribute to the environmental dissemination of ARGs in absence of extreme weather events, while rainfall acts as a major driver of widespread AMR dissemination, highlighting the anthropogenic pressure on the Lambro River and the importance of considering hydrological conditions when assessing environmental AMR dynamics.

Broad-spectrum analysis of beta-lactam resistant *Granulicatella adiacens*: from Penicillin-Binding Proteins mutational and antibiotic affinity profile to cell morphology and peptidoglycan composition

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Beta-lactam resistance in Gram-positive bacteria is mainly driven by penicillin-binding proteins (PBPs) alterations. They can also adapt their cell wall after antibiotic exposure. *Granulicatella adiacens* infections are typically treated with beta-lactam antibiotics but their resistance is increasing. This work aims to characterize *G. adiacens* PBPs and their mutations' role in beta-lactam resistance and evaluate peptidoglycan alteration determined by antibiotic stress.

Beta-lactam activity, synergy, and PBP binding were compared between IS48 clinical isolate and *G. adiacens* ATCC 49175. Affinity to ceftriaxone, ceftobiprole, and ampicillin was assessed with BocillinFL. PBP homology and mutations were analysed by sequence alignment and WGS. PBP1A was cloned and expressed in *E. coli* BL21(DE3) into pET24 and purified. Growth curves were performed in supplemented BHI with D-amino acids probes to analyse cell morphology. Peptidoglycan composition was studied *via* LC-MS after antibiotic exposure to ceftobiprole, ceftriaxone, ampicillin (1/2-fold MIC).

Normal diplococci morphology emerged only in BHI supplemented with blood and L-cysteine. IS48 was not susceptible to penicillin and ampicillin, resistant to ceftriaxone, with higher ceftobiprole MIC than the control strain. Ampicillin *plus* ceftobiprole/ceftriaxone were synergistic. Five PBPs were identified. Most IS48 PBPs carried mutations near catalytic motifs. PBP2B and PBP2 exhibited consistently low acylation levels, whereas PBP1A and PBP2A demonstrated significant inhibition. PBP1B reached IC_{50} only by double treatments in the ATCC strain and by ceftriaxone and ampicillin/ceftriaxone in IS48.

The mutations observed may affect the drug binding, impacting on the resistance phenotype. However, beta-lactam treatment may be effective due the inhibition of key bifunctional PBPs (PBP1A, PBP2A).

Prokaryotic diversity and ecosystem functioning in hypersaline environments: a metagenomic study of the Cagliari salterns

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Solar salterns are extreme environments of high scientific interest, hosting specialized microbial communities shaped by steep physicochemical gradients. Here we present the first comparative metagenomic analysis of two solar salterns in southern Sardinia: Conti Vecchi (SCV, active) and Molentargius (SM, inactive), located ~15 km apart and exposed to similar climatic conditions. This setting provides a unique opportunity to assess the impact of saltwork management on microbial diversity along a salinity gradient.

Water and sediment samples were collected from ponds with varying salinities in winter and summer. A total of 2786 metagenome-assembled genomes (MAGs) were reconstructed, 1537 from SCV and 1249 from SM, including 1131 MAGs from sediments and 1655 from water. Physicochemical analyses identified seasonal variation as the primary driver of environmental differences (p-value < 0.001), explaining 61% of total variance, although site-specific differences in thermal range and pond structure were also observed.

Benthic trophic conditions varied markedly across ponds. The medium-low salinity pond SCV-1 (57 psu) was eutrophic, with organic carbon exceeding microbial processing capacity, while the high-salinity pond SCV-17 (275 psu) showed near-complete depletion of biopolymeric carbon. Metagenomic results supported these patterns, with *Pseudomonadota* and *Bacteroidota* dominating at low salinities, and *Halobacteriota* prevailing in high-salinity ponds.

In SM, the high salinity pond SM-3 (260 psu) exhibited a striking seasonal shift, with high degradation rates and rapid turnover in winter, and reduced activity in summer, suggesting substantial changes in microbial community. Taxonomic analysis confirmed this frame, revealing an unexpected dominance of Bacteria despite the high salinity. In addition, a large fraction of MAGs could not be assigned to known taxa, suggesting unexplored novelty spanning from species to higher taxonomic levels. Overall, these findings position southern Sardinia salterns as unexplored reservoirs of prokaryotic novelty and underscore the importance of expanding metagenomic investigations to other under-characterized Mediterranean extreme environments.

Microbiome Structure and Dynamics in Novel Fixed-Bed Biofilm Systems for Medium Chain Fatty Acids Production from Organic Waste

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The microbial valorization of organic waste into medium-chain fatty acids (MCFAs) represents a promising resource recovery strategy. However, its efficiency is often limited by competition between primary fermenters and chain-elongating bacteria, with the former favored in the commonly-used fluidized-bed systems due to their higher growth rates and substrate uptake. To overcome this limitation, this study developed a novel single-stage fixed-bed biofilm reactor for the co-fermentation of food waste (FW) and waste activated sludge (WAS). Several tests were conducted under mesophilic conditions (35 °C) with a 4-day hydraulic retention time and varying organic loading rates (OLRs of 6.2, 12.4, and 18.75 $gVSL^{-1}d^{-1}$), without external pH control or the addition of external electron donors. Initially, two support-to-inoculum ratios (1:3 and 1:1 w:V) were tested using a mixture of FW and WAS at a ratio of 60:40 (on VS basis). After identifying 1:1 w:V as the best-performing ratio, additional tests were carried out using different substrates (i.e., FW:WAS at a ratio of 80:20, and FW liquid extract combined with WAS at a 60:40 ratio) combined with different feeding frequencies (i.e., 2, 3 , and 5 days per week). Control reactors without filling material were operated in parallel for each tested conditions. A comprehensive chemical and microbiological characterization of biofilm and suspended biomass was performed using complementary advanced techniques (e.g., Illumina and Nanopore sequencing, microscopy-based analysis, and real-time quantification). By promoting microbial selection and biomass retention, fixed-bed biofilm systems enhanced system stability and MCFAs production and consistently outperformed controls, representing an effective strategy for valorizing complex organic waste into high-value chemicals.

Characterization of microbial communities and functional diversity associated to native plant species *Pistacia lentiscus* L. and *Helichrysum microphyllum* subsp. *tyrrhenicum* in abandoned mining areas

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Among phytoremediation approaches, phytostabilization occurs at the root-substrate interface, where excluder-type metallophytes and associated microorganisms mitigate metal mobility and bioavailability. This study presents an integrative analysis of microbial diversity associated to two native plants of Sardinian abandoned mining areas and candidate for revegetation and restoration programmes. Spontaneous plants were studies in a Zn-Pb mine tailing dump and its surrounding areas. To deepen our understanding of the interaction among mine substrates, metals, plants, and microbes under real field conditions, a multifactorial approach was employed evaluating dehydrogenase activity, Community Level Physiological Profiling, bacterial and fungal communities through high-throughput sequencing of ribosomal genes in mine tailings, rhizosphere and roots. The studied site exhibited significant heterogeneity in environmental parameters and metal concentrations. Analysis revealed differences between the two plant species in metabolic activities and highlighted distinct abiotic drivers shaping bacterial and fungal community structures. Bacterial communities associated with the rhizosphere and roots differed between plant species, while fungal communities of both plants were dominated by the same taxa. This study represents the first comprehensive characterization of microbial communities associated with *P. lentiscus* and *H. tyrrhenicum*. Our findings demonstrate that different plant species select different microbial communities, providing critical insights into the ecological roles of root-associated microbiomes and their potential for site-specific remediation strategies. This work has been developed within the framework of the project e.INS www.einsardinia.eu (Next Generation EU- PNRR-M4 C2 I1.5 CUP F53C22000430001).