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Universidad del País Vasco

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Tesis Doctoral

DISEMINACIÓN DE RESISTENCIAS A LOS ANTIBIÓTICOS EN SUELOS AGRÍCOLAS ENMENDADOS CON RESIDUOS ORGÁNICOS

Leire Jauregui Aizpurua

Directores:

Dr. Carlos Ander Garbisu Crespo

Dra. Lur Epelde Sierra

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“Se buscan hombres para viaje peligroso.
Sueldo bajo. Frío extremo.
Largos meses de total oscuridad.
Escasas posibilidades de regresar con vida.
Honor y reconocimiento en caso de éxito”.

Ernest Shackleton

RESUMEN

Los antibióticos son herramientas indispensables para el tratamiento de las enfermedades infecciosas, tanto en salud humana como en veterinaria. Sin embargo, el uso incorrecto y abusivo de los antibióticos ha acelerado la emergencia y diseminación de las resistencias a los antibióticos, convirtiéndolo en uno de los principales problemas sanitarios a nivel mundial. El abordaje de esta creciente problemática requiere de una aproximación bajo la perspectiva *One Health*.

Las enmiendas orgánicas de origen animal y urbano son un importante reservorio de antibióticos y bacterias resistentes que albergan y transfieren genes de resistencia a los antibióticos. La fertilización de los suelos agrícolas con enmiendas orgánicas es una práctica muy común debido a su potencial agronómico, así como al hecho de ser una alternativa sostenible y económicamente viable al uso de fertilizantes de síntesis. Sin embargo, la aplicación de estas enmiendas conlleva riesgos potenciales asociados a la diseminación de residuos de antibióticos, bacterias resistentes, genes de resistencia a antibióticos y elementos genéticos móviles. El citado riesgo varía en función de numerosos factores: la abundancia del gen que concede la resistencia, la naturaleza y concentración del antibiótico específico al que confiere resistencia dicho gen, su potencial para transferirse horizontalmente, su presencia en un posible patógeno humano, etc. En este trabajo de investigación se ha evaluado el riesgo del resistoma en los agroecosistemas a lo largo de la vía de exposición que va desde las propias deyecciones animales y enmiendas orgánicas hasta los suelos enmendados y los cultivos agrícolas en ellos crecidos.

Nuestros resultados indican que (i) el empleo de enmiendas orgánicas representa una importante vía de entrada del resistoma al medioambiente agrario, aunque el riesgo asociado disminuye a lo largo de la vía de exposición "enmienda – suelo – cultivo"; (ii) la evaluación de la calidad de las enmiendas orgánicas debe incluir una caracterización físico-química y microbiológica exhaustiva, considerando los riesgos derivados de su uso en relación con la presencia de contaminantes emergentes (*e.g.*, antibióticos, genes de resistencia a los antibióticos); (iii) la adición repetida de oxitetraciclina ejerce una presión selectiva sobre las comunidades procariotas edáficas e induce su resistencia frente a dicho antibiótico a corto plazo; (iv) el origen de las enmiendas (ganadería ecológica *versus* ganadería convencional) no influye en la magnitud del resistoma en el sistema "enmienda – suelo – cultivo", al mismo tiempo que el perfil del resistoma de cada tipo de enmienda orgánica (*i.e.*, estiércol fresco, estiércol envejecido, purín) no se mantiene a lo largo de dicha vía de exposición; (v) la aplicación de lodos de depuradora urbana digeridos anaeróbicamente y deshidratados incrementa la magnitud del

resistoma y moviloma del suelo; y (vi) el tratamiento de las enmiendas orgánicas mediante digestión anaerobia y adición del biochar reduce la magnitud del resistoma y moviloma.

Se concluye que la aplicación de enmiendas orgánicas de origen animal o urbano al suelo agrícola representa una preocupante vía de entrada de resistencias a los antibióticos en los agroecosistemas. La optimización del tratamiento y manejo de las enmiendas orgánicas, previamente a su aplicación al suelo, es una opción idónea para mitigar el riesgo asociado al resistoma y, en particular, la entrada de resistencias a los antibióticos a los agroecosistemas.

LABURPENA

Antibiotikoak ezinbesteko tresnak dira giza-osasunean eta albaitaritzan bakterio-infekzioei aurre egiteko. Hala ere, antibiotikoen gehiegizko eta erabilera okerrak antibiotikoekiko erresistentzien agerpena eta barreiadura bizkortu du, mundo-mailako osasun-arazo nagusienetako bat bihurtu delarik. Areagotzen ari den arazo hau *One Health* ikuspegitik aztertzea ezinbestekoa da.

Animalia eta giza-jatorriko ongarri organikoak antibiotikoen eta bakterio erresistenteen biltegi garrantzitsuak dira, antibiotikoekiko erresistentzia geneak eduki eta transferitzen baitituzte. Ongarri organikoak nekazaritza lurzoruetan aplikatzea oso ohikoa da potentzial agronomikoa dutelako, baina baita sintesiko ongarrien alternatiba jasangarri eta ekonomikoki bideragarriak direlako. Hala ere, ongarri organikoen aplikazioak antibiotiko, bakterio erresistente, antibiotikoekiko erresistentzia gene eta elementu genetiko mugikorrak barreiatzeako arriskua dakar. Arrisku hori aldakorra da hainbat faktoreren arabera: erresistentzia genearen ugaritasuna, gene horrek erresistentzia ematen dion antibiotiko mota eta kontzentrazioa, horizontalki transferitzeko gaitasuna, eta giza patogeno posible batean aurkitzea, besteak beste. Ikerketa-lan honetan, erresistomaren arriskua agroekosistemetako esposizio-bidean zehar ebaluatu da, animalien gorozki eta ongarri organikoetatik ongarritutako lurzoru eta laboreetaraino, alegia.

Gure emaitzek adieratzen dutenez, (i) ongarri organikoen erabilera nekazaritza-ingurunean erresistomaren sarbide-puntu garrantzitsua da, honekin erlazionatutako arriskua “ongarri organikoa – lurzorua – laborea” esposizio-bidean zehar murrizten den arren, (ii) ongarri organikoen kalitatearen ebaluazioak karakterizazio fisiko-kimiko eta mikrobiologiko sakona behar du, horien erabilerak dakartzan kutsatzaile emergenteekin (antibiotikoak, antibiotikoekiko erresistentzia geneak) erlazionatutako arriskuak kontuan hartuz; (iii) oxitetraziklina behin eta berriz gehitzeak lurzoruko bakterio-komunitateei hautespen presioa egin eta antibiotiko horrekiko erresistentzia eragiten du epe laburrean; (iv) ongarri organikoen jatorriak (abeltzaintza ekologikoa *versus* abeltzaintza konbentzionala) ez du erresistomaren handitasunean eraginik “ongarri organikoa – lurzorua – laborea” esposizio-bidean, eta aldi berean, ongarri organiko bakoitzaren erresistomaren profila (simaur freskoa, biltegiratuko simaurra, minda) ez da mantentzen aipatutako esposizio-bidean; (v) lurzoruan anaerobikoki digeritutako eta deshidratatutako hiri-araztegiko lohiak aplikatzeak erresistomaren eta mobilomaren larritasuna handitzen du; eta (vi) ongarri organikoak anarobikoki digerituta edota bio-ikatza gehituta kudeatuz gero, erresistoma eta mobilomaren handitasuna murrizten da.

Laburbilduz, nekazaritza lurzoruetan animalia eta giza-jatorriko ongarri organikoen aplikazioak antibiotikoen erresistentzien sarbide-puntu kezkagarria suposatzen du agroekosistemetan. Lurzoruan aplikatu aurretik ongarri organikoen tratamendu eta kudeaketen optimizazioa aukera egokia da erresistomarekin lotutako arriskua gutxitzeko, eta bereziki antibiotikoekiko erresistentzien agroekosistemetarako sarrera murrizteko.

SUMMARY

Antibiotics are indispensable tools for the treatment of bacterial infections in human medicine and veterinary medicine. However, the inappropriate and abusive use of antibiotics has accelerated the emergence and dissemination of antibiotic resistance, becoming one of the main health problems worldwide. Tackling this growing serious problem requires a One Health approach.

Organic amendments of animal and urban origin are important reservoirs of antibiotic residues and antibiotic resistant bacteria that harbor and spread antibiotic resistance genes. The use of organic amendments as fertilizers is a very common agricultural practice due to their agronomic potential, as well as to the fact that they are a sustainable and economically-viable alternative to the use of synthetic fertilizers. Nonetheless, the application of these amendments poses potential risks associated with the dissemination of antibiotic residues, antibiotic resistant bacteria, antibiotic resistance genes and mobile genetic elements. These risks depend on multiple factors: the abundance of the antibiotic resistance gene, the nature and concentration of the specific antibiotic to which the gene confers resistance, its potential for horizontal gene transfer, its presence in a potential human pathogen, etc. In this research work, we assessed the resistome risk in agroecosystems along the exposure pathway from animal faeces and organic amendments to amended soils and the crops.

Our results indicate that (i) the use of organic amendments represents an important route of resistome entry into the agricultural environment, although the associated risk decreases along the "amendment – soil – crop" exposure pathway; (ii) the evaluation of organic amendment quality should include an exhaustive physicochemical and microbiological characterization which takes into consideration the associated risks derived from the presence of emerging contaminants (*i.e.*, antibiotic residues, antibiotic resistance genes); (iii) the repeated addition of oxytetracycline exerts a selective pressure on soil prokaryotic communities and induces their resistance to such antibiotic; (iv) the origin of the amendments (organic livestock *versus* conventional livestock) does not influence the magnitude of the resistome in the "amendment – soil – crop" system, while the resistome profile of each type of organic amendment (*i.e.*, fresh manure, aged manure, slurry) is not maintained along this exposure pathway; (v) the application of thermally-dried anaerobically-digested sewage sludge increases the magnitude of the soil resistome and mobilome; and (vi) the treatment of organic amendments by anaerobic digestion or the addition of biochar reduces the magnitude of the resistome and mobilome.

It was concluded that the application of organic amendments of animal and urban origin to agricultural soils can lead to the entry of antibiotic resistance into agroecosystems. The optimization of organic amendment treatment and management, prior to their application to agricultural soil, is an ideal option to mitigate resistome-associated risks and, in particular, the entry of antibiotic resistance into agroecosystems.

PUBLICACIONES GENERADAS

Yo, Leire Jauregui Aizpurua, declaro que:

Esta tesis doctoral ha generado cuatro artículos ya publicados en revistas indexadas JCR así como dos artículos que actualmente se encuentran bajo revisión:

1. Jauregi, L., Epelde, L., González, A., Lavín, J.L., Garbisu, C. (2021). Reduction of the resistome risk frow cow slurry and manure microbiomes to soil and vegetable microbiomes. Environ Microbiol 23, 7643-7660 (ver Capítulo 4). https://doi.org/10.1111/1462-2920.15842.
2. Epelde, L., Jauregi, L., Urra, J., Ibarretxe, L., Romo, J., Goikoetxea, I., Garbisu, C. (2018). Characterization of composted organic amendments for agricultural use. Front Sustain Food Syst 2, 44 (ver Capítulo 5). https://doi.org/10.3389/fsufs.2018.00044.
3. Jauregi, L., Artamendi, M., Epelde, L., Blanco, F., Garbisu, C. Pollution-induced tolerance of soil bacterial communities to oxytetracycline-spiked manure (ver Capítulo 6). Enviado para su publicación.
4. Jauregi, L., Epelde, L., Alkorta, I., Garbisu, C. (2021). Antibiotic resistance in agricultural soil and crops associated to the application of cow manure-derived amendments from conventional and organic livestock farms. Front Vet Sci 8, 633858 (ver Capítulo 7). https://doi.org/10.3389/fvets.2021.633858.
5. Jauregi, L., Epelde, L., Alkorta, I., Garbisu, C. (2021). Agricultural soils amended with thermally-dried anaerobically-digested sewage sludge showed increased risk of antibiotic resistance dissemination. Front Microbiol 12, 666854 (ver Capítulo 8). https://doi.org/10.3389/fmicb.2021.666854
6. Jauregi, L., González, A., Garbisu, C., Epelde, L. Organic amendments treatments for resistome risk reduction in soil-crop systems (ver Capítulo 9). Enviado para su publicación.

Asimismo, el presente trabajo ha generado un artículo en una revista no indexada por JCR:

1. Jauregi, L., Garbisu, C., Epelde, L. (2022). Antibiotikoen erresistentziak agroekosistemetan. Ekaia (ver Capítulo 1.2). https://doi.org/10.1387/ekaia.23461.

Además, de este trabajo son también fruto las siguientes comunicaciones a congresos:

1. Jauregi, L., González, A., Garbisu, C., Epelde, L. Organic amendment treatments for resistome risk reduction in soil-crop systems. Enviado a EDAR 6th International Symposium on the Environmental Dimension of Antibiotic Resistance. Gothenburg (Suecia), 2022.
2. Jauregi, L., Garbisu, C., González-Uriarte, A., Lavín, J.L., Epelde, L. Resistome risk pathways at conventional cow dairy farms. World Microbes Forum. Virtual Conference, 2021.
3. Artamendi, M., Jauregi, L., Epelde, L., Alkorta, I., Garbisu, C. Pollution-induced community tolerance in soil bacteria exposed to oxytetracycline. FEMS 8th Congress of European Microbiologists. Glasgow (Escocia), 2019.
4. Jauregi, L., Epelde, L., Alkorta, I., Garbisu, C. Antibiotic resistance in agricultural soil associated to the application of cow manure-derived amendments from ecological and conventional livestock farms. BAGECO 15th Symposium on Bacterial Genetics and Ecology. Lisboa (Portugal), 2019.
5. Garbisu, C., Epelde, L., Jauregi, L., Alkorta, I. Antibiotic resistance risk associated with the use of cow manure-derived amendments in conventional and ecological agriculture. 1st Meeting of the Iberian Ecological Society – XIV AEET Meeting-Ecology: an integrative science in the Anthropocene. Barcelona (España), 2019.
6. Jauregi, L., Urra, J., Blanco, F., Garbisu, C., Epelde, L. Diseminación de genes de resistencia a antibióticos como consecuencia de la aplicación de lodos de depuradora en suelos agrícolas. VIII Congreso Ibérico de las Ciencias del Suelo. Donostia (España), 2018.
7. Jauregi, L., Urra, J., Blanco, F., Alkorta, I., Garbisu, C., Epelde, L. Antibiotic resistant genes in agricultural soil amended with sewage sludge. 3rd Conference on Ecology of Soil Microorganisms. Helsinki (Finlandia), 2018.
8. Epelde, L., Jauregi, L., Urra, J., Garbisu, C. Reducción del riesgo de salud humana asociado al empleo de enmiendas orgánicas de origen animal en agricultura. II Simposio del Grupo de Trabajo Interacciones Planta-Suelo de la AEET. Antequera (España), 2018.

ABREVIATURAS

AIC	Akaike information criterion / Criterio de información Akaike
AMR	Antimicrobial resistance / Resistencia antimicrobiana
AQI	Amendment quality index / Índice de calidad de enmiendas
ARB	Antibiotic resistant bacteria / Bacteria resistente a antibiótico
ARG	Antibiotic resistance gene / Gen de resistencia a antibiótico
ASV	Amplicon sequence variant / Variante de secuencia de amplicón
ddPCR	Droplet digital PCR / PCR digital en gotas
EC	European Commission / Comisión Europea
EDAR	Estación depuradora de aguas residuales
FAO	Food and Agriculture Organization of the United Nations / Organización de las Naciones Unidas para la Alimentación y la Agricultura
FC	Fold change / Valor de cambio
HGT	Horizontal gene transfer / Transferencia horizontal de genes
HT-qPCR	High-throughput quantitative PCR / PCR cuantitativa de alto rendimiento
MGE	Mobile genetic element / Elemento genético móvil
MIC	Minimum inhibitory concentration / Concentración mínima inhibitoria
OIE	World Organisation for Animal Health / Organización Mundial de la Sanidad Animal
OTC	Oxytetracycline / Oxitetraciclina
OTU	Operational taxonomic unit / Unidad taxonómica operativa
PICT	Pollution induced community tolerance / Tolerancia inducida por contaminación a nivel de comunidad
PRAN	Plan Nacional frente a la Resistencia a los Antibióticos
SDG	Sustainable Development Goals / Objetivos de Desarrollo Sostenible

SEM Structural equation model / Modelo de ecuaciones estructurales

WHO World Health Organization / Organización Mundial de la Salud

ÍNDICE

1. ANTECEDENTES E INTRODUCCIÓN

1.1. Antecedentes

1.1.1. El problema de las resistencias a antimicrobianos

La aparición de la resistencia antimicrobiana (AMR) es parte intrínseca del proceso evolutivo de los microorganismos. Sin embargo, el uso incorrecto y excesivo de los antimicrobianos ha acelerado la emergencia y la propagación de las AMRs, convirtiéndolo en uno de los principales problemas sanitarios a nivel mundial. Los antimicrobianos agrupan sustancias químicas que impiden el desarrollo o causan la muerte a un amplio espectro de microorganismos, *e.g.* antibióticos, antifúngicos, antivirales y antiprotozoarios. Actualmente, carecemos de antibióticos que puedan frenar algunas infecciones causadas por bacterias multirresistentes-ampliamente resistentes-panresistentes. Según los modelos estadísticos predictivos de Antimicrobial Resistance Collaborators (2022), en 2019, hubo 4,95 millones de muertes asociadas a la AMR bacteriana, incluyendo 1,27 millones de muertes atribuibles a la AMR bacteriana. Si no se toman medidas, se estima que la cifra podría aumentar a 10 millones de muertes anuales para el año 2050, superando así las muertes por cáncer. Además, provocaría una pérdida en producción económica mundial que ascendería a 100 billones de dólares para 2050 (O'Neill, 2016).

1.1.2. Historia de los antibióticos

La *época dorada* del descubrimiento de nuevas clases de antibióticos estuvo comprendida entre 1950 y 1970 (Figura 1.1), principalmente mediante el aislamiento de organismos productores de antibióticos a partir de muestras de suelo (Hutchings et al., 2019). Sin embargo, desde la época dorada apenas se han descubierto nuevas clases de antibióticos. En consecuencia, el procedimiento general para combatir las AMRs se ha basado en la modificación de los antibióticos existentes (Chopra et al., 2002). La resistencia antimicrobiana surge de promedio a los cinco años de la comercialización del antimicrobiano en cuestión (Harbarth et al., 2015). En una entrevista de 1945 Alexander Fleming dijo que "la persona imprudente que juega con el tratamiento de la penicilina es moralmente responsable de la muerte del hombre que sucumbe a la infección por el organismo resistente a la penicilina. Espero que este mal pueda ser evitado". Actualmente, es evidente que el *mal no ha podido ser evitado* como refleja el hecho de que la Organización Mundial de la Salud (WHO) incluyó las AMRs como uno de los 10 principales problemas de salud mundial (WHO, 2020).

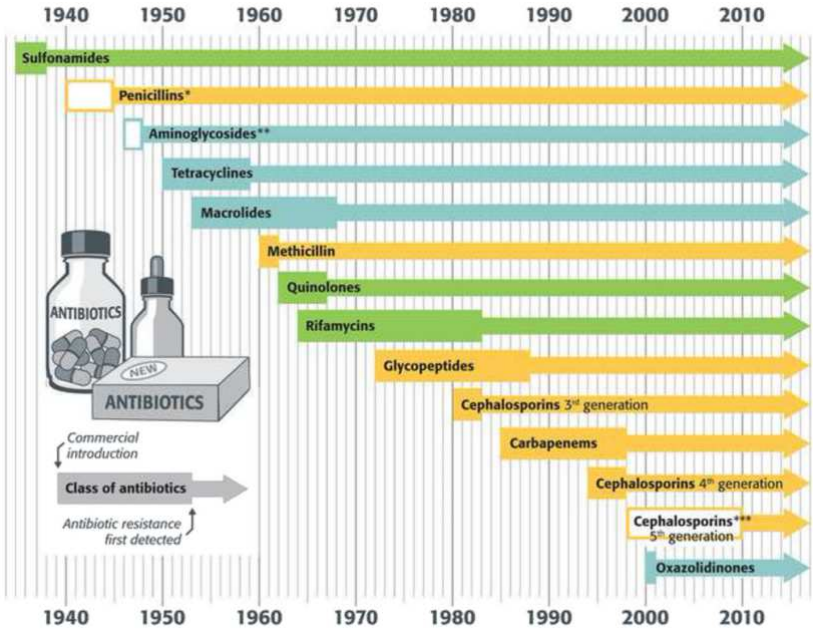


Figura 1.1. Comercialización (lado izquierdo del rectángulo) y primera detección de bacterias resistentes (lado derecho del rectángulo) para algunas clases de antibióticos. Fuente: Harbarth et al., 2015.

1.1.3. Acciones internacionales, nacionales y locales contra las AMRs

La Comisión Europea (EC) publicó su primer Plan de Acción en 2011, abarcando el periodo 2011-2016, contra la creciente amenaza de las AMRs. La evaluación de este plan identificó la necesidad de comprender el papel que desempeña el medioambiente en la emergencia y transmisión de las resistencias a través de los residuos animales y humanos. La comprensión científica de las vías de exposición a los antimicrobianos contribuye a explorar las medidas necesarias para identificar y reducir los riesgos asociados.

En 2015, la WHO adoptó el Plan de Acción Mundial sobre las AMRs (WHO, 2016), el cual fue adoptado posteriormente por la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) y la Organización Mundial de Sanidad Animal (OIE). El plan destacó la necesidad de tomar medidas armonizadas e inmediatas a escala mundial, debido a que las AMRs suponen una amenaza para varios de los Objetivos de Desarrollo Sostenible (ODS) referentes a la salud, la seguridad alimentaria, el bienestar animal y el desarrollo socioeconómico. En dicho plan se establecieron cinco objetivos: (i) mejorar la concienciación y la comprensión con respecto a las AMRs a través de una comunicación, educación y formación efectivas; (ii) reforzar los conocimientos y la base científica a través de la vigilancia y la investigación; (iii) reducir la incidencia de las infecciones con medidas

eficaces de saneamiento, higiene y prevención de las infecciones; (iv) utilizar de forma óptima los medicamentos antimicrobianos en la salud humana y animal; y (v) preparar argumentos económicos a favor de una inversión sostenible que tenga en cuenta las necesidades de todos los países, aumentando la inversión en nuevos medicamentos, medios de diagnóstico, vacunas y otras intervenciones. El esquema establecido con el enfoque *One Health* propuso un marco de seguimiento y evaluación, además de establecer tareas y responsabilidades de los gobiernos de los países, las organizaciones de la alianza tripartita (WHO, FAO, OIE) y otras asociaciones nacionales e internacionales.

En 2017, la EC emprendió su segundo Plan de Acción contra las AMRs, en el que se incluían acciones específicas para hacer de la Unión Europea una región de mejores prácticas y abordar mejor el papel que ejerce el medioambiente, desde el enfoque *One Health* que resalta la interdependencia de la salud humana, animal y ambiental (COM/2017/339 final). Ese mismo año, el informe publicado por la Organización de las Naciones Unidas incluyó las AMRs entre uno de los seis temas de interés ambiental con consecuencias de alcance mundial, remarcando la importancia de investigar la dimensión ambiental de las AMRs (PNUMA, 2017).

En 2019, la EC adoptó el enfoque estratégico de la Unión Europea en materia de productos farmacéuticos en el medioambiente, en el que se subraya el impacto de los antimicrobianos empleados en salud humana y sanidad animal en el suelo, acelerando la aparición, el desarrollo y la propagación de bacterias resistentes (COM/2019/128 final). Sin embargo, el enfoque estratégico plantea varias lagunas de conocimiento relativas a la evaluación de riesgos, al seguimiento de los puntos críticos y a los posibles efectos de la presencia combinada de antimicrobianos y otras sustancias químicas en el medioambiente.

En 2020 se lanzó la estrategia “De la Granja a la Mesa” (Farm to Fork), enmarcada en el Pacto Verde Europeo, pretendiendo impulsar la transición hacia un sistema alimentario sostenible, justo y respetuoso con el medioambiente, basándose en una economía circular climáticamente neutra (COM/2020/381 final). Dentro de sus objetivos concretos, la EC tomará medidas específicas para el año 2030 para: (i) reducir un 50% las ventas de los antimicrobianos utilizados en ganadería y acuicultura; (ii) reducir como mínimo un 20% el uso de fertilizantes; y (iii) alcanzar un 25% de tierras agrícolas dedicadas a la agricultura ecológica.

En 2021, el segundo Plan de Acción de la FAO sobre las AMRs 2021-2025 marcó una hoja druta para focalizar los esfuerzos mundiales en los sectores alimentario y agrícola (FAO, 2021). El principal objetivo de este plan es acelerar el progreso en el desarrollo y la posterior aplicación de los planes de acción nacionales para combatir las AMRs. Propone el desarrollo de una nueva herramienta con fines de vigilancia multisectorial para evaluar la capacidad existente de los laboratorios para el análisis de residuos antimicrobianos en el suelo. Además, para minimizar la propagación de AMRs en los sectores de alimentación y agricultura, incide en la necesidad de elaborar directrices en la gestión de los residuos agrícolas (*e.g.*, aguas residuales, estiércol, biosólidos) y en las prácticas de gestión sostenible del suelo.

En España, en 2014 se adoptó el Plan Nacional frente a la Resistencia a los Antibióticos (PRAN) que abarcaba el periodo 2014-2018 (AEMPS, 2014). El PRAN fue aprobado por el Consejo Interterritorial del Sistema Nacional de la Salud y por la Conferencia Intersectorial de Agricultura como respuesta a la petición de la EC de desarrollar planes a nivel nacional. El plan se estructuró en seis líneas estratégicas comunes para la sanidad humana y veterinaria, con una perspectiva integral: (i) vigilancia del consumo y de la resistencia a los antibióticos; (ii) controlar las resistencias bacterianas; (iii) identificar e impulsar medidas alternativas y/o complementarias de prevención y tratamiento; (iv) definir prioridades en materia de investigación; (v) formación e información a los profesionales sanitarios; y (vi) comunicación y sensibilización de la población. En 2019 se adoptó el segundo PRAN 2019-2021 (AEMPS, 2019). Cabe destacar que se formó un grupo de trabajo encargado de analizar el posible impacto sobre la salud humana y animal de la presencia de genes de resistencia a antibióticos (ARGs) y antibióticos en el medioambiente.

En la Comunidad Autónoma del País Vasco se puso en marcha el Programa de Actuación frente a las Resistencias Antimicrobianas 2017-2020 (RAM-Euskadi). El programa se estructuró en los siguientes objetivos bajo la perspectiva *Una sola salud*: (i) mejorar la vigilancia y contro; (ii) prevenir enfermedades animales; (iii) promover la investigación; y (iv) mejorar la comunicación y formación. El Departamento de Sanidad Animal de Neiker, en coordinación con RAM-Euskadi, publicó dos guías de recomendaciones para un uso prudente de antibióticos en ganado bovino de leche; una dirigida a ganaderas y ganaderos, y otra a veterinarias y veterinarios (Neiker, 2021a; Neiker, 2021b).

1.1.4. Papel del medioambiente en la diseminación de las AMRs

A pesar de que se desconoce la magnitud de la contribución del riesgo del resistoma (colección de genes que contribuyen a la resistencia) ambiental a las bacterias patógenas importantes en el ámbito clínico, es evidente que el medioambiente ofrece diversas rutas para que la transmisión de las resistencias a los humanos. Teniendo en cuenta que todas las clases de antibióticos comercializados han encontrado resistencia en al menos algunos de los patógenos a los que se dirigen, se deduce que el medioambiente ya almacena factores de resistencia para todos los antibióticos (Larsson & Flach, 2022). En este sentido, es necesario realizar un seguimiento de las AMRs en el medioambiente, así como evaluar el impacto derivado del uso de antimicrobianos en los ecosistemas para cuantificar los riesgos asociados a su exposición.

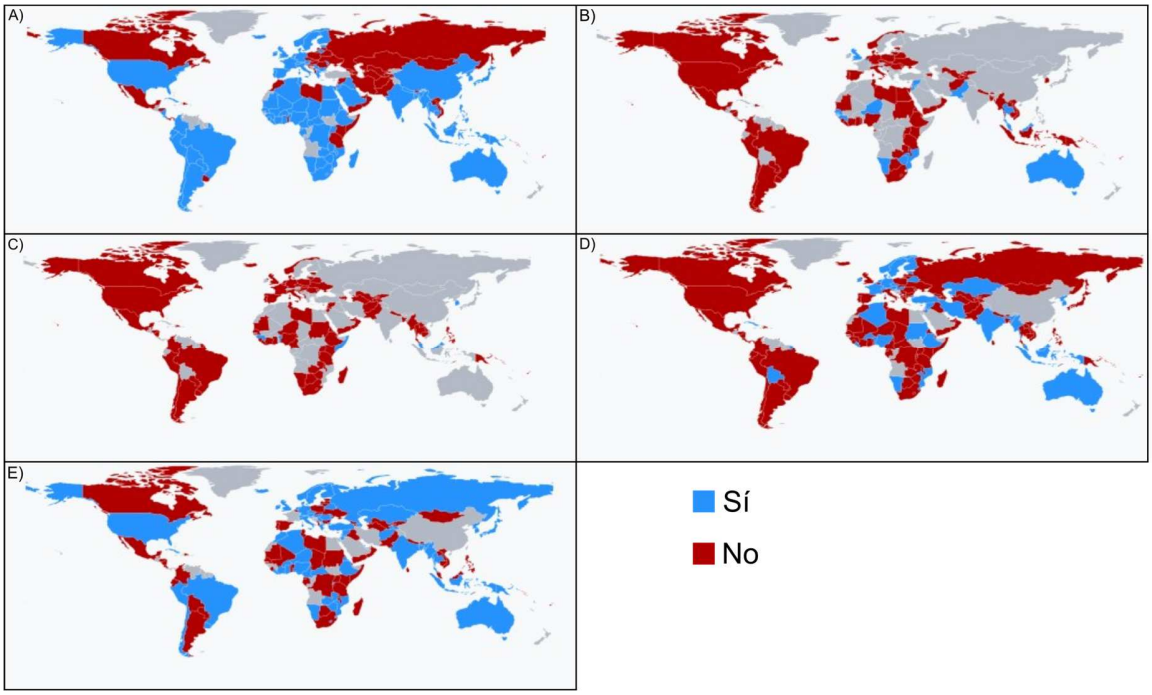


Figura 1.2. Encuesta de autoevaluación de los países sobre las AMRs para el periodo 2020-2021. A) ¿Participa el sector del medioambiente activamente en el desarrollo y aplicación de los Planes de Acción nacionales contra las AMRs? B) ¿Ha establecido o ha comenzado a aplicar el país un sistema integrado de vigilancia multisectorial que incluya las AMRs y el uso de antimicrobianos desde el sector del medioambiente? C) Países que utilizan los datos asociados a antimicrobianos y/o las AMRs para modificar la estrategia nacional y/o informar la toma de decisiones desde el sector del medioambiente. D) ¿Se ha realizado una evaluación nacional de los riesgos de propagación de las AMRs en el medioambiente? E) Países que disponen de legislación y/o normativa para evitar la contaminación medioambiental con antimicrobianos. Fuente: adaptación de Global Database for the Tripartite Antimicrobial Resistance (AMR) Country Self-Assessment Survey (TrACSS).

La urgente puesta en marcha de los planes internacionales y nacionales contra las AMRs presenta regulaciones incompletas respecto al papel que juega el medioambiente en la perspectiva *One Health* (Figura 1.2). Es necesaria la estandarización de los métodos de monitorización de (i) los antibióticos y sus metabolitos; (ii) el resistoma; y (iii) el moviloma (conjunto de genes presentes en elementos genéticos móviles), en diversos compartimentos ambientales, para así cuantificar y evaluar los riesgos asociados. Por otro lado, para determinar los niveles de seguridad, es imprescindible conocer los valores de referencia de las AMRs existentes de forma natural en el medioambiente.

En esta línea, en mayo de 2022, se publicaron dos informes vinculados a la primera de las tres fases fijadas por el grupo de trabajo de resistencias en el medioambiente a nivel estatal dentro del PRAN, con el objetivo de conocer mejor el papel del medioambiente en la emergencia y diseminación de las AMRs. En dicha fase se identificaron los puntos de emisión de determinantes de resistencias, para en un futuro seguir con la fase 2 centrada en estudiar los datos de monitorización y finalizar con la fase 3 dedicada a la evaluación de metodologías de análisis de riesgos. Cabe destacar que éstos fueron los primeros informes vinculados exclusivamente a la intervención del medioambiente en la diseminación de las AMRs como parte de una estrategia a largo plazo. El primer informe publicado reveló que la principal causa de la diseminación de resistencias en el medioambiente es la actividad humana (PRAN, 2022a). Se evaluaron varias fuentes de emisión de antimicrobianos (industriales, urbanas, agrícolas), rutas de dispersión de resistencias antimicrobianas (efluentes de depuración, biosólidos, purines y deshechos de granja, escorrentía y filtraciones, aerosoles y partículas en suspensión, fauna silvestre y sinantrópica) y la exposición potencial para humanos y animales a través de varios compartimentos medioambientales (agua, suelo, aire). El segundo informe se centró en estudiar el comportamiento en el medioambiente de 13 antibióticos seleccionados por su relevancia (PRAN, 2022b). Se reveló que los factores intrínsecos y extrínsecos de los antibióticos sirven para predecir la probabilidad de fenotipos resistentes con relevancia clínica en los compartimentos medioambientales (agua, sedimento, suelo, biota).

1.1.5. Consumo de antimicrobianos en medicina humana y veterinaria

A nivel mundial, se estima que el consumo bruto de antimicrobianos alcanzará las 236.757 toneladas en 2030 (Figura 1.3). La contribución de los sectores se mantendrá relativamente constante: 20%, 74% y 6%, en humanos, animales terrestres y acuicultura, respectivamente (Schar et al., 2020).

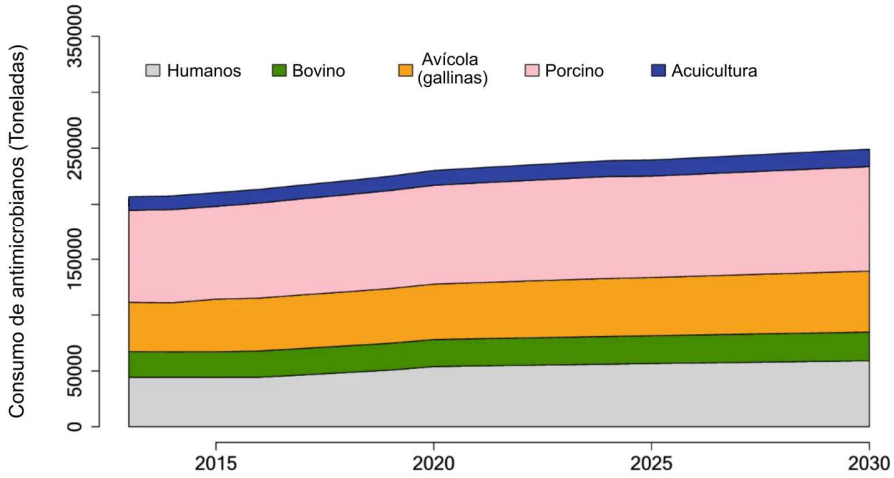


Figura 1.3. Consumo mundial de antimicrobianos en el periodo 2013-2030. Fuente: adaptación de Schar et al., 2020.

En Europa, de acuerdo con el informe de ESVAC publicado en 2021, el volumen total de ventas de antimicrobianos destinados a ganadería disminuyó un 43% entre 2011 y 2020 (EMA, 2021a). El volumen total de ventas se situó en 5.507 toneladas en 2020. En términos relativos, España se sitúo en el séptimo país con mayor consumo de antimicrobianos de Europa (Figura 1.4). Por otro lado, en 2018, el consumo medio de sustancia activa por kg de biomasa en ganadería fue menor que en humanos (105 *versus* 133, respectivamente) en 29 países de la UE/EEE (OECD, 2022).

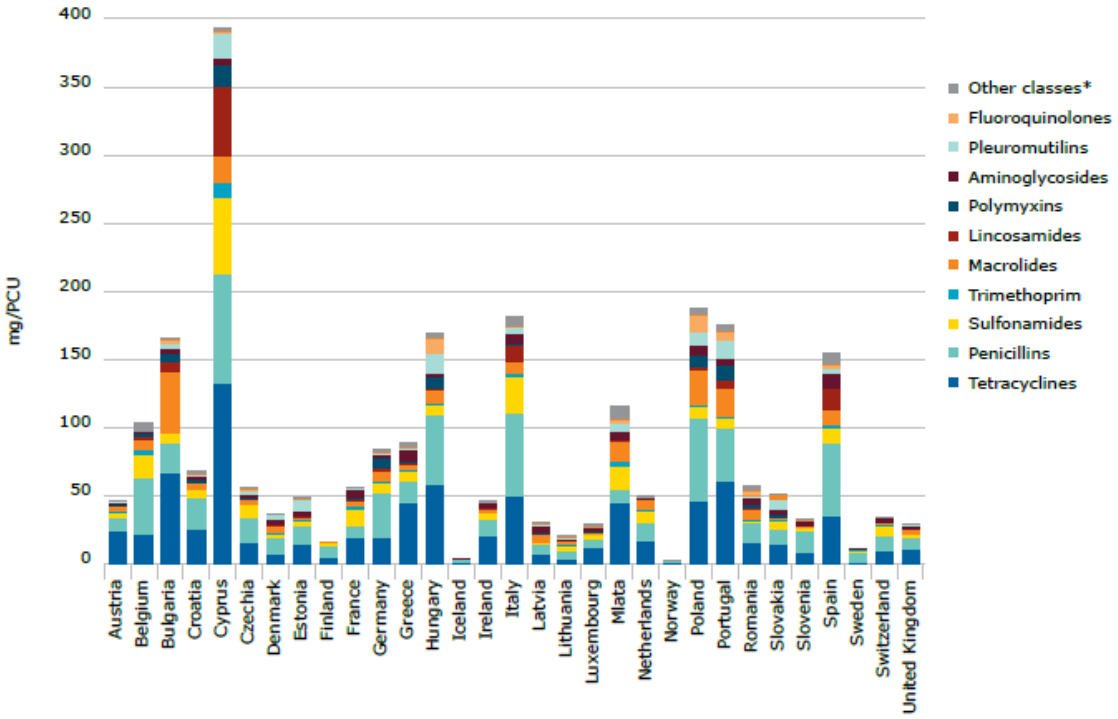


Figura 1.4. Ventas de antimicrobianos destinados a ganadería en 31 países europeos en 2020 expresados en mg PCU^{-1}. La Unidad de corrección de la población (PCU) es una unidad teórica desarrollada por la Agencia Europea de Medicamentos que tiene en cuenta la población animal de un país durante un año, así como el peso estimado de cada especie en el momento del tratamiento antimicrobiano. *Other classes incluye: anfenicoles, cefalosporinas, otras quinolonas y otros. Las diferencias entre países se pueden deber a la demografía animal, la aparición de enfermedades bacterianas, la selección de antimicrobianos, pautas de dosis, fuentes de datos y hábitos de prescripción de las veterinarias y los veterinarios. Fuente: EMA (2021a).

En España, las ventas de antimicrobianos en ganadería en 2020 se redujeron un 63% en comparación con las de 2014 (EMA, 2021a). Las penicilinas, seguidas de las tetraciclinas y lincosamidas, son las clases de antibióticos que más se emplean en veterinaria (Figura 1.5). Según el PRAN 2019-2021 (AEMPS, 2019), se observó una reducción del 7.2% del consumo total de antibióticos en salud humana entre 2015 y 2018; y una reducción en las ventas de antibióticos en el ámbito veterinario del 32% entre 2014 y 2017. El descenso puede ser atribuible a los planes nacionales e internacionales para hacer frente a las AMRs.

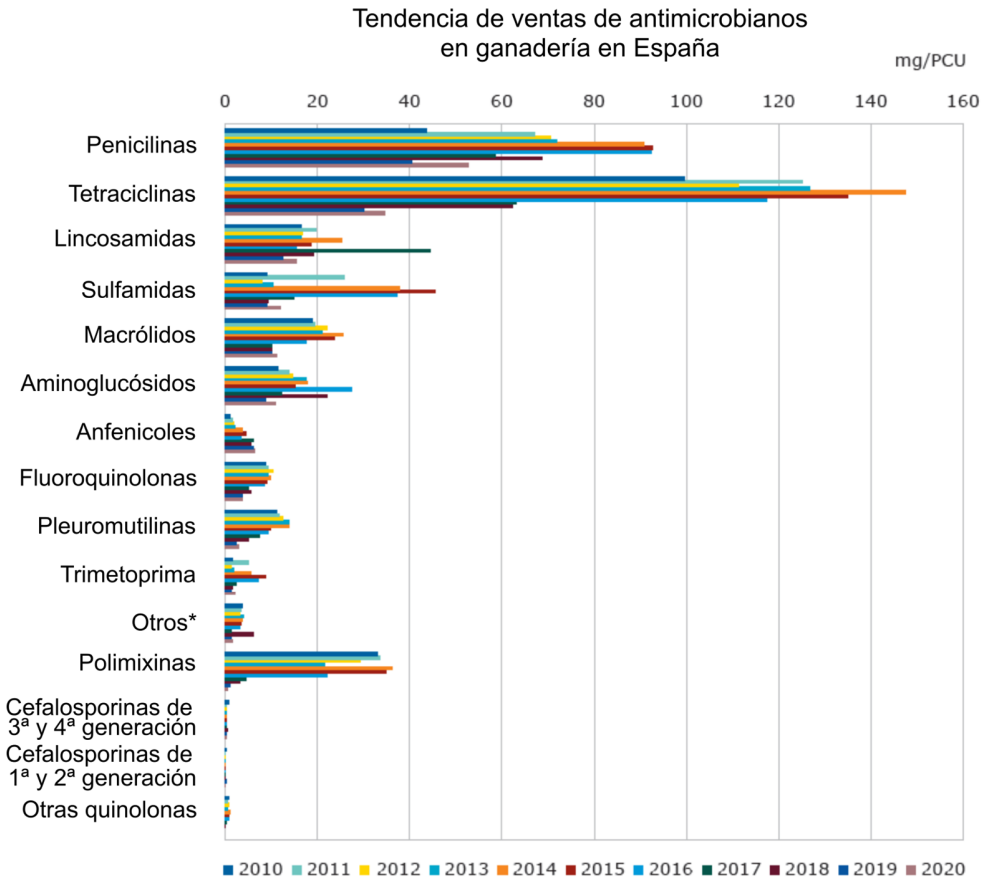


Figura 1.5. Ventas de antimicrobianos destinados a ganadería en España en el periodo 2010-2020 expresadas en mg PCU^{-1}. *Otros: bacitracinas, rifaximinas y espectinomicinas. Fuente: adaptación de EMA (2021b).

1.1.6. Listado de patógenos prioritarios resistentes a los antibióticos

La WHO publicó la lista de patógenos prioritarios resistentes a los antibióticos con el fin de promover la investigación y el desarrollo de nuevos compuestos antibióticos y, en última instancia, para reunir requisitos urgentes de salud pública en relación al creciente problema mundial de las AMRs (WHO, 2017a). Los criterios de selección de los patógenos se basan en: (i) la mortalidad de las infecciones provocadas; (ii) la duración de la estancia hospitalaria; (iii) la frecuencia de la resistencia a los antibióticos existentes; (iv) la facilidad de transmisión entre humanos, animales y humanos, alimentos y humanos; (v) la prevención de la infección; (vi) las opciones terapéuticas efectivas existentes; y (vii) si se están investigando y desarrollando nuevos antibióticos para tratar las infecciones causadas (WHO, 2017a). Los patógenos incluidos en prioridad crítica son bacterias multirresistentes; y en la lista de prioridad elevada y media se incluyen bacterias cuya resistencia va en aumento (Tabla 1.1.).

Tabla 1.1. Lista de patógenos prioritarios de la WHO para la I+D de nuevos antibióticos.

PATÓGENOS PRIORITARIOS	RESISTENCIA
Prioridad 1: Crítica	
Acinetobacter baumannii	Carbapenémicos
Pseudomonas aeruginosa	Carbapenémicos
Enterobacteriaceae: *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp., *Morganella* spp.	Carbapenémicos, cefalosporinas de 3ª generación
Prioridad 2: Elevada	
Enterococcus faecium	Vancomicina
Staphylococcus aureus	Meticilina, vancomicina
Helicobacter pylori	Claritromicina
Campylobacter spp.	Fluoroquinolonas
Salmonellae	Fluoroquinolonas
Neisseria gonorrhoeae	Cefalosporinas de 3ª generación, fluoroquinolonas
Prioridad 3: Media	
Streptococcus pneumoniae	Sin sensibilidad a la penicilina
Haemophilus influenzae	Ampicilina
Shigella spp.	Fluoroquinolonas

1.1.7. Antimicrobianos de importancia crítica

La WHO publicó una guía actualizada sobre los antimicrobianos de importancia crítica para la medicina humana, con el fin de optimizar y priorizar el uso de los antimicrobianos en humanos y en animales y así proteger su eficacia bajo la perspectiva *One Health* (WHO, 2019a). Se incluyeron 35 clases de antimicrobianos y se priorizaron en función de su importancia médica basándose en los siguientes criterios:

1. Criterio 1: la clase antimicrobiana es única o una de las limitadas disponibles para tratar infecciones bacterianas graves en personas.
2. Criterio 2: la clase antimicrobiana se utiliza para tratar infecciones causadas por bacterias que tienen el potencial de transmitirse a los humanos a través de fuentes no humanas y por bacterias con potencial de adquirir genes de resistencia a antibióticos (ARGs) de fuentes no humanas.
3. Prioridad 1: se utiliza para tratar un gran número de personas con infecciones para las que se dispone de antimicrobianos limitados.
4. Prioridad 2: se utiliza con alta frecuencia en medicina humana o en determinados grupos de alto riesgo.
5. Prioridad 3: se utiliza para tratar infecciones humanas en las que hay evidencias que son causadas por la transmisión de bacterias resistentes o de ARGs de fuentes no humanas.

Si la clase de antimicrobiano cumple los criterios 1 y 2 se considera un *antimicrobiano de importancia crítica*. Además, si cumple las tres prioridades, se considera de *la más alta prioridad* (cefalosporinas de 3ª, 4ª y 5ª generación; glucopéptidos; macrólidos; polimixinas; quinolonas); en caso contrario, se califica de *alta prioridad* (aminoglucósidos; ansamicinas; carbapenem; algunas penicilinas; etc.). Si cumple sólo uno de los criterios, se considera un *antimicrobiano muy importante* (lincosamidas; sulfamidas; tetraciclinas; etc.). Por último, la clase de antimicrobiano que no cumple ningún criterio se califica como *antimicrobiano importante* (nitromidazoles; pleuromutilinas; etc).

1.1.8. Listado de genes de resistencia a antibióticos

Algunos estudios metagenómicos y meta-análisis de qPCR de alto rendimiento (HT-qPCR) recientes proponen rangos de riesgo de ARGs en los que incluyen genes de alto riesgo para monitorizar en el medioambiente. Los estudios metagenómicos de algunos autores (Zhang et al., 2021; Zhang et al., 2022) se basan en el enriquecimiento de los ARGs asociados a entornos humanos, la movilidad, la patogenicidad del huésped y las resistencias asociadas a los antimicrobianos de importancia crítica (Tabla 1.2 y Tabla 1.3). En el meta-análisis llevado a cabo por Abramova et al. (2022), se proponen varios ARGs candidatos para la vigilancia del resistoma medioambiental (Tabla 1.4). Los ARGs propuestos son comunes de encontrar en el medioambiente y en patógenos humanos.

Tabla 1.2. Lista de ARGs de alto riesgo según Zhang et al. (2021). FCA: fluoroquinolona, quinolona, florfenicol, cloranfenicol, anfenicol; MLSB: macrólido, lincosamida, estreptogramina B.

Aminoglu-cósidos	β-lactámicos			FCA	MLSB	Multi-droga	Tetra-ciclinas	Trimeto-primas	Otros
aac(3)-II	*bacA*	*GES-11*	*OXA-10*	*catA*	*ermB*	*EmrB-QacA*	*tetL*	*dfrA1*	*fosB*
aac(3)-VI	*blaZ*	*IMP-4*	*OXA-4*	*catB*	*ermC*	*mdtE*	*tetM*	*dfrA12*	*fusB*
aac(6')-I	*CMY-111*	*KPC-2*	*SHV*	*cmlA*	*ermT*	*mdtL*		*dfrA14*	*mcr-1*
aadE	*CMY-4*	*KPC-4*	*SHV-1*	*floR*	*lnuA*	*mepA*		*dfrA15*	*vanY*
ant(2'')-I	*CMY-6*	*KPC-6*	*SHV-5*	*qnrA*	*lnuB*	*norA*		*dfrA17*	
aph(3')-I	*CTX-M-129*	*mecA*	*TEM*	*qnrB*	*mphA*	*TolC*		*dfrA25*	
aph(3')-III	*CTX-M-15*	*mecR1*	*TEM-1*	*qnrS*	*mphB*			*dfrA5*	
aph(6)-I	*CTX-M-2*	*NDM-5*	*VEB-3*		*msrA*			*dfrB1*	
rmtF	*CTX-M-24*	*NDM-6*	*VIM-1*						
rmtG	*CTX-M-55*	*OXA-1*	*VIM-2*						

Tabla 1.3. Lista de ARGs de alto riesgo según Zhang et al. (2022). FCA: fluoroquinolona, quinolona, florfenicol, cloranfenicol, anfenicol; MLSB: macrólido, lincosamida, estreptogramina B.

Amino-glucósidos	β-lactámicos	FCA	MLSB	Multidroga				Tetraci-clinas	Otros
aac(3)-IId	*CfxA2*	*catI*	*ermA*	*acrB*	*evgS*	*MexF*	*PmpM*	*emrK*	*bacA*
aac(6')-Ib7	*CfxA3*	*emrA*	*ermB*	*acrE*	*gadW*	*mexH*	*poxtA*	*emrY*	*eptA*
aac(6')-Ie-aph(2'')-Ia	*CfxA4*	*emrB*	*ermC*	*acrF*	*gadX*	*mexI*	*ramA*	*tet(40)*	*mdtG*
aad(6)	*CfxA5*	*emrR*	*ermF*	*acrS*	*H-NS*	*mexK*	*sdiA*	*tet(A)*	*pmrF*
aadA5	*CTX-M-15*	*mdtH*	*ermT*	*adeF*	*LAP-2*	*mexQ*	*smeB*	*tet(B)*	*sul1*
acrD	*FOX-5*	*mdtK*	*ermX*	*adeJ*	*lsaA*	*mexW*	*smeD*	*tet(C)*	*sul2*
ant(2'')-Ia	*mecA*	*patA*	*macB*	*arlR*	*lsaC*	*mexY*	*smeE*	*tet(D)*	*Ugd*
ant(3'')-IIa	*mecI*	*patB*	*Mef(En2)*	*arlS*	*lsaE*	*mgrA*	*smeF*	*tet(K)*	*yojl*
ant(4')-Ib	*mecR1*	*qacA*	*mphA*	*AxyX*	*marA*	*msrA*	*smeS*	*tet(L)*	
ant(6)-Ia	*OXA-1*	*qacB*	*mphB*	*AxyY*	*mdsB*	*msrC*	*tolC*	*tet(W/N/W)*	
aph(3')-Ia	*TEM-1*	*qnrS1*	*mphC*	*baeR*	*mdtE*	*mtrA*	*vgaA*	*tet(X3)*	
aph(3'')-Ib	*TEM-112*		*mphE*	*baeS*	*mdtF*	*mtrC*		*tet(X4)*	
aph(3')-IIIa	*TEM-135*		*rlmA(II)*	*ceoB*	*mdtM*	*mtrD*		*tet32*	
aph(6)-Id	*TEM-163*			*cpxA*	*mel*	*norA*		*tetA(P)*	
kdpE	*TEM-196*			*CRP*	*mexA*	*opmB*		*tetM*	
	TEM-206			*efmA*	*mexB*	*oprM*		*tetO*	
	TEM-214			*efrA*	*mexC*	*oprZ*		*tetQ*	
	TEM-95			*efrB*	*mexD*	*oqxA*		*tetW*	
				evgA	*mexE*	*oqxB*		*tetX*	

Tabla 1.4. Lista de ARGs más reportados y abundantes en ensayos de qPCR de alto rendimiento según Abramova et al. (2022). FCA: fluoroquinolona, quinolona, florfenicol, cloranfenicol, anfenicol; MLSB: macrólido, lincosamida, estreptogramina B.

Amino-glucósidos	β-lactámicos	FCA	Inte-grasa	MLSB	Multi-droga	Sulfa-midas	Tetra-ciclinas	Transpo-sasas	Gluco-péptidos
aadA1	*blaCTX-M*	*floR*	*intl1*	*ereA*	*mexF*	*sul1*	*tetA*	*tnpA4*	*vanA*
aadA2	*blaSHV*	*qnrS*		*ermB*	*qacEdelta*	*sul2*	*tetB*	*tnpA5*	
strB	*blaTEM*			*ermF*		*sul3*	*tetC*	*IS613*	
							tetG		
							tetH		
							tetM		
							tetO		
							tetW		
							tetX		

1.2. Antibiotikoen erresistentziak agroekosistemetan

Antibiotic resistance in agroecosystems

Laburpena

Antibiotikoak ezinbesteko tresna bilakatu dira medikuntza eta albaitaritzan bakterio-infekzioei aurre egiteko. Antibiotikoen gehiegizko eta erabilera okerrak osasun-erronka garrantzitsuenetako bat bizkortu du, bakterioek antibiotikoekiko erresistentzia zabala garatu baitute. Mundu mailako arazo hau Osasun Bakarraren ikuspegitik aztertu behar da, antibiotikoekiko erresistentziak gizakien, animalien eta ingurumenaren artean elkarbanatu, barreiatu eta ugaritzen baitira. Adibidez, lurzoruetan ongarri organikoak aplikatzeak berauen osasuna eta uzten etekina hobetu ditzake. Hala ere, praktika honek arriskuak dakartza, gizaki nahiz abereek guztiz metabolizatu ez dituzten antibiotikoak lurzorura iritsi baitaitezke. Antibiotikoaz gain, antibiotikoekiko erresistenteak diren geneak, bakterioak eta elementu genetiko mugikorrak barreiatzea dakar mota honetako ongarri organikoen erabilerak. Izan ere, lurzoruetan etengabe antibiotikoak sartzeak mehatxu potentziala sortzen du bertako bakterioentzat; antibiotikoekiko bakterio erresistenteak ugaritu eta bertako bakterio-komunitateen bioaniztasuna aldatzen dute. Barreiadura zabal honen ondorioz, gero eta gehiago, antibiotikoen erabilera ez da eraginkorra izaten ari bakterio erresistente patogenoen infekzioen tratamenduetan. Berrikuspen honetan, lehenik, antibiotiko nahiz antibiotikoekiko gene erresistenteen jatorria eta mekanismoak azaltzen dira. Jarraian, lurzoruetan egiten den giza edo animalia jatorriko ongarri organikoen erabilera aipatzen da, antibiotiko eta erresistentziak ingurumenean barreiatzeko sarbide garrantzitsuak direlako. Ondoren, antibiotikoekiko erresistentziek ingurumenean duten eragina azaltzen da, esposizio-bidea jarraituz kultiboetatik gizakietara iritsi arte. Azkenik, abereetatik eratorritako ongarri organikoetan antibiotikoekiko erresistentzien arriskua gutxitzeko asmoz erabilgarriak izan litezkeen kudeaketa-prozesuak proposatzen ditugu.

Abstract

Antibiotics have become an indispensable tool to combat bacterial infections in medicine and veterinary medicine. The abuse and misuse of antibiotics has led to the emergence of one of the most important health challenges, as bacteria have developed widespread resistance to antibiotics. This global problem must be analysed from a One Health perspective, as antibiotic resistance is shared, spread and multiplied between humans, animals and the environment. For example, the application of

organic fertilisers to soils can improve soil health and crop yields. However, this practice carries risks, as antibiotics not fully metabolised by humans or animals can find their way to soil. In addition to the antibiotic, the use of such organic fertilisers involves the spread of antibiotic-resistant genes, antibiotic resistant bacteria and mobile genetic elements. Indeed, the continuous introduction of antibiotics into soils creates a potential threat to indigenous bacteria; antibiotic-resistant bacteria multiply and alter the biodiversity of indigenous bacterial communities. This wide dispersal makes the use of antibiotics increasingly ineffective in the treatment of pathogenic resistant bacterial infections. This review first tries to explain the origin and mechanisms of antibiotic-resistant genes and antibiotics. Next, the use of organic fertilisers of human or animal origin in soils is mentioned, as they are a major source for the spread of antibiotics and antibiotic resistance in the environment. The environmental impact of antibiotic resistance is then explained, following the exposure pathway from crops to humans. Finally, we propose management processes that could be useful to reduce the risk of antibiotic resistance in livestock-derived organic fertilisers.

1.2.1. Antibiotikoak eta antibiotikoekiko erresistentziak

1.2.1.1. Antibiotiko eta antibiotikoekiko gene erresistenteen jatorria

Antibiotikoak ingurumeneko bakterio eta onddoek jariatutako edo sintetikoki ekoitzitako substantzia kimikoak dira. Antibiotikoek bakterioak hil (bakterizidak) edota haien hazkundea eragozten dute (bakteriostatikoak). Erabilera klinikoko antibiotiko gehienak lurzoruko bakterioetatik eratorriak dira, zuzenean produktu natural gisa edo horien deribatu erdisintetiko moduan. Bereziki, aktinobakteria filumekoak dira garrantzi klinikoa duten antibiotiko gehienen sintesiaren erantzuleak (Durand et al., 2019). Antibiotikoekiko erresistentzia oso hedatuta dago naturan, antibiotikoak sortzen dituzten bakterioek auto-erresistentzia mekanismoak baitituzte (D'Costa et al., 2011). Antibiotikoekiko erresistentzia geneak, glaziarretan, permafrostean eta baita gizakiak ia inoiz bisitatu ez dituen kobazulo isolatuetan ere aurkitu izan dira (Pawlowski et al., 2016; Mindlin & Petrova, 2017; Van Goethem et al., 2018).

Mikrobiologian, mikroorganismoen hazkuntza inhibitzen duen gutxieneko antibiotiko kontzentrazioari gutxieneko kontzentrazio inhibitzailea deritzo. Normalki, ingurumenean aurkitzen diren antibiotikoen kontzentrazioak erabilera klinikoan (dosi terapeutikoak) erabiltzen direnak baino askoz baxuagoak dira (Sengupta et al., 2013; Larsson & Flach, 2021). Antibiotikoek ingurumenean,

halako kontzentrazio baxuetan, hainbat funtzio burutzen dituzte: bakterioen arteko komunikazioa, espezieen arteko lehia, ostalari-parasito elkarrekintza, birulentziaren modulazioa, SOS erantzuna, mutagenesia eta biofilmen eraketa (Davies et al., 1990; Sengupta et al., 2013). Beraz, lurzoruko organismoek sortutako antibiotikoek komunitate bakterianoen populazioa eta bizimodua erregulatzen dute (Baquero et al., 2013). Hala ere, ingurumenean antibiotikoen kontzentrazioa gutxieneko kontzentrazio inhibitzailearen azpitik aurkitzen denean ere, fenotipo erresistenteak hauta ditzakete (Gullberg et al., 2014). Horrez gain, antibiotikoek hormesia eragin dezakete; dosi baxuetan bakterioak estimulatu eta dosi altuetan inhibitu ditzaketelako (Davies et al., 2006). Inhibizioaren ondorioa ez da nahitaez bakterio lehiakorrak desagerraraztea, ekosistema partekatuetan bakterio batzuen gain-hazkuntza ekiditea baizik, eta horrela, populazio anitza bermatzea (Baquero et al., 2013).

Bestalde, metalek antibiotikoekiko erresistentzien agerpena, mantentzea eta hedapena ekar dezakete ko-hautespen mekanismoen bidez (Baker-Austin et al., 2006). Metalekiko eta antibiotikoekiko erresistentzia hautaketa bi mekanismo nagusitan bereizten da: (i) ko-erresistentzia, erresistentzia determinatzaile desberdinak elementu genetiko berean aurkitzen direnean; eta (ii) erresistentzia gurutzatua, metalekiko zein antibiotikoekiko erresistentziaren erantzulea determinatzaile genetiko bera denean (Baker-Austin et al., 2006). Metalek agente selektibo gisa joka dezakete antibiotikoekiko erresistentziak sustatzeko orduan, kutsatzaile metalikoen izaera eta kontzentrazioa, antibiotiko mota eta honen ekintza-mekanismoaren arabera, besteak beste. Metalek hautespen presio garrantzitsua burutzen dute lurzoruan, bai denbora-epe luzeetan kutsadura kronikoak eragiten dituztenean baina baita denbora-epe laburretan akutuak direnean ere (Dickinson et al., 2019; Wang et al., 2021a).

1.2.1.2. Antibiotikoen ekintza-mekanismoak eta erresistentzia-mekanismoak

Antibiotiko batek bere ekintza itua lortu behar du, behar besteko kontzentrazio eta denboran, hazkuntza ekidin edo bakterioen heriotza eragin ahal izateko. Antibiotikoen ekintza-mekanismoak bost itutan sailkatzen dira: bakterioen (i) pareta zelularra, (ii) mintz zelularra, (iii) proteinen sintesia, (iv) ADN eta ARN-ren erreplikazioa eta, (v) aziko folikoaren metabolismoa (Wang et al., 2021a). Honez gain, bakterioek hainbat erresistentzia-mekanismo erabil ditzakete antibiotikoen eraginari aurre egiteko: (i) antibiotikoa bakterioan sar dadin saihestu (pareta edo mintz zelularra iragazgaitz bihurtuz), (ii) antibiotikoa aldatzen edo inaktibatzen duten entzimak sortu, (iii) antibiotikoen itua aldatu edo babestu, bien arteko elkarrekintza saihestuz eta, (iv) antibiotikoa bakterioaren kanpoaldera bota efluxu-ponpen

bidez (Wright, 2010). Gainera, bakterioek dituzten erresistentzia-mekanismoak berezkoak edo eskuratutakoak izan daitezke (Van Hoek et al., 2011). Berezko erresistentziak, sortzez komunitate edo espezie bereko bakterio guztiek dituzte. Bestetik, eskuratutako erresistentziak bakterio batzuetan daude bakarrik. Bakterio gram-negatiboak berez gram-positiboak baino erresistenteagoak dira pareta zelularren ezaugarriei eta droga anitzeko fluxu ponpa askoren adierazpenari esker (Nikaido, 1998). Pareta zelularrak iragazkortasun-hesi gisa jarduten du, antibiotikoak bere ituetara iristea eragotziz. Droga anitzeko fluxu ponpek antibiotikoen zelula barneko kontzentrazioa eraginkortasunez murrizten dute.

Antibiotikoekiko erresistentzia geneek (AEGek) antibiotikoen presentzian bakterioek biziraun eta hazteko ahalmena bideratzen dute. Geneen transferentzia guraso bakterioetatik bakterio alabetara gertatzen denean, prozesuari transferentzia bertikala deritzo. Bakterioek mutazio-tasa handia dute banatze prozesuan eta ausazko mutazio batek antibiotikoaren presentzian bizirauteko ahalmena ematen badio, populazio bakteriano tolerante bat agertzea ahalbidetuko du, populazio sentikorra desplazatuz (Schmitt et al., 2005). Mutazioek beraz, egokitzeko estrategia ebolutiboa irudikatzen dute. Bestalde, geneen transferentzia horizontala (GTH) ahaide ez diren bi bakterioren arteko material genetikoaren transferentzian datza. Bakterio hartzaileek azkar eta eraginkortasunez eskura ditzakete aurkako ingurumenetan biziraupena ahalbidetu ditzaketen geneak, betiere ingurumen berean bizi badira. Hiru transferentzia-mekanismo mota daude bakterioen artean material genetikoa elkarbanatzea ahalbidetzen dutenak: transformazioa, transdukzioa eta konjugazioa. Transformazioan, bakterioek ingurumenean aske dagoen ADN zuzenean hartzen dute. Transdukzioa birusen bitarteko bakterioen arteko trukaketa genetikoa da. Azkenik, bakterioen arteko kontaktu zuzenaren bidez ematen da konjugazioa. Plasmido eta transposoi konjugatiboak dira transferentzia honen erantzule. Konjugazioan, bakterio hartzaileak, AEGak barneratzeaz gain, jasotako AEGak transferitzeko gaitasuna hartzen du.

Ingurumen, animalia eta giza osasunean izan ditzaketen eraginak aztertzeko, bereziki garrantzitsua da AEGak eskuratzeko, mobilizatzeko eta barreiatzeko plataforma genetikoetan arreta jartzea. Horien artean, aipatzekoak dira hurrengo elementu genetiko mugikorrak (EGMak): integroiak (AEGak atzeman eta metatu ditzakete) eta plasmido eta transposoiak (mobilizatu eta beste bakterio batzuetara AEGak sakabanatu ditzakete).

1.2.2. Ongarri organikoen erabilera nekazaritzan

Lurzorua funtsezko baliabide natural mugatua da eta ezinbesteko zerbitzu ekologikoz hornitzen gaitu; elikagaiak birziklatu, ura eta airea araztu eta, karbonoa biltegiratzen du, besteak beste. Gure elikagaien % 99 lurzoruetatik dator eta munduko biodibertsitatearen laurdena baino gehiago lurzoruetan dagoela estimatzen da (Orgiazzi et al., 2016). Hortaz, lurzorua medio oso aberatsa da organismoen ugaritasun eta aniztasunari dagokionez. Gainera, lurzoruak etengabeko elkarrekintza du biosferako gainerako konpartimentuekin, hainbat nitxo ekologikoren arteko material genetikoaren trukea gertatzen delarik (Forsberg et al., 2012). Ongarri organikoak nekazaritza lurzoruetan aplikatzea ohiko praktika jasangarria da. Gainera, ongarri organikoak aplikatzeak lurzoruen (i) egitura eta agregatuak hobetu, (ii) ura atxikitzeko gaitasuna handitu, (iii) materia organikoa eta elikagaien edukia handitu, (iv) higadura eta degradazioa kontrolatu, (v) mikroorganismoen aktibitatea eta landareen hazkundea sustatu eta, (vi) klima aldaketaren aurrean, karbonoa biltegiratu dezakete (Bulluck et al., 2002; Ros et al., 2003; Diacono & Montemurro, 2011; Park et al., 2011). Ondorioz, ongarri organikoek lurzoruen osasuna eta uzten etekina hobetzeko ahalmena dute (Epelde et al., 2018; Urra et al., 2020).

Munduko egungo giza-populazioa 7,7 bilioitan finkatzen da eta 2050 urterako 9,6 bilioitara iritsiko dela estimatzen da (UN, 2019). Giza-populazioaren gorakada elikagaien ekoizpenaren igoerarekin lotuta dago. Geroz eta handiagoa den elikagaien eskaria asetzeko, abeltzaintza eta nekazaritza intentsiboetara jotzeko joera dago. Zoritxarrez, kudeaketa intentsibo hauek eragin larria dute hainbeste zerbitzu eskaintzen dizkiguten ekosistemetan. Egoera honek ekoizpen sistema jasangarriagoen aldeko trantsizioa eskatzen du, ziklo ekologikoen funtzionamendu eta printzipioetara gerturatzen dena. Honen adibide dira bioekonomia eta ekonomia zirkularraren paradigmak, non bio-produktu eta hondakinak modu eraginkorrean integratzen diren sistema aktibo baliotsu gisa, berrerabilpen eta kudeaketa jasangarrian oinarrituz (EMA, 2016). Bestalde, nekazaritza birsortzailea prozesu ekologiko naturaletan oinarritzen da, praktika iraunkorrak sustatuz, lurraren emankortasuna zainduz, bioaniztasuna bultzatuz, ingurumena babestuz eta animalien ongizatea bermatuz. Gainera, nekazaritza eta abeltzaintza-ekoizpen ekologikoa Europako araudi batek erregulatzen du (Commission Regulation 889/2008), sintesiko produktu kimikoen erabilera baztertu eta ongarri organikoen balorizazioa bultzatuz (Misselbrook et al., 2012). Oro har, lurzoruetan aplikatzen diren ongarri organikoen kopurua gero eta handiagoa da nekazaritza birsortzaile edo ekologikoaren hedapenaren ondorioz. Europar Batasuneko nekazaritza ekologikoaren azalera % 70-ean handitu da azken

hamarkadan (European Commission, 2019). Euskal Autonomia Erkidegoan ere goranzko joera du nekazaritza ekologikoak eta ekoizpen ekologikoa nekazaritza-azalera osoaren % 4,3 da gaur egun (Consejo de Agricultura y Alimentación Ecológica de Euskadi, 2020).

1.2.2.1. Ongarri organiko motak

Ongarri organikoen jatorriaren (abereak, urbanoa, nekazaritza, industria), materiaren egoeraren (likidoa, solidoa), kudeatzeko moduaren (heltzea, konpostajea, digestio anaerobikoa, etab.) eta aplikazio dosiaren arabera, eragin agronomikoa desberdina izan daiteke. Ongarri organikoen artean egun gehien erabiltzen direnak animalia nahiz gizakien gorozki eta gernu jatorrikoak dira.

Mundu mailan, abereetatik eratorritako eta lurzoruetan aplikaturiko nitrogeno organiko kopurua 116 milioi tona ingurukoa izan zen 2018. urtean (FAO, 2020). Kopuru hau laborantza lurretan erabiltzen diren ongarri sintetikoen kopuruaren antzekoa da (FAO, 2020). Aipatzekoa da lurzoruetan aplikatutako abere-ongarri kopurua bazkatzearen ondorioz larreetan geratzen den kopurua baino handiagoa dela (8,4 *versus* 4,8 milioi tona nitrogeno), ganadu ekoizpen intentsiboaren nagusitasuna eta bazka lur mugatuak azaleratuz (FAO, 2020).

Hondakin uren araztegietan tratamendutik sortutako produktu erdi-solido birziklagarriak dira araztegiko lohiak. Nekazaritza lurzoruetan lohien erabilera arautua dago EB-n (Directiva 86/278/CEE), gizakien, animalien eta landareen metal astunen toxikotasuna murrizteko asmoz. Euskal Autonomi Erkidegoan lohi hauek nekazal lurzoruetan aplikatzea oso mugatuta dago (453/2013 Dekretua). Beste lurralde batzuetan, lohiak nekazaritzako lurzoruetan erabili aurretik, metodo egokiekin (biologikoa, kimikoa, termikoa eta biltegiratzea, besteak beste) tratatu behar dira, ondoriozko osasun arriskuak murrizteko. Hondakin uren araztegietan aplikatzen diren prozesu, lohi gordinaren kantitate eta jatorriaren arabera, lohien konposizioa aldakorra da. Dena dela, lohien aplikazioak lurzoruen emankortasuna eta landare nahiz lurzoruko mikrobiota osasuntsuak areagotu ditzake (Samara et al., 2017; Tyrrell et al., 2019), oinarrizko elikagaiak (nitrogenoa, fosforoa, potasioa) eta materia organikoa emendatzen baititu.

1.2.3. Gizaki, animalia eta ingurumenaren osasuna: Osasun Bakarra

Gaur egun aurre egin beharreko gaixotasun infekziosoen dimentsioak eta mundu mailako ondorioek ez dute aurrekari historikorik. Gaixotasun infekzioso hauek jatorri zoonosikoa dute nagusiki, era

naturalean animalietatik gizakietara transmititzen diren gaixotasunak dira. Ildo honetan, Osasun Bakarra (One Health ingelesez) kontzeptuak ideia hau laburbiltzen du: giza osasuna, animalien osasuna eta ingurumenaren osasuna berez lotuta eta elkarren menpe daudela. Norberaren osasunak guztion osasunari eragiten dio, eta alderantziz. Mundu mailako estrategia honek ekosistema iraunkor orekatuago baten alde egiten du. Mehatxu hauen konplexutasuna diziplina anitzeko taldeek jorratzea nahitaezkoa da, betiere Osasun Bakarraren ikuspuntutik lan eginez.

1.2.3.1. Antibiotikoen erabilera eta erresistentziak

Medikuntzan nahiz albaitaritzan antibiotikoak ezinbesteko tresnak dira bakterio-infekzioak tratatzeko. Gehienbat bakterio-infekzioak tratatzeko erabiltzen diren arren, infekzioak prebenitzeko eta abereen hazkuntza sustatzeko ere erabiltzen dira. Antibiotikoek abereen elikagaiak xurgatzeko gaitasuna handitzen laguntzen dute eta horrela gizenketa prozesua azkartzen da. Dena dela, Munduko Osasun Erakundeak ez du gomendatzen prebentziorako erabilera (WHO, 2017b). Gainera, Europar Batasunak abereen hazkuntzaren sustapenerako antibiotikoen erabilera debekatu egin zuen 2006an (Regulation 1831/2003).

2018. urteko antibiotikoen salmenta, soilik ustiategietako abereak kontuan hartuta, honela multzokatu zen Europako 31 herrialdetan, antibiotiko familiaren arabera: tetraziklinak, penizilinak eta sulfonamidak % 31, % 29 eta % 8-a suposatu zuten, hurrenez hurren (EMA, 2020). Gainera, abere ustiategietan estimatutako antibiotikoen kontsumo globala 131.109 tonakoa izan zen 2013an (Van Boeckel et al., 2017). Abeltzaintza eta nekazaritza sektoreek duten antibiotiko kontsumoa gizakiok dugun elikagai eskariarekin loturik dagoenez, abere ekoizleen antibiotikoen kontsumoa > 200.000 tonatan zenbatesten da 2030 urterako (Van Boeckel et al., 2017).

Azken hamarkadetan, giza eta animalia osasunean eta nekazaritzan antibiotikoen gehiegizko erabilerak eta erabilera desegokiak mundu mailako osasun arriskua eragin dute. Bakterioek antibiotikoen eraginpean bizirauteko eta hazteko gai direnean antibiotikoekiko erresistentzia sortzen da. Hori gertatzen denean, bakterio erresistenteek infekzioa eragiten jarraitzen dute antibiotikoen presentzian ere. Geroz eta antibiotiko gehiagorekiko erresistente edo multierresistente diren bakterio gehiago daude, eta hauek sortzen dituzten gaixotasunak tratatzeak zailtasun larriak dakartza giza eta animalia osasunean. Osasunaren Mundu Erakundeak 2019an antibiotikoen aurkako erresistentzia munduko giza osasunerako 10 mehatxu nagusienen artean sartu zuen (WHO, 2019b). Berriki

argitaratutako kalkulu batzuen arabera, 2019an 4,95 milioi heriotza egon ziren bakterio erresistenteei lotuta (Antimicrobial Resistance Collaborators, 2022). Neurririk hartu ezean, 2050.ean urteko 10 milioi heriotza inguru eta ekoizpen ekonomikoaren 100 bilioi dolarreko galera metatua espero dira (O'Neill, 2016).

1.2.3.2. Antibiotiko eta erresistentziak gorotz, ongarri organiko eta lurzoruetan

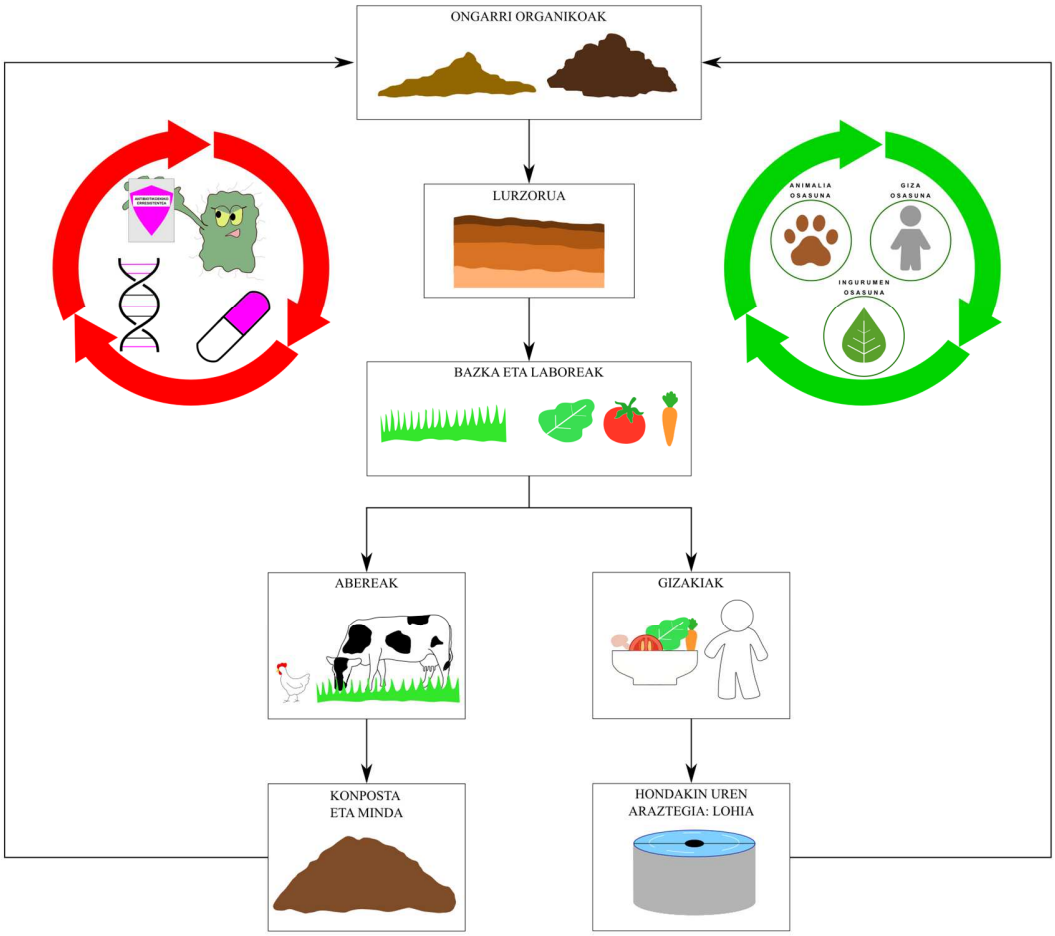
Abere gorozkietan antibiotikoak 1-10 mg kg^{-1}-ko tartean aurkitu ohi diren arren (Kumar et al., 2005), kasu batzuetan erraz gainditzen da aipatutako tarte hori. Adibidez, ondorengo antibiotiko kontzentrazioak detektatu dira ikerketa batzuetan: 1420, 764, 216 eta 91 mg kg^{-1}, fluorokinolona, tetraziklina, β-laktamiko eta sulfamida, hurrenez hurren (Gilbertson et al., 1990; Martínez-Carballo et al., 2007; Zhao et al., 2010; Pan et al., 2011). Hondakin uren araztegietako lohietan aurkitu ohi den antibiotiko kontzentrazioa abere gorozkietan aurkitzen dena baino txikiagoa izan ohi da (Jones-Lepp et al., 2007). Halaber, lohietan honako antibiotiko kontzentrazioak ere detektatu dira: 21.335, 8.326, 158 eta 133 µg kg^{-1} fluorokinolona, tetraziklina, makrolido eta trimetoprima, hurrenez hurren (Li et al., 2013a; Cheng et al., 2014; Göbel et al., 2005).

Abereei emandako antibiotikoak ez dira osoki metabolizatzen eta gorozki eta gernuetan iraizten dira, bai jatorrizko antibiotiko gisa edota metabolito aktibo gisa (Sarmah et al., 2006). Antibiotiko klase, antibiotiko dosi, antibiotikoa emateko forma, abere espezie eta aberearen adinaren arabera aldakorra bada ere, batazbeste emandako antibiotiko kopuruaren % 30-90 iraizten da (Kumar et al., 2005; Sarmah et al., 2006; Motoyama et al., 2011). Kopuru esanguratsu hauek gorozki eta gernuetatik ingurumenera iristen dira, batik bat abereetatik eratorritako ongarriak lurzoruetan aplikatzen direnean (1.6 irudia). Antibiotikoen erdi-bizitza antibiotikoaren kontzentrazioa hasierakoaren erdira gutxitzeko behar den denbora bezala definitzen da. Parametro farmakozinetiko honek antibiotikoek ingurumenean izan dezaketen iraunkortasuna eta hautespen presioa azal dezake. Esaterako, aminoglukosido, β-laktamiko, makrolido eta sulfamidek < 30 eguneko erdi-bizitza dute; aldiz, fluorokinolona eta tetraziklinek 100 egunekoa izan dezakete (Boxall et al., 2004).

Immobilizazio edo degradazio urriaren ondorioz, antibiotiko asko ingurumenean mantentzen dira (Chenxi et al., 2008). Lurzoruan antibiotikoak hainbat prozesu biotiko nahiz abiotikoren menpe daude, hala nola eraldaketa, degradazioa, adsorzioa-desortzioa, landareen xurgapena, lurgaineko jariatzea eta lurpeko uretara iristea (Tolls, 2001; Kuchta et al., 2009; Duan et al., 2017). Lurzoruetan

neurtutako antibiotikoen kontzentrazioak ng-mg gutxi batzuen artekoak izan ohi dira lurzoru kg bakoitzeko. Hala ere, abereengandik eratorritako ongarri organikoekin ongarritutako eta abereen bazkarako erabilitako lursailetako lurzoruetan antibiotiko kontzentrazio altuagoak izan ohi dira. Esaterako, kontzentrazio maximo hauek detektatu ziren abereetatik eratorritako ongarriak jaso zituzten lurzoruetan: 86, 760 eta 204 µg kg^{-1} tetraziklina, sulfamida eta fluorokinolona, hurrenez hurren (Wei et al., 2016; An et al., 2015; Uslu et al., 2008).

Gainera, abereen hesteetan antibiotikoek bakterio erresistenteen hautaketa bultzatzen dute. Nahikoa da antibiotikoen kontzentrazioa ppb mailan aurkitzea AEGak mantendu eta transferentziaren prozesua laguntzeko (Gullberg et al., 2014). Beraz, abereetatik eratorritako ongarri organikoak, antibiotikoen kutsadura iturri izateaz gain, antibiotikoekiko erresistente diren bakterioen biltegi ere badira, bakterioek potentzialki AEGak hedatzeko gaitasuna dutelarik (Zhu et al., 2013).



1.6. irudia. Antibiotiko, antibiotikoekiko erresistentzia gene eta antibiotikoekiko bakterio erresistenteen esposizio-bidea animalia eta giza jatorriko ongarri organikoak lurzoruan aplikatzerakoan.

1.2.3.3. Erresistoma ingurumenetik gizakietara

Aipatutako hesteetako bakterio horietako batzuk patogenoak izan daitezke gizakientzat, eta mehatxua nabarmen handitzen da bakterio hauek AEGak eskuratzen badituzte. Ingurumenean AEGen presentzia antibiotiko bakterio ekoizleetara mugatzen ez denez (Aminov, 2009), eta lurzoruan dagoen bakterio biodibertsitate hain handia kontutan hartuz, lurzoruari lotutako erresistoma (AEG eta euren aitzindarien bilduma) AEGen biltegi garrantzitsua da. Lurzoruko bakterio komunitate anitzek inbasioen aurkako oztopo biologiko gisa ere jardun dezakete, bakterio aniztasuna eta AEGen ugaritasuna negatiboki erlazionatuta baitaude (Chen et al., 2019a; Jauregi et al., 2021a). Zoritxarrez, jarduera antropogenikoek (nekazaritzaren intentsifikazioa, basoen ustiapena, etab.) lurzoruko mikroorganismoen aniztasun oparoa murrizten dute. Lurzorutik bertan ekoitzitako laboreetara zabaldu daiteke erresistomaren arriskua, eta handik gizakietara (1.6. irudia). Batez ere gordinik kontsumitzen diren barazkiek suposatzen dute ingurumenetik eratorritako erresistoma gizakira iristeko esposizio biderik arriskutsuena (Marti et al., 2013), sukaldatze prozesuek arrisku hau asko murrizten baitute.

Bestalde, AEG baten jatorrizko bakterio ostalaritik giza patogeno baterako transferentzia hainbat botila-lepok mugatzen dute. Lehenak lotura ekologikoarekin du zerikusia: AEG bat transferitzeko bakterio emaile zein hartzaileak habitat bera elkarbanatu edota, bakterio-kate batek emailea eta hartzailea elkartu behar ditu. Dena den, transferentzia kateak arrakasta izateko nahitaezkoa da erresistentzia determinatzaileak hautaketa positiboa izatea (Martínez, 2012). Jarraitzeko, fundatzaile efektua deritzon fenomenoa gertatu daiteke, bakterio hartzailera iristen den lehen AEGa soilik nagusitzen denean (Baquero et al., 2009). Hirugarren botila-lepoa, gaitasun kostuarekin erlazionatuta dago: erresistentzia determinatzaile bat eskuratzeak gaitasun kostu bat gehitzen die bakterioei (Andersson & Levin, 1999). Hortaz, hautespen presiorik ezean, bakterio sentikorrek gainditu egingo dituzte erresistenteak. Alabaina, konpentsazio-mutazioak gertatzen direnean, bakterioetan erresistentziak mantentzearen kostua murrizten da (Martínez et al., 2011).

1.2.4. Ongarri organikoen kudeaketarako alternatibak

Azaldu dugunez, lurzoruan animalia edo giza jatorridun ongarri organikoak (simaurra eta minda) kudeaketa-prozesu zuzentzailerik gabe aplikatzeak antibiotiko, antibiotikoekiko erresistentziadun

bakterio, AEG, EGM eta patogenoen ugaritasuna eta aniztasuna handitzea ekar dezake (Chen et al., 2019b). Egoera honen aurrean, erresistomaren barreiadura murrizteko ezinbestekoa da ingurumenerako sarbide-puntuetan ahalik eta neurri prebentibo eta zuzentzaile zorrotzenak aplikatzea. Ondoren, erresistomaren arriskua ahalik eta gehien gutxitzeko erabili litezkeen ongarri organikoen maneiu-prozesuak proposatzen ditugu. Gainera, hainbat ikerketatan oinarrituta, proposatutako ongarri organikoen kudeaketa-prozesuen joerak laburbildu ditugu (1.5. taula).

1.2.4.1. Konpostajea

Konpostajea abereen simaurra ongarri organiko bihurtzeko mikrobio-prozesu aerobiko kontrolatua da; prozesua aldakorra da konpostaje-teknologia eta baldintzen arabera (Liang et al., 2003). Konpostaje-prozesuak antibiotikoen kontzentrazioa esanguratsuki murrizten du, batez ere fase termofiloan (Youngquist et al., 2016). Prozesuan zehar bakterio ostalaria deuseztatzearen emaitza ere bada AEGen ugaritasunaren murrizketa (Wang et al., 2015a). Dena den, konpostajean AEG eta EGMen ugaritasunaren gorako edo beherako joera oso gene-espezifikoa dela dirudi (Xu et al., 2018).

1.2.4.2. Digestio anaerobikoa

Digestio anaerobikoa oxigenorik gabeko ingurunean ematen den mikrobioen deskonposizio-prozesua da. Tradizionalki, hondakin uren araztegietan erabili izan den prozesua da, bertako solidoen masa murriztu eta biogasa edo bio-produktuak sortzeko. Bestalde, antibiotiko mota, tenperatura eta digestio-denboraren arabera, antibiotikoen ezabatze-tasa % 0 eta 100 bitartekoa izan daiteke (Mohring et al., 2009). Esaterako, digestio anaerobioaz AEGen ugaritasuna % 21 murriztu zela ondorioztatu zuten analisi metagenomikoan oinarritu zen ikerketa batean (Yang et al., 2014). Hala ere, beste zenbait ikerketen arabera, digestio anaerobikoak AEGen ugaritasunean dituen ondorioak ez dira sendoak (Wang et al., 2017; Zhang et al., 2018).

1.2.4.3. Bio-ikatza

Bio-ikatza biomasaren pirolisitik sortutako karbono materiala da. Azalera espezifiko handia eta katioiak trukatzeko gaitasun nabarmena du. Hortaz, ura, elikagaiak eta kutsatzaileak atxikitzen ditu lurzoruan aplikatzen denean (Ahmad et al., 2014) eta mikrobioentzako habitata eman dezake (Ogawa & Okimori, 2010). Bio-ikatza ongarri gisa aplikatu ohi izan da, batez ere, lurzoruen emankortasuna

handitzeko. Dena dela, geroz eta praktika ohikoagoa da lurzoruen osasuna ikuspuntu orokorrago batetik hobetzeko, kutsadura murrizteko eta klima-aldaketa arintzeko ere (Guo et al., 2020).

1.2.4.4. Agortutako onddo-substratua

Onddo fresko kg bat ekoiztean, bost kg onddo substratu erabiltzen dira [adibidez, Espainiak 166.010 tona onddo ekoizten ditu urtean (FAOSTAT, 2020a)]. Beraz, agortutako onddo-substratua kantitate handitan sortzen da eta urte askoan zabortegietan metatu da, ingurumen arazo bihurtuz. Agortutako onddo-substratua onddoen ekoizpenetik eratorritako bio-produktua da. Ekonomia zirkularrerako trantsizioa sustatzeaz gain, abereen elikagai gisa, lurzoruen bioerremediaziorako, ongarrien erresistoma murrizteko eta entzimak ekoizteko ere erabiltzen da (Grimm & Wösten, 2018; Hu et al., 2019).

1.2.4.5. Nanopartikulak

Zero balentzia duten burdinazko nanopartikulek azalera-bolumen erlazio handia dute eta erreaktibitate handia aurkezten dute kutsatzaile askoren aurrean (Ken & Sinha, 2020). Lurpeko urak eta lurzoruak berreskuratzeko erabiltzen dira, hidrokarburo aromatiko poliziklikoak, metalak, antibiotikoak, bakterioak eta birusak murrizteko ahalmena baitute (Shi et al., 2012; Xia et al., 2014; Li et al., 2017a; Anza et al., 2019). Ongarri organikoetan zero balentzia duten burdinazko nanopartikulen aplikazioak bakterioen mintz zelularra kaltetu dezakete, antibiotikoak eta AEGen ugaritasuna murriztuz (Wang et al., 2019; Qiu et al., 2022).

1.5. taula Ongarri organikoen kudeaketa-prozesuen ondorioz emandako antibiotikoen murrizketa portzentaia eta murrizketa joerak, hainbat ikerketatan oinarrituz.

KONPOSTAJEA				
Antibiotikoa	**Jatorria**	**Egunak**	**Murrizketa (%)**	**Erreferentzia**
Doxiziklina, klortetraziklina	Txerri simaurra, oilo simaurra	21, 40	99,8-100	(Ho et al., 2013; Selvam et al., 2012)
Enrofloxazina, norfloxazina, ziprofloxazina	Txerri simaurra, oilo simaurra	56, 40	69->99,9	(Ho et al., 2013; Selvam et al., 2012)
Florfenikola	Behi simaurra, lohia	21	95-99	(Mitchell et al., 2015)
Sulfadiazina, sulfadimetoxina, sulfametazina	Txerri simaurra, oilo simaurra, behi	3, 40	97-100	(Ho et al., 2013; Selvam et al., 2012; Mitchell et al., 2015)

	simaurra, lohia			
Eritromizina, tilosina	Oilo simaurra, behi simaurra, lohia	21, 40	96->99,9	(Ho et al., 2013; Mitchell et al., 2015)
Trimetoprima	Oilo zirina	40	>99,9	(Ho et al., 2013)
AEG	**Jatorria**	**Egunak**	**Joera**	**Erreferentzia**
ermA, ermB, ermF, ermX	Behi simaurra	30	2,0 log kopia murriztu-2,0 log kopia igo	(Xu et al., 2018)
dfrA7	Behi simaurra	40	% 3,7-9,8 igo	(Qian et al., 2016)
tetB, tetC, tetL, tetM, tetW	Behi simaurra	30	3,2 log kopia murriztu-3,8 log kopia igo	(Xu et al., 2018)
tetC, tetL, tetM, tetQ, tetW, tetX	Behi simaurra	40	% 35,1 murriztu-% 41,5 igo	(Qian et al., 2016)
sul1, sul2	Behi simaurra	30	0,5-0,7 log kopia murriztu	(Xu et al., 2018)
sul1, sul2	Behi simaurra	40	% 4,3-30,8 igo	(Qian et al., 2016)
DIGESTIO ANAEROBIKOA				
Antibiotikoa	**Jatorria**	**Egunak**	**Murrizketa (%)**	**Erreferentzia**
Klortetraziklina, oxitetraziklina	Txerri simaurra	31	53-92	(Álvarez et al., 2010)
Sulfadiazina, sulfamerazina, sulfametazina, sulfametoxazola, sulfametoxina, sulfametoxipiridiazina, sulfatizola	Txerri simaurra	34	0-100	(Mohring et al., 2009)
AEG	**Jatorria**	**Egunak**	**Joera**	**Erreferentzia**
Aminoglukosidoa, β-laktamikoa, bankomizina, fenikola, kinolona, MLSB, droga anitza, sulfamida, tetraziklina	Lohia		% 70,3 murriztu-% 1050 igo	(Yang et al., 2014)
qnrA	Txerri simaurra	90	3,8-27,4 log kopia igo	(Zhang et al., 2018)
tetA, tetB, tetC, tetG, tetL, tetM, tetO, tetQ, tetW, tetX	Txerri simaurra	49, 90	1,5 log kopia murriztu-2,8 log kopia igo	(Wang et al., 2017; Zhang et al., 2018)
intl1, intl2, ISCR1, Tn916/1545	Txerri simaurra	49, 90	<1 log kopia murriztu-0,08 log kopia igo	(Wang et al., 2017; Zhang et al., 2018)
BIO-IKATZA				
Antibiotikoa	**Jatorria**	**Dosia**	**Murrizketa (%)**	**Erreferentzia**
Oxitetraziklina	Lurzorua, letxuga hostoa	% 2	10,7-39,0	(Chenxi et al., 2008)
AEG-EGM	**Jatorria**	**Dosia**	**Joera**	**Erreferentzia**
tetC, tetG	Lurzorua	% 2	% 32,5-84,3 murriztu	(Chenxi et al., 2008)
tetX	Lurzorua	% 2	1,5 log kopia igo	(Chenxi et al., 2008)
ermG, ermX	Lurzorua	% 2	Mantendu	(Chenxi et al., 2008)
sul1, sul2	Lurzorua	% 2	Mantendu	(Chenxi et al., 2008)

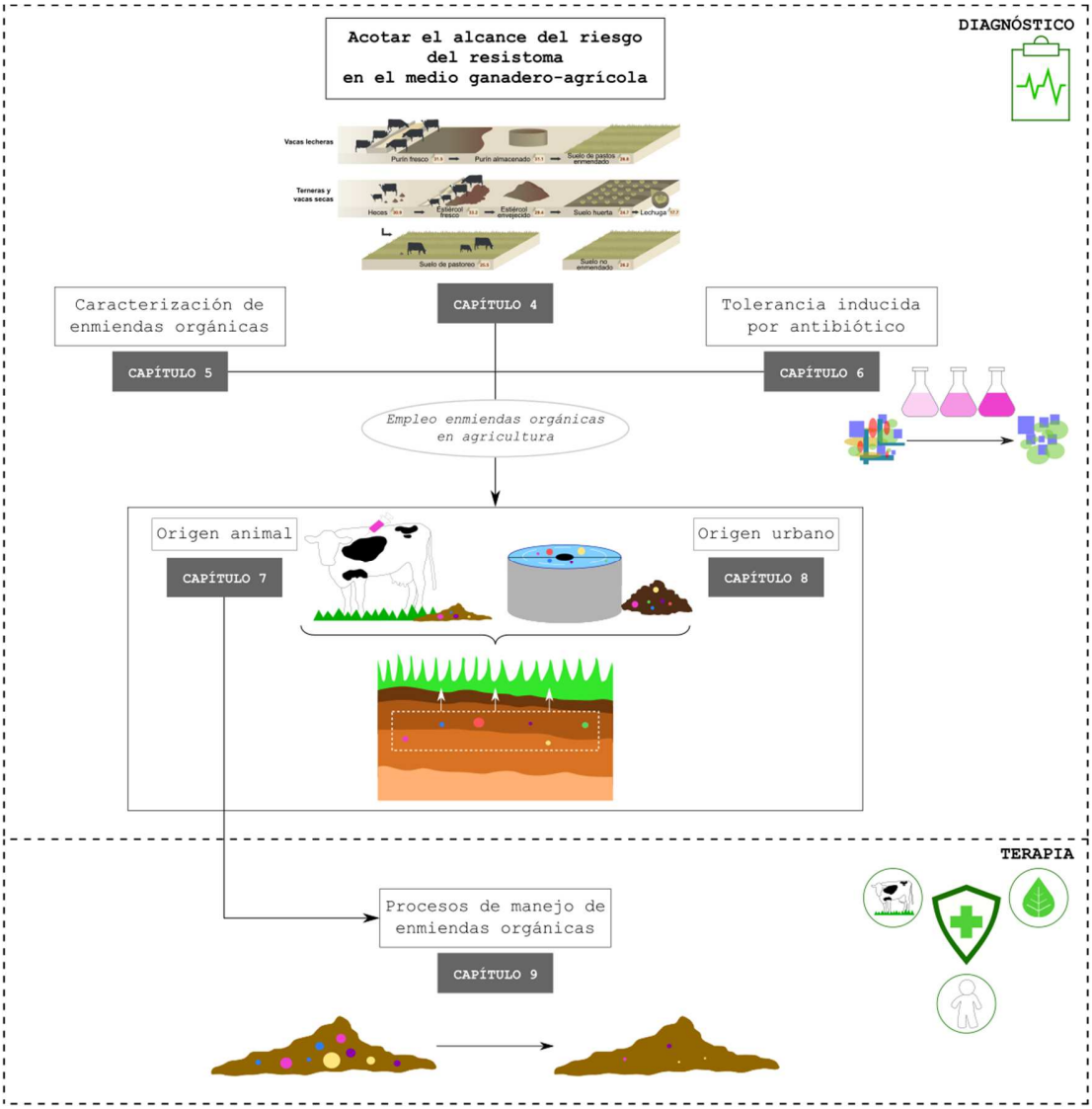
intl1	Lurzorua	% 2	Mantendu	(Chenxi et al., 2008)
89 AEG	Lurzorua	% 0.5	Esanguratsuki murriztu AEG kopia absolutuak	(Chen et al., 2018)
AGORTUTAKO ONDDO-SUBSTRATUA				
AEG-EGM	**Jatorria**	**Joera**		**Erreferentzia**
aac(6')-Ib-cr, dfrA7, ermQ, ermX, intl1, ISCR1, sul2, tetC, tetG, tetW, Tn916/1545	Txerri simaurra	0,03-1,67 log kopia murriztu		(Hu et al., 2019)
ermF, sul1, tetX	Txerri simaurra	0,08-0,87 log kopia igo		(Hu et al., 2019)
blaTEM, blaCTX, ermB, ermC, ermF, sul1, sul2, tetB, tetG, tetL, tetM, tetO, tetZ,	Lurzorua	AEG-n abundantzia murriztu oilo zirinarekin alderatuz		(Peng et al., 2021)
NANOPARTIKULAK				
Antibiotikoak	**Jatorria**	**Dosia**	**Murrizketa (%)**	**Erreferentzia**
Makrolidoa	Oilo simaurra	300-600 nZVI mg kg^{-1}	-57,1-100	(Qiu et al., 2022)
Sulfamida	Oilo simaurra	300-600 nZVI mg kg^{-1}	-950-100	(Qiu et al., 2022)
Tetraziklina	Oilo simaurra	300-600 nZVI mg kg^{-1}	-53,9-90,4	(Qiu et al., 2022)
AEG-EGM	**Jatorria**	**Dosia**	**Joera**	**Erreferentzia**
tetC, tetG, tetW	Oilo simaurra	300-600 nZVI mg kg^{-1}	% 22,6-76,8 murriztu	(Qiu et al., 2022)
sul1, sul2	Oilo simaurra	300-600 nZVI mg kg^{-1}	% 25,3-74,4 murriztu	(Qiu et al., 2022)
blaOXA-1, blaTEM, ermB, ermF, intl1, mefA, sul1, sul2, tetM, tetO, tetQ, tetW, tetX	Janari hondakinak	2000-5000 nZVI mg L^{-1}	% 4,3-43,4 murriztu	(Wang et al., 2019)

1.2.5. Aurrera begira

Antibiotikoekiko erresistentzia mehatxu globaltzat hartu behar da, ez bakterioek ezta geneek ere ez dituztelako oztopo geografikoak errespetatzen. Arazo honen aurrean, nahitaezkoa da antibiotikoen ordezko estrategiak garatzea. Hala, abeltzaintzan, funtsezkoa da abereen gaixotasunen agerraldiak prebenitzea. Horretarako higienea, txertaketa, bio-segurtasuna eta ongizatea bermatu behar dira, abereen osasun orokorra indartzeko asmoz. Aipatutako neurri hauek antibiotikoen erabilera gutxitzea ekarriko lukete, eta ondorioz, abereen hesteetan nahiz gorozkietan hautespen presioa murriztea. Prebentzioarekin batera, gaixotasunen detekzio eta diagnostiko goiztiarra egiteak antibiotiko tratamendu egokiena ezartzea ahalbidetuko luke.

Bukatzeko, antibiotikoek eragindako kutsadura arazo larria denez gizaki, animalia eta ingurumenarentzat, Osasun Bakarraren ikuspegitik neurriak hartzea ezinbestekoa da. Hala ere, oraindik ez dago argi ingurumenean bakterio erresistenteak eta AEGak egoteak zenbateraino laguntzen duten klinikoki garrantzitsuak diren bakterioetan erresistentziak eskuratzen eta zabaltzen. Gai horiei heltzeko, funtsezkoa da ingurumeneko erresistentziak hobeto identifikatu eta kuantifikatzea. Ingurunean modu naturalean dauden AEGen kontzentrazioak ezagutzea segurtasun-mailak zehazteko aurrebaldintza bat da, eta giza jardueraren ondorioz ingurune bat AEGekin zenbateraino aberastu daitekeen jakiteko informazioa ere emango luke. Honen harira, oraindik ez dago erresistentzien monitorizazio sistematikorik ingurumenean, ezta ingurune gehienetarako erreferentziazko daturik ere. Horrelako zerbaitek gizakien eta abereen osasunerako arriskurik handienak zein ingurunek dituzten adieraztea ahalbidetuko luke. Gainera, ingurumenetik erresistentzien barreiatzea prebenitu edo atzeratzeko neurriak hartzeko aukera emango liguke. Artikulu honetan azaldu den moduan, gizakien eta abereen gorozkiak lurzoruetan ongarri organiko moduan erabili aurretik, erresistomaren arriskua ahalik eta gehien murriztu beharko litzateke, barreiadura mugatu eta bakterio patogeno erresistenteak sortzeko probabilitatea gutxitzeko.

1.3. Justificación secuencial del trabajo



2. HIPÓTESIS Y OBJETIVOS

2.1. Hipótesis general

La aplicación de enmiendas orgánicas de origen animal o humano puede mejorar la salud de los suelos agrícolas y aumentar el rendimiento de los cultivos. Sin embargo, esta práctica agrícola conlleva, a su vez, riesgos asociados a la posible contaminación ambiental con antibióticos y sus productos de transformación y, sobre todo, con bacterias resistentes a los antibióticos. Los antibióticos ejercen una presión selectiva sobre las bacterias ambientales expuestas, la cual puede dar lugar a la emergencia y propagación de resistencias bacterianas. El principal riesgo reside en la posibilidad de que las bacterias ambientales resistentes a los antibióticos transmitan dicha resistencia a bacterias potencialmente patógenas. Aunque la aplicación de enmiendas orgánicas representa potencialmente una vía de entrada de resistencias a antibióticos en los agroecosistemas, el tratamiento y manejo adecuado de dichas enmiendas puede reducir considerablemente el citado riesgo.

2.2. Hipótesis específicas que sustentan los diferentes capítulos

Capítulo 4: El riesgo del resistoma se reduce a lo largo de la vía de exposición "deyecciones – enmienda – suelo – cultivo".

Capítulo 5: El origen y los procesos de compostaje de las enmiendas orgánicas influyen en el potencial de riesgo de contaminación, así como en su calidad.

Capítulo 6: El gradiente de concentración de antibiótico (oxitetraciclina) al que se exponen las comunidades bacterianas se traduce en un gradiente de tolerancia.

Capítulo 7: El origen (de ganadería convencional *versus* de ganadería ecológica) y el tipo (estiércol fresco *versus* estiércol almacenado *versus* purín) de enmienda orgánica influyen en el resistoma en el sistema "enmienda – suelo – planta".

Capítulo 8: Los suelos enmendados con lodos de depuradora presentan una magnitud del resistoma mayor que los no enmendados.

Capítulo 9: Los procesos de manejo de las enmiendas orgánicas pueden reducir el resistoma en el sistema "enmienda – suelo – planta".

2.3. Objetivo general

El objetivo general de esta tesis doctoral fue evaluar la contribución potencial de la aplicación de enmiendas orgánicas de origen animal o humano a los suelos agrícolas en la emergencia y diseminación de resistencias a antibióticos, mediante la monitorización en diversas matrices del ámbito agroganadero de antibióticos, bacterias resistentes a antibióticos, genes de resistencia a antibióticos y genes asociados a elementos genéticos móviles.

2.4. Objetivos específicos asociados a los diferentes capítulos

Capítulo 4: Evaluar el riesgo del resistoma, moviloma y patogenoma a lo largo de la vía de exposición que va desde los purines y estiércoles hasta los suelos y vegetales en explotaciones agroganaderas.

Capítulo 5: Realizar una caracterización fisicoquímica y biológica de enmiendas orgánicas de cara a la selección de aquellas que presenten una mejor calidad y menor riesgo para su empleo en agricultura.

Capítulo 6: Evaluar los efectos de la adición repetida de oxitetraciclina, a través de su presencia en enmienda orgánica de origen animal (estiércol de vacuno), en el desarrollo de la tolerancia a dicho antibiótico por parte de las comunidades bacterianas edáficas.

Capítulo 7: Estudiar en condiciones controladas el resistoma y el moviloma del sistema "enmienda – suelo – planta" (lechuga y trigo) en respuesta a la aplicación de distintas enmiendas orgánicas (estiércol fresco, estiércol envejecido, purín) de origen convencional o ecológico.

Capítulo 8: Evaluar el efecto de la dosis y el tiempo transcurrido desde la última aplicación de lodo de depuradora sobre (i) las propiedades fisicoquímicas y biológicas del suelo agrícola; y (ii) la presencia y abundancia de genes de resistencia a antibióticos y genes asociados a elementos genéticos móviles en los suelos enmendados.

Capítulo 9: Evaluar la eficacia de diferentes tratamientos y manejos (digestión anaerobia, ozonización, aplicación de biochar, adición de nanopartículas de hierro cero valente, adición de substrato agotado de hongos) de enmiendas orgánicas de origen animal para reducir el resistoma y moviloma en el sistema "enmienda – suelo – planta".

3. MATERIALES Y MÉTODOS

En este capítulo se describen (i) los parámetros analíticos y (ii) las herramientas estadísticas más relevantes empleadas en este trabajo para cuantificar y evaluar el riesgo del resistoma ambiental.

3.1. Parámetros analíticos

Los parámetros para evaluar y cuantificar el riesgo asociado a la resistencia a los antibióticos en muestras ambientales pueden dividirse principalmente en (i) métodos cultivables y (ii) métodos no cultivables o moleculares. Cada tipo de método tiene sus ventajas e inconvenientes y ninguno proporcionará por sí solo una visión completa. Sin embargo, al combinar varios métodos, es posible obtener información amplia y útil sobre el riesgo y el potencial de diseminación de los ARGs en el medio ambiente.

3.1.1. Métodos cultivables

El cultivo permite investigar directamente rasgos funcionales, como el desarrollo de la tolerancia a los antibióticos de comunidades microbianas. Por otro lado, uno de los inconvenientes de los métodos cultivables es que sólo una pequeña parte de los microorganismos son cultivables en los medios artificiales utilizados en los laboratorios.

3.1.1.1. Concentración mínima inhibitoria

La concentración mínima inhibitoria (MIC) es la concentración más baja de un antibiótico a la que se inhibe el crecimiento de un microorganismo o comunidad de microorganismos. Para la determinación de la MIC se emplean dos tipos de métodos: métodos de dilución y métodos de gradiente. En el Capítulo 7 de este trabajo se llevó a cabo un método de gradiente empleando los MIC Test Strips de Liofilchem®, aunque los resultados obtenidos no se muestran en la versión final de la publicación. Este sistema consiste en tiras porosas impregnadas con un gradiente predefinido de concentración de antibiótico. Al colocarlas en una placa de agar previamente inoculada, el gradiente exponencial preformado de antibiótico se transfiere a la matriz de agar. Tras la incubación de las placas, se forma una elipse de inhibición simétrica centrada a lo largo de la tira.

Se utilizaron estas tiras para cuantificar los valores de MIC de cinco antibióticos (meropenem, estreptomicina, sulfametoxazol, tetraciclina y vancomicina) de las comunidades bacterianas extraídas

de las enmiendas orgánicas, suelos enmendados y plantas. Los antibióticos seleccionados tienen diferentes modos de acción y se utilizan ampliamente en veterinaria y medicina humana. Para ello, se colocaron cinco tiras MIC Test Strips de Liofilchem™ en placas de agar Müller-Hinton de 150 mm previamente inoculadas. Para la preparación del inóculo bacteriano, se añadió 1 g PS de enmienda orgánica o suelo o 1 g PS de material vegetal molido a 200 ml y 20 ml de agua destilada estéril, respectivamente, en matraces cónicos de 150 ml, y se agitó (con una barra magnética recubierta de teflón) durante 15 minutos a 200 rpm en un agitador orbital. Se inocularon 2 ml de esta suspensión (cada muestra se repitió dos veces) en las placas de agar Müller-Hinton. Al cabo de 15 minutos, se colocaron las tiras en forma radial con unas pinzas estériles, a una distancia equidistante entre sí. Las placas inoculadas se incubaron durante 24 horas a 28 ºC en la oscuridad. Posteriormente, se anotó la concentración de cada antibiótico que daba lugar a la inhibición del crecimiento bacteriano (el punto en el que el borde de la elipse de inhibición cruzaba la tira).

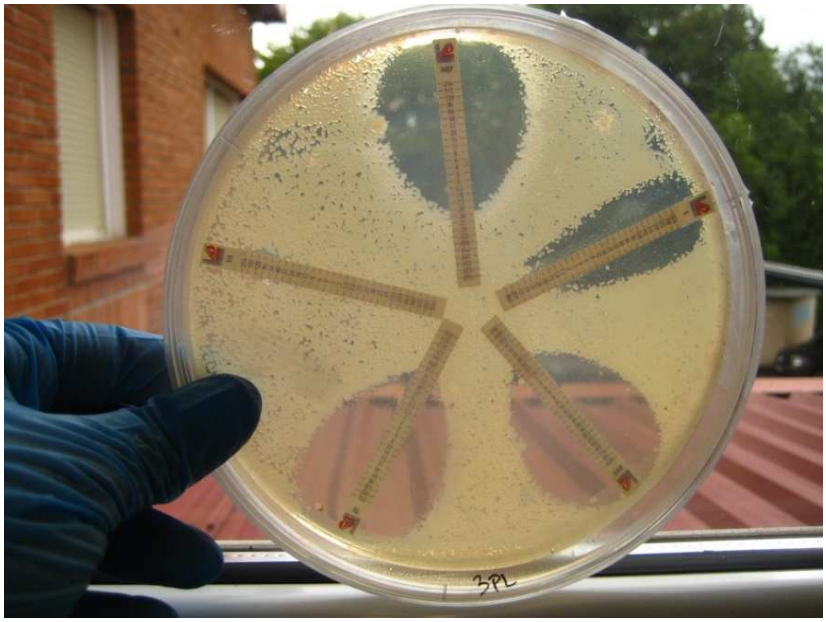


Figura 3.1. Ejemplo del crecimiento de las comunidades bacterianas de una muestra ambiental tras 24 horas de incubación. Cada tira MIC Test Strips de Liofilchem® tiene un gradiente de concentración de un antibiótico específico.

3.1.1.2. Tolerancia inducida por antibióticos a nivel de comunidad

El método de la tolerancia inducida por contaminantes a nivel de comunidad (PICT) se basa en el principio de que una comunidad microbiana responderá a la presión de selección ejercida por un

contaminante. La respuesta puede ser por cambios en (i) la composición microbiana hacia especies más tolerantes, (ii) la abundancia de genes resistentes y/o (iii) la fisiología. El procedimiento de determinación del parámetro PICT se divide en dos fases. En la primera fase, denominada fase de selección, la comunidad se expone al agente contaminante. En la segunda fase, denominada fase de detección, se determina la tolerancia de la comunidad al mismo contaminante que en la fase de selección. Si la tolerancia de las comunidades expuestas al contaminante supera a la tolerancia de las comunidades control, se deduce que el contaminante ha ejercido una presión selectiva en la comunidad. Si bien hay otros métodos para medir el PICT por métodos no cultivables, en el Capítulo 6 de este trabajo la determinación del PICT se llevó a cabo utilizando placas Biolog Ecoplates^{TM}, que incluyen 31 sustratos de carbono ambientalmente relevantes (Figura 3.2.).

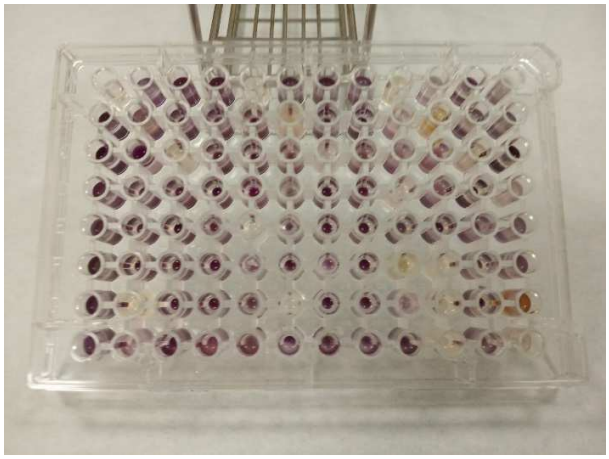


Figura 3.2. Placa Biolog EcoPlate^{TM} a la que se ha inoculado una muestra a diferentes concentraciones de oxitetraciclina en la fase de detección.

3.1.2. Métodos no cultivables

Los avances en tecnologías moleculares han permitido la creación de métodos independientes de los cultivos para estudiar las comunidades microbianas y su genética. La técnica que está en la base de la mayoría de estos métodos es la PCR, mediante la cual se pueden amplificar segmentos específicos del DNA. El primer paso para la medición de estos parámetros es la extracción del DNA de las comunidades bacterianas presentes en las muestras ambientales, que en el presente trabajo se realizó con kits comerciales. El DNA de las muestras de las enmiendas orgánicas y suelos se extrajo con los kits de extracción PowerSoil DNA Isolation (MoBio Laboratories) y Qiagen DNeasy PowerSoil Pro

(Qiagen) partiendo de 250 mg por muestra; y de las muestras de plantas utilizando el kit innuPREP DNA (Analytik Jena), siguiendo las instrucciones de los fabricantes.

3.1.2.1. PCR cuantitativa a tiempo real

La PCR cuantitativa a tiempo real (qPCR) consiste en la detección fluorescente de la acumulación de copias en cada ciclo de amplificación de la PCR. El número de copias detectado en la fase exponencial temprana es proporcional a la concentración presente en la muestra inicial, permitiendo la cuantificación relativa o absoluta de los genes diana. La abundancia de los ARGs y elementos genéticos móviles (MGEs) en el DNA ambiental se cuantifica al amplificar un gen diana mediante un conjunto de cebadores diseñados específicamente para dicho gen. La cuantificación relativa se basa en la relación entre las abundancias de un gen de interés (ARG, MGE) y uno estructural universalmente conservado (*e.g.*, 16S rRNA). En cambio, la cuantificación absoluta consiste en cuantificar las copias del gen diana en base a una recta patrón de concentración conocida. En el Capítulo 5 se realizó la cuantificación relativa del gen *intl1* en las enmiendas orgánicas. En el Capítulo 7 se cuantificó la abundancia absoluta del gen 16S rRNA en las enmiendas orgánicas, suelos enmendados y plantas.

3.1.2.2. PCR digital en gotas

La PCR digital en gotas (ddPCR) es un método para realizar PCR digital basado en la tecnología de gotas en una emulsión agua-aceite. La tecnología consiste en fraccionar una muestra en 20.000 gotas y realizar una reacción de PCR de las moléculas diana en cada gota generada. Esta partición permite la medición de miles de eventos de amplificación independientes dentro de una sola muestra. La ddPCR detecta la fluorescencia al final de la amplificación, identificando el número de gotas positivas y determinando la concentración, expresada en copias absolutas por reacción, mediante la distribución de Poisson. En el Capítulo 9 de este trabajo se cuantificaron 4 ARGs y 2 MGEs en las enmiendas orgánicas, suelos y plantas.

Figura 3.3. Equipos utilizados en la cuantificación de ARGs y MGEs mediante ddPCR.

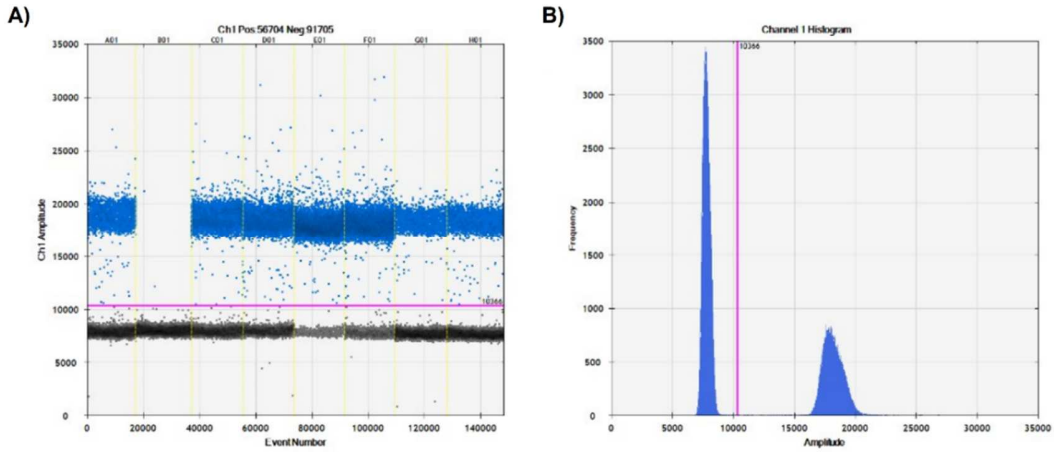


Figura 3.4. A) Gráfico del número de gotas con PCR positiva (banda azul) y negativa (banda negra). B) Histograma de las microgotas negativas (pico de la izquierda) y de las positivas (pico de la derecha).

3.1.2.3. qPCR de alto rendimiento

La qPCR de alto rendimiento (HT-qPCR) es un tipo de qPCR que sirve para monitorizar simultáneamente un número elevado de genes. Mediante cebadores que previamente han sido diseñados, validados y que tengan una temperatura de hibridación similar, se mide la presencia y la abundancia relativa de los genes diana. En los Capítulos 7 y 8 de este trabajó se cuantificaron 95 ARGs y MGEs mediante HT-qPCR, utilizando la tecnología qPCR BioMark^{TM} HD con chips de 48.48 y 96.96 Dynamic Array IFCs de Fluidigm, en colaboración con la unidad de secuenciación y genotipado de SGIker (UPV/EHU). En el Capítulo 9, se cuantificaron 95 (suelos) y 23 (plantas) ARGs y MGEs (aquellos que fueron detectados en un análisis previo realizado con 384 genes). La tecnología empleada fue SmartChip qPCR (TakaraBio), en colaboración con Resistomap Oy (Helsinki, Finlandia). En el Capítulo 9 también se presenta una comparación de la sensibilidad de los métodos ddPCR y HT-qPCR.

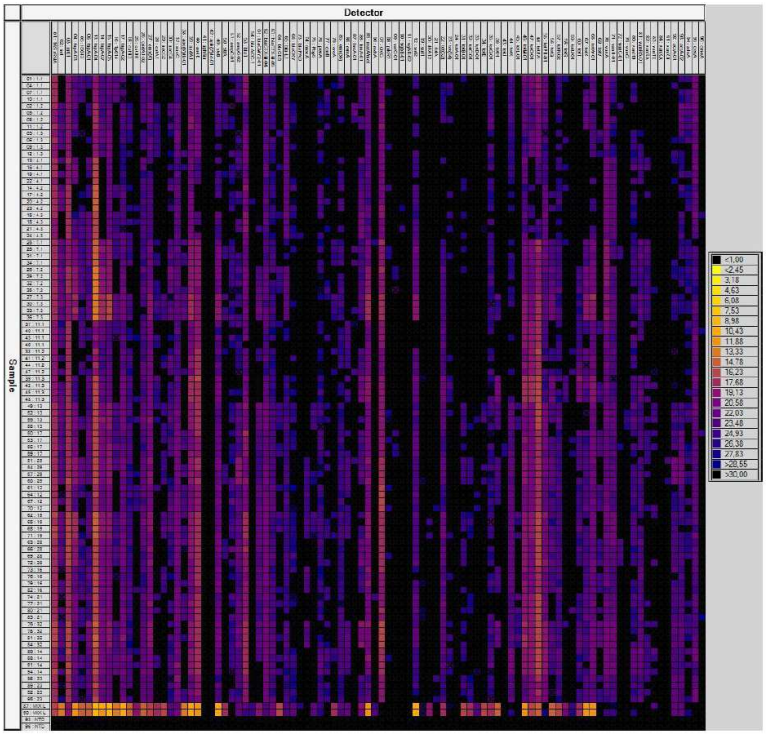


Figura 3.5. Representación gráfica de los datos de amplificación obtenidos mediante HT-qPCR.

Las abundancias relativas de los genes evaluados por HT-qPCR se suelen expresar en (i) fold change (FC) y/o (ii) número de copias del gen diana. Hay dos formas principales para calcular el FC: el primer método (ecuaciones 1.1 y 1.2) es apropiado para determinar el efecto del tratamiento con relación a un gen control (*e.g.*, 16S rRNA) y cuando no existe un tratamiento control común (Livak & Schmittgen, 2001). El segundo método para calcular el FC (ecuaciones 2.1 y 2.2) es adecuado en el caso de que se disponga de un tratamiento control (Livak & Schmittgen, 2001).

$$\Delta C_T = C_{T(gen\ diana)} - C_{T(16S\ rRNA)} \qquad [1.1]$$

$$FC = 2^{-\Delta C_T} \qquad [1.2]$$

$$\Delta\Delta C_T = \Delta C_{T(tratamiento)} - C_{T(control)} \qquad [2.1]$$

$$FC = 2^{-\Delta\Delta C_T} \qquad [2.2]$$

El número de copias del gen diana se calcula a partir de las ecuaciones 3.1 y 3.2. En la ecuación 3.1, se calcula la abundancia relativa de un gen (Looft et al., 2012). Para calcular las copias absolutas del gen diana, previamente se necesitan obtener las copias absolutas del gen 16S rRNA [ecuación 3.2

(Zhu et al., 2017a)]. En el Capítulo 7 de este trabajo, las abundancias absolutas del gen 16S rRNA se obtuvieron vía qPCR.

$$GR = 10^{\frac{(31-C_T)}{(10/3)}} \qquad [3.1]$$

$$GA_{gen\ diana} = \frac{GA_{16S\ rRNA} \times GR_{gen\ diana}}{GR_{16S\ rRNA}} \qquad [3.2]$$

Siguiendo las ecuaciones antes descritas, es frecuente presentar los resultados en abundancia relativa respecto al gen 16s rRNA, proporcionando información sobre la abundancia de genes por bacteria y permitiendo la comparación entre entornos y muestras. La otra opción es presentar los valores absolutos en relación con el tamaño de muestra, por lo que el valor estará directamente relacionado con la densidad celular de la muestra. Aunque la comparación entre distintas muestras es más difícil de esta última forma, también es cierto que es un buen reflejo del riesgo, ya que una alta densidad celular puede aumentar la diseminación de la resistencia a los antibióticos.

El método de HT-qPCR presenta la ventaja de que es muy útil para estimar la abundancia de los genes seleccionados en una muestra, y probablemente por esto es el más utilizado para la identificación y cuantificación de ARGs y MGEs en muestras ambientales. Sin embargo, no proporciona información sobre qué bacterias albergan los genes detectados, si están asociados a MGEs o si los genes son funcionales. Otra limitación es que este método sólo se centra en un número definido de genes, mientras que, por ejemplo, los métodos de secuenciación metagenómica incluyen todos los posibles genes presentes en las muestras (aunque sólo se podrán identificar aquellos presentes en las bases de datos).

3.1.2.4. Secuenciación metagenómica

La secuenciación metagenómica consiste en la secuenciación no dirigida de todos los genomas microbianos presentes en una muestra, permitiendo analizar simultáneamente el perfil taxonómico y el potencial funcional de las comunidades microbianas. Este método se empleó en el Capítulo 4 del presente trabajo. El análisis se llevó a cabo en el Centro Nacional de Análisis Genómico (CNAG-CRG, España) mediante la tecnología Illumina NovaSeq6000, con una longitud de lectura de 2 × 150 pares de bases y obteniendo > 20 Gb por muestra.

La secuenciación metagenómica permite explorar, en cierta medida, el contexto genético, y a su vez el huésped microbiano puede ser identificado. En el Capítulo 4, con el fin de evaluar el riesgo del resistoma en el medio ganadero-agrícola, se empleó una herramienta bioinformática denominada MetaCompare (Oh et al., 2018). Esta herramienta proporciona un índice de riesgo que puede servir para priorizar los esfuerzos de mitigación de la diseminación de ARGs, MGEs y patógenos humanos. Brevemente, los contigs obtenidos de la secuenciación metagenómica se clasifican en tres categorías: (i) contigs en los que se encuentran ARGs, (ii) contigs en los que se encuentran simultáneamente ARGs y MGEs y, (iii) contigs en los que se encuentran conjuntamente ARGs, MGEs y patógenos humanos (Figura 3.6.).

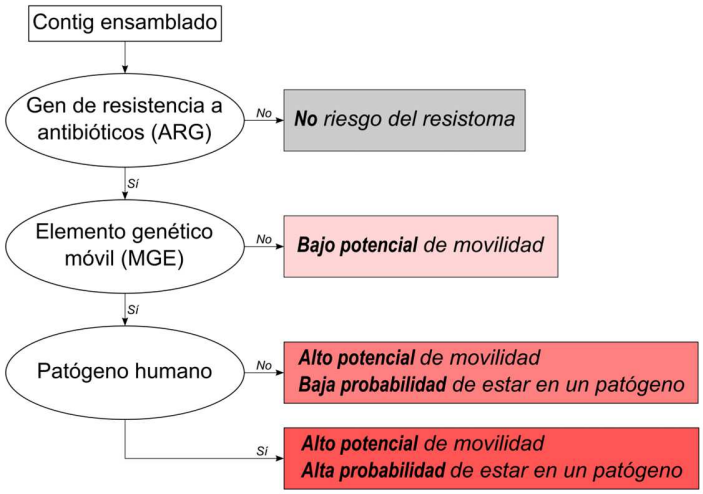


Figura 3.6. Categorización de contigs propuesta por MetaCompare. Adaptado de Oh et al., 2018.

Sin embargo, la sensibilidad de la metagenómica depende en gran medida de la profundidad de secuenciación y, por tanto, la estimación de la abundancia y la diversidad de ciertos genes (por ejemplo, los ARG) suele verse afectada, por lo que los métodos basados en PCRs pueden permitir una detección más sensible.

3.1.2.5. Secuenciación de amplicones

La secuenciación de amplicones es un método de alto rendimiento ampliamente utilizado para estudiar la diversidad y la composición taxonómica de las comunidades microbianas. La técnica se basa en la amplificación por PCR de regiones cortas e hipervariables de genes conservados. En una primera PCR,

se amplifica el gen diana mediante cebadores específicos. En una segunda amplificación, se incluye una secuencia nucleotídica específica para cada muestra, lo que permitirá la identificación de la muestra tras el proceso de secuenciación. Los amplicones obtenidos después de cada PCR se purifican mediante microesferas magnéticas AMPure XP de Agencourt. La secuenciación se realiza utilizando tecnologías de secuenciación de alto rendimiento. A continuación, las lecturas obtenidas se procesan mediante diversos softwares bioinformáticos. Durante el procesamiento de los datos, las secuencias de amplicones se agrupan en unidades taxonómicas operativas (OTUs) o en variantes de secuencias de amplicones (ASVs). Tanto los OTUs como las ASVs obtenidas se comparan después con bases de datos para determinar la identidad de los microorganismos. En este trabajo, la secuenciación de amplicones se empleó para estudiar la diversidad y composición de organismos procariotas, secuenciando el gen 16S rRNA. En los Capítulos 7 y 8 la secuenciación se llevó a cabo por la tecnología Illumina MiSeq V2 en las instalaciones de Tecnalia (Miñano) y, en el Capítulo 9 en la unidad de secuenciación y genotipado de SGIker (UPV/EHU, Leioa).

3.2. Herramientas estadísticas

La magnitud de los datos generados con las metodologías descritas más arriba precisa de análisis estadísticos apropiados para poder dar respuesta a las preguntas científicas planteadas. A continuación, se describen brevemente dos análisis estadísticos empleados en este trabajo.

3.2.1. Análisis de redes

Los análisis de redes consisten en estructuras que se representan mediante nodos (las variables de estudio) y aristas (la relaciones entre los nodos). La modularidad es una propiedad de la red que describe la tendencia de los nodos a agruparse. Las redes con alta modularidad presentan conexiones densas entre los nodos del mismo módulo y, conexiones débiles y escasas entre los nodos de otros módulos. Los análisis de redes se han convertido en herramientas populares para investigar asociaciones e interacciones, aunque no está de más recordar que la correlación no implica causalidad. La naturaleza de los datos y el objetivo del estudio condicionan la elección del tipo de análisis. En este trabajo se han empleado dos tipos de análisis que se describen a continuación.

3.2.1.1. Análisis de redes basado en correlaciones

Este análisis se basa en medidas de (di)similitud que correlacionan diferentes variables entre sí. Los coeficientes de correlación obtenidos revelan la magnitud de la relación colineal de las variables. La construcción del análisis comienza con el cálculo de los coeficientes de correlación por pares entre variables del conjunto de datos. Una vez que se construye la matriz de correlaciones con coeficientes de correlación significativos, la red se puede visualizar con plataformas como Gephi. En los Capítulos 8 y 9 de este trabajo se determinaron los posibles taxones hospedadores de ARGs y MGEs mediante este tipo de análisis de redes.

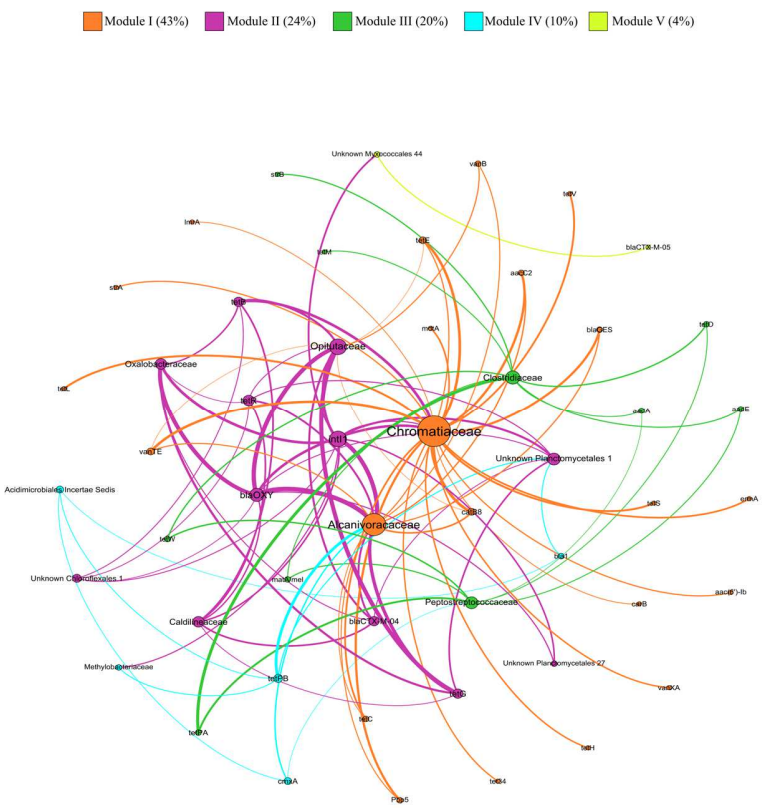


Figura 3.7. Ejemplo de un análisis de redes basado en correlaciones entre las abundancias de ARGs, MGEs y 12 familias de bacterias multirresistentes.

3.2.1.2. Análisis de redes basado en co-ocurrencia

Este análisis permite explorar e identificar patrones o marcadores en conjuntos de datos grandes y complejos. Se puede realizar con el paquete *cooccur* de R en el que aplica el modelo probabilístico de

co-ocurrencia que clasifica las parejas de variables en asociaciones positivas, negativas y aleatorias. Una red basada en co-ocurrencias requiere un número relativamente grande de muestras para detectar asociaciones sólidas, ya que puede resultar difícil determinar si los patrones de co-ocurrencia son estadísticamente significativos o no. En el Capítulo 4 de este trabajo se empleó esta herramienta con datos de presencia-ausencia de los ARGs y MGEs anotados en la secuenciación metagenómica. Brevemente, el algoritmo del modelo calculó las frecuencias observadas y esperadas de co-ocurrencia entre cada par de ARGs. La frecuencia de co-ocurrencia esperada se basó en que la distribución de cada ARG era aleatoria e independiente de los otros ARGs anotados. Se construyó la matriz de correlaciones calculando todas las posibles correlaciones de Spearman entre los ARGs que ocurrieron, al menos, en la mitad de las muestras, y finalmente el resultado se visualizó en la plataforma Gephi.

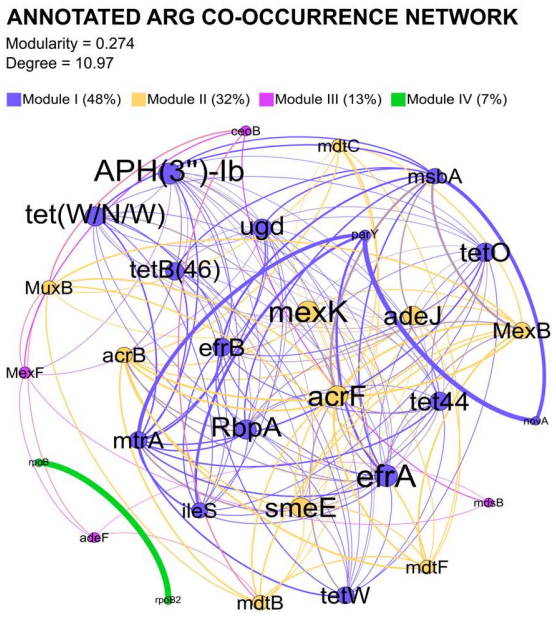


Figura 3.8. Ejemplo de un análisis de redes basado en co-ocurrencia de ARGs.

3.2.2. Modelos de ecuaciones estructurales

La comprensión de los sistemas requiere la capacidad de examinar influencias y respuestas simultáneas. Los modelos de ecuaciones estructurales (SEM) pueden definirse en su sentido más básico como el uso de dos o más ecuaciones estructurales para modelar una relación multivariante. Las relaciones multivariantes se refieren a las que implican influencias y respuestas simultáneas. Una definición alternativa es que representa dependencias o asociaciones estadísticas sujetas a

interpretación causal. Aunque los resultados de los análisis de ecuaciones estructurales pretenden reflejar dependencias causales, no son los resultados estadísticos *per se* los que demuestran la causalidad, sino el hacer una interpretación causal, que a su vez depende principalmente de la experiencia previa y del conocimiento sustantivo. El SEM tiene una enorme flexibilidad y precisa de importantes requisitos para su uso adecuado.

En el SEM, existen cuatro tipos de variables: observadas, latentes, compuestas y de error. En este trabajo se han empleado las dos primeras variables. Las variables observadas representan aquellas que se han medido directamente. Las variables latentes representan las que no han sido medidas. La variable latente suele estar asociada a una o más variables indicadoras observadas. Este tipo de modelo de ecuaciones estructurales se empleó en el Capítulo 8 del presente trabajo. En este trabajo, previo al análisis del SEM, se llevó a cabo un análisis de componentes principales con diversas variables, con el objetivo de crear predictores. Así, se seleccionaron para el SEM los dos ejes principales que explicaron la mayor varianza. La capacidad de modelar las variables latentes se ha convertido en una capacidad integral del SEM. Permite que el modelo tenga un contenido teórico y empírico explícitamente vinculado. Para comparar las matrices de covarianza observadas y predichas, se emplearon las pruebas de separación dirigidas. Dichas pruebas evalúan la suposición de que la estructura causal refleje los datos. Los coeficientes estandarizados de la magnitud y el signo de la relación entre las variables se estimaron mediante el algoritmo de máxima verosimilitud. Posteriormente, se seleccionaron aquellos modelos en los que el C de Fisher se situó por encima del nivel de significación ($P > 0.05$). Por último, el criterio de información de Akaike (AIC) hace referencia a una probabilidad logarítmica penalizada. Se utilizó como medida del ajuste de un modelo. Al comparar dos modelos, cuanto menor sea el AIC, mejor será el ajuste.

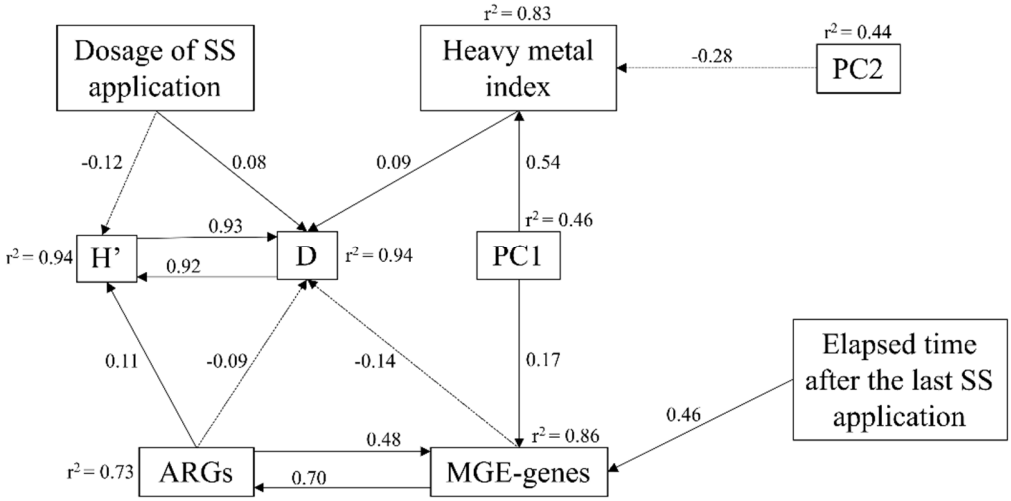


Figura 3.9. Ejemplo de representación de un modelo de ecuaciones estructurales. Las flechas indican relaciones positivas y negativas mediante líneas continuas y discontinuas, respectivamente. Los números junto a las flechas representan los coeficientes de regresión estimados estandarizados. Los valores r^2 indican la proporción de varianza explicada para cada variable.

4. REDUCTION OF THE RESISTOME RISK FROM COW SLURRY AND MANURE MICROBIOMES TO SOIL AND VEGETABLE MICROBIOMES

Abstract

In cow farms, the interaction between animal and environmental microbiomes creates hotspots for antibiotic resistance dissemination. A shotgun metagenomic approach was used to survey the resistome risk in five dairy cow farms. To this purpose, ten environmental compartments were sampled: three of them linked to productive cows (fresh slurry, stored slurry, slurry-amended pasture soil); six of them to non-productive heifers and dry cows (faeces, fresh manure, aged manure, aged manure-amended orchard soil, vegetables-lettuces, and grazed soil); and, finally, unamended control soil. The resistome risk was assessed using MetaCompare, a computational pipeline which scores the resistome risk according to possible links between antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), and human pathogens. The resistome risk decreased from slurry and manure microbiomes to soil and vegetable microbiomes. In total (sum of all the compartments), 18,157 ARGs were detected: 24% related to ansamycins, 21% to multidrugs, 14% to aminoglycosides, 12% to tetracyclines, 9% to β-lactams, and 9% to MLSBs (macrolide-lincosamide-streptogramin B). All but two of the MGE-associated ARGs were only found in the animal dejections (not in soil or vegetable samples). Several ARGs with potential as resistome risk markers (based on their presence in hubs of co-occurrence networks and high dissemination potential) were identified. As a precautionary principle, improved management of livestock dejections is necessary to minimise the risk of antibiotic resistance.

4.1. Introduction

Antimicrobial resistance is a growing serious problem with potentially dramatic human health and economic consequences. Actually, in 2019, the World Health Organization (WHO) listed antimicrobial resistance as one of the top ten threats to global human health (WHO, 2019b). In this respect, O'Neill (2016) attributed 700,000 annual deaths to antimicrobial resistance and, if no action is taken, estimated around 10 million deaths and a cumulative 100 trillion USD loss of economic output by 2050. However, regarding the antibiotic resistant bacteria (ARB) which harbor the antibiotic resistance genes (ARGs), not all of them present the same risk for human health. Most attention must be paid to those bacterial strains that show resistance to several antibiotics (*multiresistant:* acquired non-susceptibility to at least one agent in three or more antimicrobial categories; *extensively resistant:* non-susceptibility

to at least one agent in all but two or fewer antimicrobial categories; *panresistant:* non-susceptibility to all agents in all antimicrobial categories) (Magiorakos et al., 2012). In 2017, the WHO released a catalogue of twelve families of antibiotic-resistant priority pathogens that pose the greatest threat to human health. This catalogue included the infamously known ESKAPE pathogens, responsible for many dangerous nosocomial infections, against which new antibiotics are urgently needed (Tacconelli et al., 2018).

Antimicrobials have traditionally been used for medical, veterinary and, to a lesser extent, agricultural purposes. Nonetheless, Van Boeckel et al. (2017) reported that more than 73% of all antimicrobials are administered to animals for veterinary or food-producing purposes. The global consumption of antimicrobials in 2013 for food animal production was estimated at an outstanding 131,109 tonnes (Van Boeckel et al., 2017). Moreover, as the demand for animal protein (*e.g.*, livestock products) is increasing globally, the consumption of antimicrobials is projected to rise to 200,235 tonnes by 2030 (Van Boeckel et al., 2017).

Dairy cows are commonly treated with antibiotics for the prevention and treatment of bacterial infections. Relevantly, the antibiotics administered to cows are not fully metabolized and are then excreted, unchanged or as active metabolites, in the animal urine and feces (Sarmah et al., 2006), thus potentially reaching a variety of environmental compartments which can later act as environmental sinks. The amount of antibiotic thus excreted may vary from 30 to 90% of the dose administered (Kumar et al., 2005; Sarmah et al., 2006).

The application of organic amendments of animal origin (*e.g.*, slurry, fresh or composted manure) to agricultural soil as fertilizers is a common practice that can enhance both soil quality and crop yield (Epelde et al., 2018; Urra et al., 2019a, 2020). However, this frequent agricultural practice can also lead to environmental contamination with antibiotics and their transformation products, as well as with ARB harboring ARGs (Chee-Sanford et al., 2009). These emerging contaminants are known to exert a selective pressure on exposed environmental bacteria (Heuer et al., 2008), potentially resulting in the emergence and dissemination of antibiotic resistance in different environmental compartments, with potential risks for human health and ecosystem functioning.

Horizontal gene transfer (HGT) of ARGs is the main mechanism responsible for the spread of antibiotic resistance among bacteria (*e.g.*, for the transfer of antibiotic resistance from environmental

bacteria to pathogenic bacteria) (von Wintersdorff et al., 2016). Horizontal gene transfer is largely mediated by specialized mobile genetic elements (MGEs), such as plasmids, integrative conjugative elements, integrons, phages, transposons, etc., which are involved in intracellular and intercellular gene mobility (Heuer et al., 2011; Jechalke et al., 2014). All these MGEs are collectively known as the mobilome (Gillings, 2013).

In agricultural and livestock settings, it is essential to quantify the extent of the antibiotic resistome, as well as its potential links with the mobilome and pathogenome, across the different animal and environmental microbiomes (*e.g.*, animal dejections: slurry, manure; organically amended soils; crops) (van Hoek et al., 2011; Martínez et al., 2015; Oh et al., 2018; Zhang et al., 2021). Interestingly, the recent advances in next generation sequencing technologies and bioinformatics have revolutionized our capacity to study and understand microbiomes, and, in particular, the resistome, mobilome and pathogenome. Pertinently, the MetaCompare tool is a computational pipeline which can quantify and prioritize the resistome risk, defined as the potential for ARGs to be associated with MGEs and mobilize to potential human pathogens based on metagenomic data, among environmental samples (Oh et al., 2018). In this computational pipeline, each ARG is evaluated based on relative abundance, mobility, and presence within a pathogen (Oh et al., 2018).

The main objective of this study was to survey the antibiotic resistome risk (from metagenomics data on the resistome, mobilome and pathogenome) in different environmental compartments associated with conventional dairy cow farms, as well as to identify genes with potential as resistome risk markers. We hypothesized a reduction of the antibiotic resistome risk from cow slurry and manure microbiomes to soil and vegetable microbiomes (in other words, a reduction of the resistome risk along the studied agricultural pathways: “animal dejections – amended soils – crops”). In particular, we sought to answer the following questions: (1) Which is the resistome risk in the studied environmental compartments from the dairy cow farms?; (2) Which are the main types of ARGs and associated MGE-genes and priority human pathogens in those environmental compartments?; (3) Which are the links, if any, between antibiotic resistance and prokaryotic community composition?; and (4) Which genes, if any, could be used as resistome risk markers, based on their presence in hubs of co-occurrence networks and high dissemination potential?

4.2. Materials and methods

4.2.1. Samplings

In this study, we assessed the antibiotic resistome risk in five conventional cow dairy farms located in the Basque Country (north of Spain). Ten different environmental compartments were sampled in each of the studied farms as follows (Figure 4.5): three compartments associated with productive cows, *i.e.*, fresh slurry, stored slurry, and slurry-amended pasture soil; six compartments related to non-productive heifers and dry cows, *i.e.*, faeces, fresh manure, aged manure, aged manure-amended orchard soil, vegetables (lettuce plants grown in the aged manure-amended orchard soil), and grazed soil from plots where the non-productive heifers and dry cows regularly grazed; and, finally, unamended soil as control soil. Fresh slurry was collected at the inlet of the slurry ponds. Stored slurry was sampled in the middle of the slurry ponds. Faeces were obtained by placing a polyethylene bag in the rectum of non-productive heifers and dry cows at the time of defecation. Fresh manure was collected from the cow beddings (made from faeces, urine and wheat straw). Aged manure was sampled from manure piles that had been stored for 6 months. All soil samples were composed of 20 cores (0-10 cm soil depth) randomly taken and, then, thoroughly mixed. Vegetables (*i.e.*, lettuces) were collected from orchards amended with aged manure. Samples of fresh and stored slurry were collected in polyethylene barrels. The rest of samples were collected in plastic bags. All samples were immediately transferred to the laboratory. Soil samples were sieved to <2 mm. Finally, all samples were stored at 4ºC until use.

4.2.2. Physicochemical properties

The physicochemical properties of the studied soils (*i.e.*, slurry-amended pasture soil, aged manure-amended orchard soil, grazed soil and unamended control soil) and animal dejections (*i.e.*, fresh slurry, stored slurry, faeces, fresh manure and aged manure) were determined according to standard methods (MAPA, 1994). The following parameters were measured: organic matter (OM) content, pH, electrical conductivity (EC), total nitrogen (N), Olsen phosphorus (P), potassium (K^+), cation exchange capacity (CEC), texture, and nitrate (NO_3^-) and ammonium (NH_4^+) content (the last four parameters were measured only in soil samples). Besides, inductively coupled plasma-optical emission spectrometry (ICP-OES) was used for the determination of metal concentrations (cadmium, cobalt, chromium, copper, nickel, lead and zinc) following aqua regia digestion (McGrath & Cunliffe, 1985).

4.2.3. Metagenomics analysis

DNA was extracted in triplicate from soil (0.25 g DW) and animal dejection samples (0.25 g DW) using the Power Soil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). DNA was extracted in triplicate from vegetable samples (lettuces) using the innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). DNA concentration was determined with a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). The extracted DNA was pooled and stored at -20 ºC until use.

Shotgun metagenomic sequencing, including PCR library preparation, was carried out by the Centro Nacional de Análisis Genómico (CNAG-CRG, Spain), using an Illumina NovaSeq6000 with a paired-end 2×150 read length and >20 Gb per sample protocol. Sequence data were submitted to the European Nucleotide Archive under accession number PRJEB46212.

Quality Control of sequenced samples was performed by FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimming by TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were used as input for SqueezeMeta pipeline (Tamames & Puente-Sánchez, 2019) in sequential mode (-m sequential) with a minimum identity of 60% (-miniden 60) using 16 threads (-t 12) and an amount of RAM of 64 Gb (-canumem 64).

The antibiotic resistome risk, defined as the potential for ARGs to be associated with MGE-genes and mobilize to potential human pathogens based on metagenomic data, was calculated using the MetaCompare computational pipeline (Oh et al., 2018). Before being used as input for the Metacompare tool, sequences were preprocessed using MEGAHIT (Li et al., 2015a) for contig assembly and Prodigal (Hyatt et al., 2010) for gene prediction. Assembled scaffolds, derived from the metagenomics reads, were analyzed with BLASTX against CARD, ACLAME and PATRIC databases for ARGs, MGEs, and human pathogen-like sequences, respectively. The number of assembled contigs corresponding to ARGs, MGEs, and human pathogen-like sequences were normalized by the total number of assembled contigs in each sample (Oh et al., 2018).

Furthermore, we closely studied the response of some ARGs of particular interest due to their dissemination potential. These ARGs met the following three criteria: (i) they encode for acquired

antibiotic resistances according to van Hoek et al. (2011); and (ii) they were classified in Rank I (Rank 1 includes those ARGs most at risk of dissemination amongst pathogens) by Zhang et al. (2021); and (iii) they were shown to be associated to MGEs in our study. Finally, priority pathogens associated with plasmids were classified according to their transmissibility following Smillie et al. (2010).

4.2.4. Statistical analysis

Chao1 index was used to estimate the α-diversity (richness) of ARGs and prokaryotic taxa (order level) using reshape2 (Wickham, 2007) and vegan (Oksanen et al., 2015) R packages. Games-Howell test was used for *post hoc* comparisons among the studied environmental compartments regarding their physicochemical properties, Chao1 index values, and the composition of the 30 most abundant prokaryotic orders. Statistical tests were considered significant at $p < 0.05$) with the rstatix package (Kassambara, 2021).

Multiple pie chart visualizations were conducted using dplyr (Wickham et al., 2021), moonBook (Moon, 2015), and webr (Moon, 2020) packages. Principal component analysis (PCA) of the presence/absence of ARGs and prokaryotic composition data were performed using FactoMineR package (Lê et al., 2008), and visualized with ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020) packages.

To visualize co-occurrence patterns between ARGs in the network interface, a correlation matrix was constructed by calculating all possible pairwise Spearman's correlations ($p < 0.05$) between ARGs which occurred in ≥ 50% of the samples, using cooccur (Griffith et al., 2016), reshape2 (Wickham, 2007) and tidyverse (Wickham et al., 2019) packages. This preliminary filtering step removed poorly represented ARGs that were detected in only a limited number of samples and, thus, reduced the artificial association bias (Li et al., 2015b). Network visualization was conducted on Gephi platform (V 0.9.2).

4.3. Results

4.3.1. Antibiotic use in the studied dairy cow farms

In Farm 1, the most common disease treated with antibiotics was mastitis, followed by lameness. In this farm, the most commonly used antibiotics were fluoroquinolones (in 81% of the cases) and β-

lactams (in 78% of the cases) for mastitis and lameness, respectively. In Farm 2, the main disease treated with antibiotics was mastitis, followed by metritis: β-lactams and fluoroquinolones were administered in 49% of the mastitis cases, and β-lactams in 81% of the metritis cases. In Farm 3, the main disease treated with antibiotics was lameness, followed by mastitis: β-lactams were used in 88% of the lameness cases, and fluoroquinolones in 94% of the mastitis cases. In Farm 4, the most common disease treated with antibiotics was lameness, followed by other unspecified diseases: β-lactams were administered in 57% of the lameness cases, and tetracyclines in 53% of the unspecified disease cases. We did not have access to information about diseases and antibiotic use in Farm 5.

4.3.2 Physicochemical properties of the environmental compartments

Regarding soil physicochemical properties, the following average values were found for slurry-amended pasture soil, aged manure-amended orchard soil, grazed soil, and unamended control soil, respectively (Supplementary Table 4.1): organic matter content = 6.8, 11.5, 9.3 and 9.6%; cation exchange capacity = 22.2, 30.2, 26.7 and 27.4 mEq 100 g^{-1}; pH = 6.8, 6.4, 6.9 and 7.1; and C/N = 8.0, 7.9, 7.6 and 8.3. No statistically significant differences were found among the studied soils.

In relation to the physicochemical properties of the animal dejections (Supplementary Table 4.2), fresh manure had a higher organic C content than faeces, as well as a higher dry matter content than fresh slurry. No other statistically significant differences were found among the animal dejections.

4.3.3 Which are the main types of ARGs and associated MGE-genes and priority human pathogens in the studied environmental compartments?

In total (sum of all the environmental compartments), 18,157 ARG subtypes, conferring resistance across 23 classes of antibiotics, were annotated (Fig. 4.1A). A considerable percentage of the ARGs detected in fresh slurry and stored slurry were associated to ansamycin (*e.g.*, rifamycin) (25 and 21%, respectively) and tetracycline (17 and 18%, respectively) resistance through the following mechanisms: antibiotic target alteration and replacement for ansamycin resistance, and antibiotic target protection for tetracycline resistance. Similarly, a substantial percentage of the ARGs found in faeces was related to ansamycin (25%) and β-lactam (20%) resistance, via antibiotic target alteration and replacement for ansamycin resistance, and inactivation for β-lactam resistance. A large percentage of the ARGs found in fresh manure corresponded to multidrug (resistance against, at least, two classes of antibiotic; 25%) and aminoglycoside (21%) resistance, via antibiotic efflux for multidrug resistance and inactivation for

aminoglycoside resistance. In aged manure, an important percentage of the detected ARGs referred to multidrug (33%) and ansamycin (21%) resistance through antibiotic efflux for multidrug resistance, and antibiotic target alteration and replacement for ansamycin resistance. A high percentage of the ARGs found in grazed soil and slurry-amended pasture soil was connected with ansamycin resistance (51 and 55%, respectively) via antibiotic target alteration and replacement. In unamended control soil and aged manure-amended orchard soil, a significant percentage of the observed ARGs pertained to ansamycin (45 and 52%, respectively) and multidrug (25 and 23%, respectively) resistance via antibiotic target alteration and replacement for ansamycin resistance, and efflux for multidrug resistance. Finally, in vegetables (lettuce plants grown in the aged manure-amended orchard soil), a high percentage of the ARGs referred to multidrug resistance (48%) through antibiotic efflux.

Among the 18,157 annotated ARGs, 1,031 unique ARG subtypes (*i.e.*, those with a single antibiotic resistance ontology number assigned by CARD, removing from the database the duplicate sequences for each resistance gene in every environmental compartment) were detected (Fig. 4.1B). In fresh slurry, faeces, fresh manure, and aged manure, the unique ARG subtype corresponded to β-lactam (28, 60, 47 and 29%, respectively) resistance, mainly via antibiotic inactivation. In stored slurry, the unique ARG subtypes were linked to other (35%) and β-lactam (24%) resistance through target protection and inactivation, respectively. Finally, multidrug resistance was the main unique ARG subtype in slurry-amended pasture soil, aged manure-amended orchard soil, grazed soil, unamended control soil, and vegetables (36, 38, 38, 41 and 55%, respectively), in all cases via antibiotic efflux. These multidrug ARGs showed 83, 87, 83 and 87% resistance to at least one of the most commonly used antibiotics (*i.e.*, β-lactams, fluoroquinolones and tetracyclines) on Farm 1, Farm 2, Farm 3 and Farm 4, respectively (Supplementary Table S3, see online).

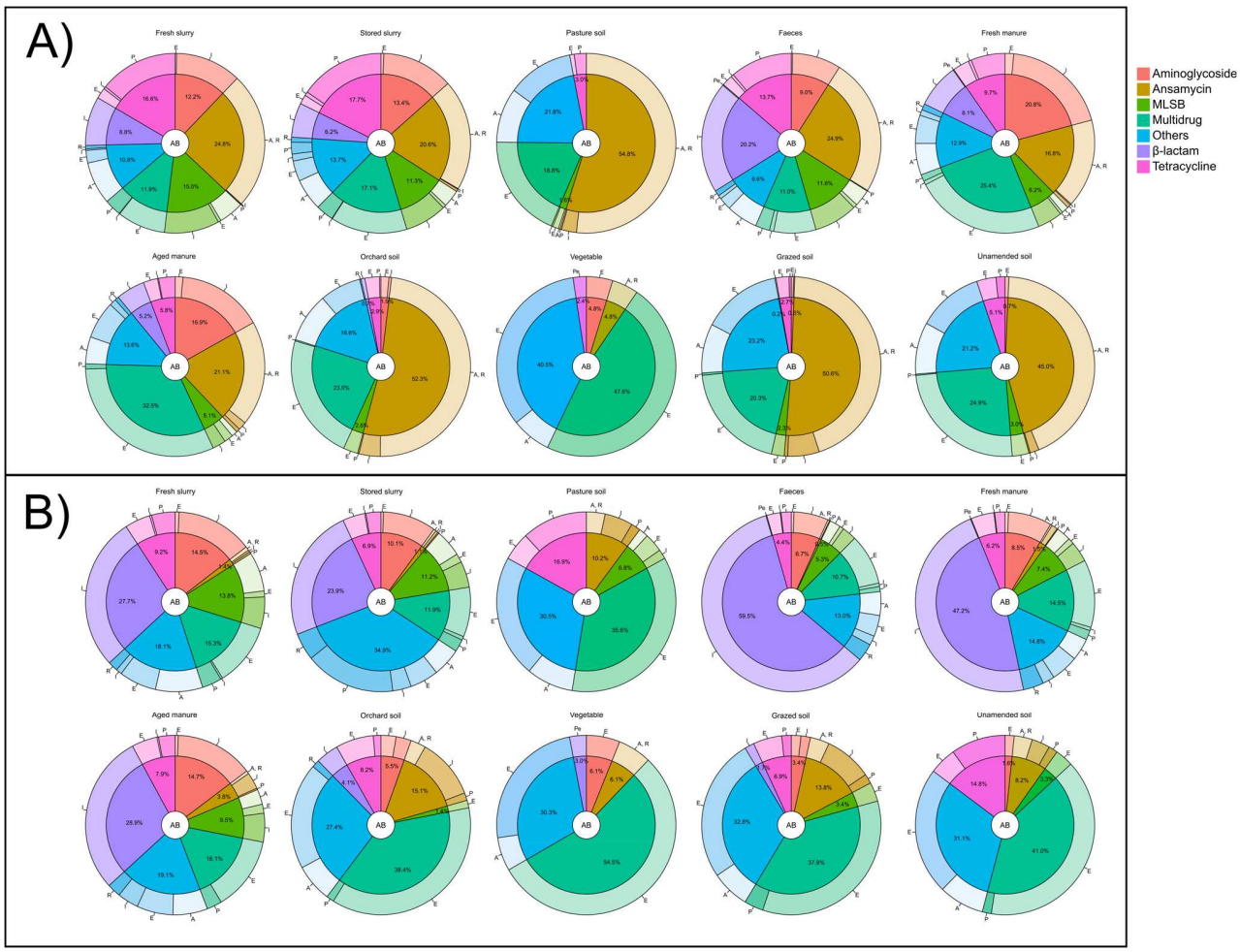


Figure 4.1. Profile of (A) total and (B) unique ARGs and mechanism of resistance annotated along the studied environmental compartments. A: antibiotic target alteration; E: antibiotic efflux; I: antibiotic inactivation; P: antibiotic target protection; Pe: reduced permeability to antibiotic; R: antibiotic target replacement. MLSB: macrolide-lincosamide-streptogramins B. "Others" included the following antibiotics: acridine dye; aminocoumarin; antibacterial free fatty acids; bicyclomycin: diaminopyrimidine; disinfecting agents and intercalating dyes; fluoroquinolone; fosfomycin; mupirocin; nitroimidazole; nucleoside; peptide; phenicol; pleuromutilin; sulfonamide, triclosan.

Figure 4.2 shows those ARGs associated to MGE-like gene sequences which were detected in the studied environmental compartments (which will be called hereinafter as MGE-associated ARGs). In animal dejections (Fig. 4.2A), the main MGE-associated ARG type referred to aminoglycoside resistance (from 32% in faeces to 70% in aged manure). Regarding unique ARGs associated to MGE-like gene sequences (Fig. 4.2B), aminoglycoside resistance was the most abundant in fresh slurry, stored slurry, and aged manure (30, 26 and 35%, respectively), while the most abundant in faeces and fresh manure was β-lactam (56 and 55%, respectively) resistance. The most common mechanism of resistance was antibiotic inactivation. No ARGs associated to MGE-like gene sequences were detected

in soil samples, except for two MGE-associated ARGs that were detected in aged manure-amended orchard soil.

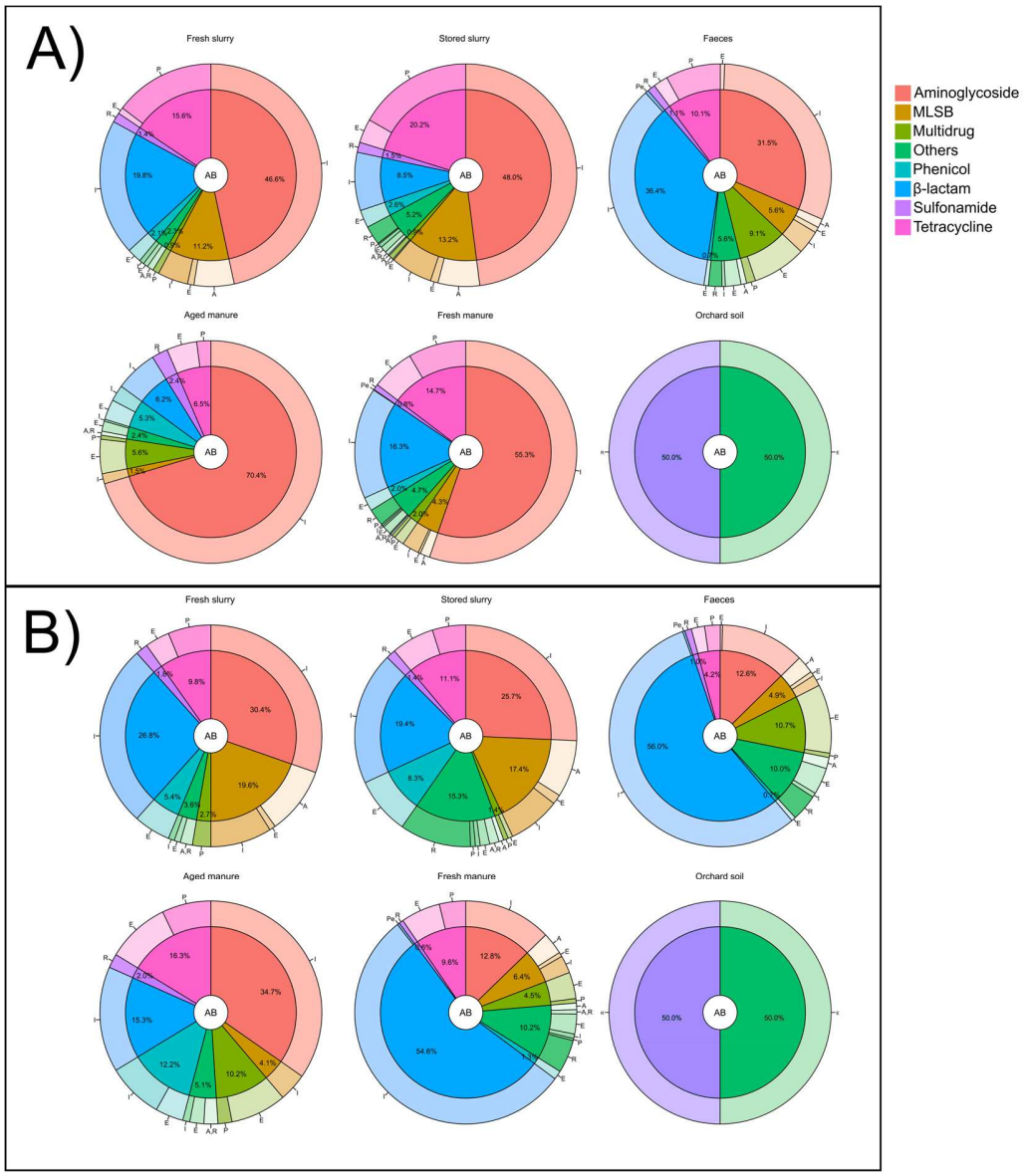


Figure 4.2. Profile of (A) total and (B) unique MGE-associated ARGs and mechanism of resistance annotated along the studied environmental compartments. Only those environmental compartments in which ARGs and associated MGE-genes were detected are shown. A: antibiotic target alteration; E: antibiotic efflux; I: antibiotic inactivation; P: antibiotic target protection; Pe: reduced permeability to antibiotic; R: antibiotic target replacement. MLSB: macrolide-lincosamide-streptogramins B. "Others" included the following antibiotics: acridine dye; aminocoumarin; ansamycin; diaminopyrimidine; disinfecting agents and intercalating dyes; fluoroquinolone; fosfomycin; nitroimidazole; nucleoside; peptide.

Figure 4.3A shows the distribution of samples, according to the presence/absence of ARGs, in a principal component analysis (PCA). Animal dejection samples appeared spatially much more dispersed than soil and vegetable samples. The PCA separated soils and vegetables from fresh slurry, stored slurry, and fresh manure. Chao1 index was calculated to estimate the ARG richness of each environmental compartment (Fig. 4.4A). Animal dejections tended to show higher Chao1 values than soil and vegetable samples. However, when performing multiple pairwise comparisons, no statistically significant differences among environmental compartments were detected.

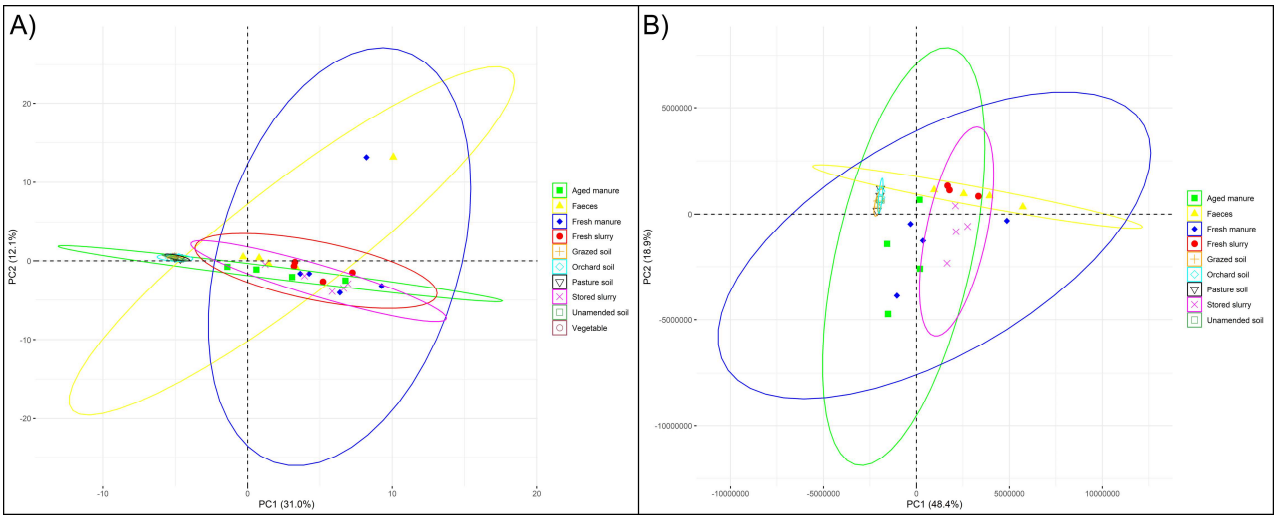


Figure 4.3. Biplot of principal component analysis of (A) presence/absence of ARGs and (B) prokaryotic community composition along the studied environmental compartments.

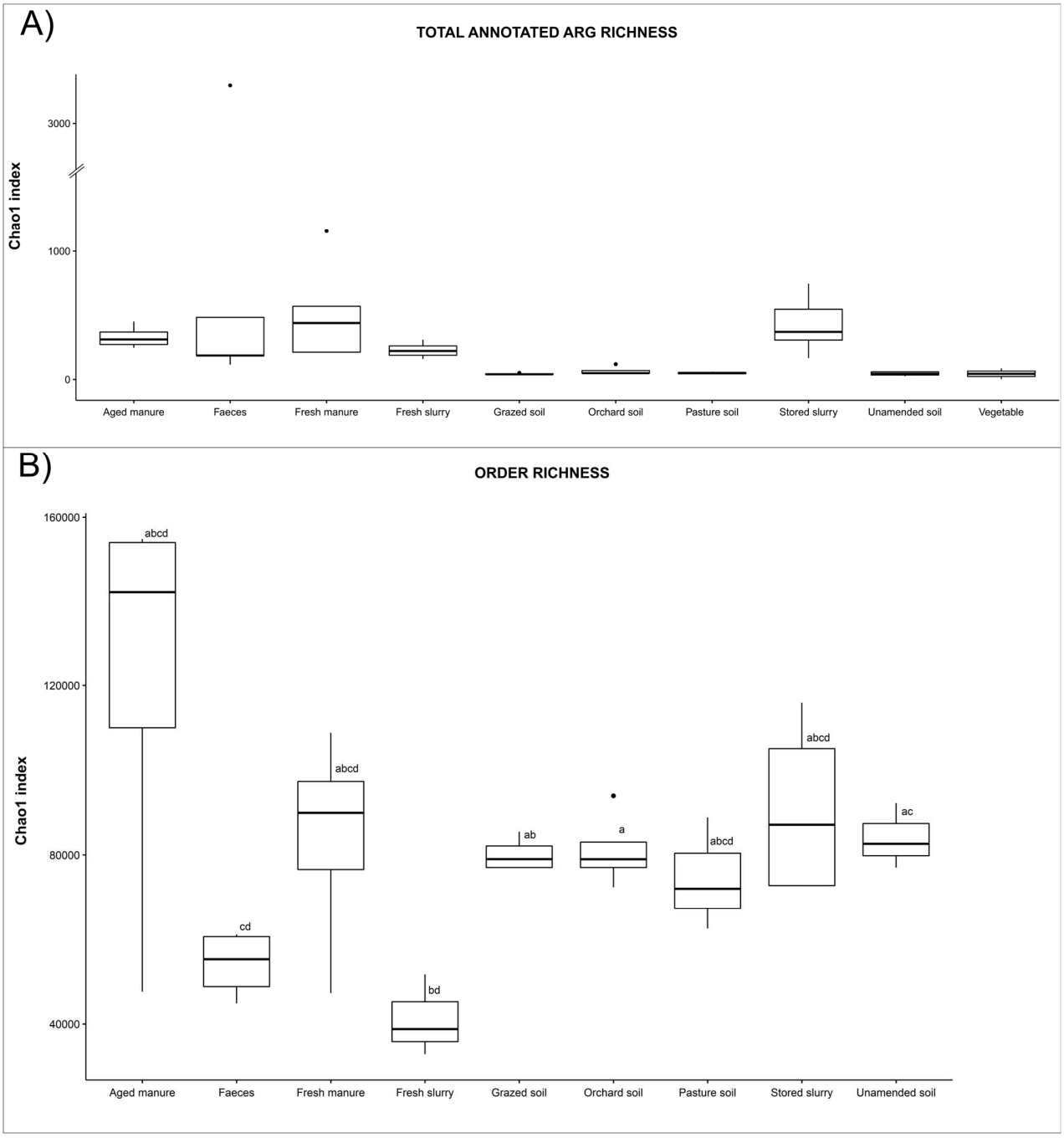


Figure 4.4. Chao1 index of (A) ARGs and (B) prokaryotic orders along the studied environmental compartments. Values with different letters are significantly ($p < 0.05$) different according to Games-Howell test.

From the ACLAME database, we selected those MGE-associated ARGs that were linked, as the highest identity (Supplementary Table S4, see online), to a pathogen included in the WHO priority list (Tacconelli et al., 2018). Among the pathogens classified as critical, the plasmid pRAY, associated

with multiple aminoglycoside resistance genes, was detected in *Acinetobacter sp. SUN*. In *Pseudomonas aeruginosa*, plasmids pBS228, pMATVIM-7 and Rms149 were identified. These three plasmids were associated to a multiresistant profile in at least one annotated contig. The most common ARG detected in *P. aeruginosa* was *ANT(3'')-IIa* (multiple resistances to carbapenem were also found). In *Klebsiella pneumoniae,* the most prevalent plasmids were pKPN4, pKPN5 and pJHCMW1. These plasmids were associated to cephalosporin and carbapenem resistance. The most abundant ARGs detected in *K. pneumoniae* were *aadA15* and *ANT(3'')-IIa.* Regarding *Escherichia coli*, the most common plasmids detected here were pMUR050, pLEW517, p1658/97 and NR1. Plasmid pMUR050 was related to MLSB (macrolide-lincosamide-streptogramin B) resistance. The other three plasmids were associated to a multiresistant profile in at least one annotated contig and, in addition, to cephalosporin and carbapenem resistances. The most common ARG detected in *E. coli* was *ANT(3'')-IIa.* In *Enterobacter* spp., the most abundant plasmid was R751, followed by pENTE01. Both plasmids showed a multiresistant profile in at least one annotated contig and, in addition, cephalosporin and carbapenem resistance. The most prevalent ARGs detected in *Enterobacter* spp. were *aadA15* and *ANT(3'')-IIa.* Plasmids R478 and Rts1 were detected in *Serratia* spp. and *Proteus* spp., respectively. Both plasmids presented multiresistance in at least one annotated contig. The most common ARG detected in *Serratia* spp. was *qacH*. In *Enterococcus faecium*, the most prevalent plasmid was pRUM. No resistance to vancomycin was detected in *E. faecium*. The most common ARGs in *E. faecium* were *aad(6)* and *ANT(6)-Ib*. In *Staphylococcus aureus,* the most prevalent plasmids were pT48, pE194 and pWBG738, associated to resistance to multiple MLSB. In *Campylobacter*, plasmids pTet and pCC31 were detected, both associated with multiple tetracycline resistance genes. Regarding *Salmonella* spp., the most common plasmids were pSC138 and R46. These plasmids were associated to multiresistant profile in at least one annotated contig. The most prevalent ARGs associated to *Salmonella* spp. were *aadA15* and *ANT(3'')-IIa*. Finally, in *Shigella* spp., a pathogen classified with medium priority, the most abundant plasmids were R100 and pSS046_spA. Plasmid pCP301, one of the least common plasmids in *Shigella* spp., was associated with fluoroquinolone resistance. The most prevalent ARGs in *Shigella* spp. were *aadA15* and *ANT(3'')-IIa*.

Regarding the classification of MGEs according to their transmissibility (conjugative, mobilizable and non-mobilizable) (Smillie *et al.*, 2010), the most common MGEs detected in *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *Enterobater* sp., *Serratia* sp., *Proteus* sp., *Campylobacter* and

Shigella sp. were conjugative plasmids (Supplementary Table S5, see online). By contrast, mobilizable plasmids were the most common MGEs in *Acinetobacter* sp. and *E. faecium*.

4.3.4 Which is the resistome risk in the studied environmental compartments from the dairy cow farms?

Resistome risk scores along the studied environmental compartments (Fig. 4.5, Supplementary Table 1.6) were obtained with the MetaCompare computational pipeline and reflects the potential for ARGs to be associated with MGEs and mobilize to potential human pathogens. Overall, the following resistome risk gradient was observed: animal dejections > soils > plants; in other words, a decreasing trend from cow slurry and manure microbiomes to soil and vegetable microbiomes (*i.e.*, a reduction along the studied agricultural pathways). Fresh manure (33.2^{ab}; values with different letters are significantly different according to Games-Howell test) showed the highest resistome risk, followed by fresh slurry (31.5^{abc}), stored slurry (31.1^{a}), faeces (30.9^{abcd}), and aged manure (29.4^{abcd}). On the contrary, vegetables-lettuces (17.7^{d}) showed the lowest resistome risk, followed by aged manure-amended orchard soil (24.7^{c}), grazed soil (25.5^{bc}), unamended control soil (26.2^{abcd}), and, finally, slurry-amended pasture soil (26.8^{abc}). Then, stored slurry had a significantly higher resistome risk than vegetables, orchard soil and grazed soil. Instead, the risk score of vegetables was lower than fresh manure, both fresh and stored slurry, orchard soil, grazed soil and pasture soil. Besides, orchard soil risk was lower than that of fresh manure and stored slurry.

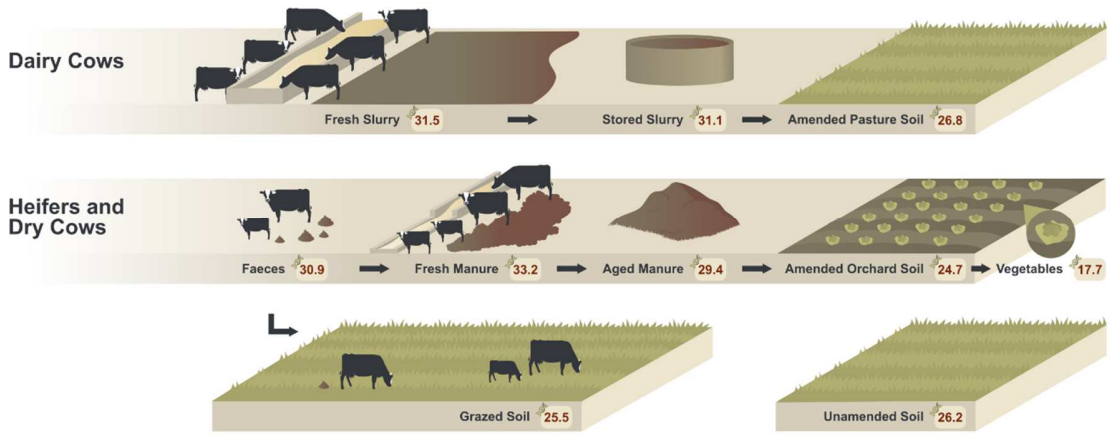


Figure 4.5. Resistome risk scores for the 10 studied environmental compartments. The dairy cows pathway is associated to productive cows, while the heifers and dry cows pathway is associated to non-

productive cows; the unamended soil was used as a control. The values are mean risk scores from the five cow dairy farms assessed.

4.3.5 Which are the links, if any, between antibiotic resistance and prokaryotic community composition?

Regarding the composition of the prokaryotic communities present in the environmental compartments, Figure 4.3B shows the PCA spatial distribution of all the samples based on order-level relative abundances. As abovementioned for ARGs data (Fig. 4.4A), animal dejection samples appeared more dispersed than soil samples. The PCA separated all soils from both fresh and stored slurry. We found 98 common prokaryotic orders out of a total of 1,024 orders identified in all the environmental compartments. Supplementary Figure 4.1 shows the relative abundance of the 30 most abundant prokaryotic orders. The most abundant prokaryotic order in fresh slurry, stored slurry and faeces was Clostridiales. Faeces had a higher relative abundance of Bacteroidales and Clostridiales compared to aged manure and soils (Supplementary Table 7, see online). The most abundant taxon in soil samples corresponded to unclassified Actinobacteria, whose relative abundance was significantly higher in aged manure-amended orchard soil and grazed soil than in fresh slurry, stored slurry, faeces and fresh manure. Aged manure-amended orchard soil showed a higher relative abundance of Rhizobiales than all the other animal dejections.

Finally, the Chao1 index was significantly lower in faeces than in aged manure-amended orchard soil and grazed soil (Fig. 4.4B). This index was significantly lower in fresh slurry compared to aged manure-amended orchard soil and unamended control soil.

4.3.6 Which genes, if any, could be used as resistome risk markers, based on their presence in hubs of co-occurrence networks and high dissemination potential?

A first network analysis was conducted to explore co-occurrence patterns among ARGs (Fig. 4.6A). The constructed network consists of 31 nodes (ARG subtypes), 170 edges, a modularity of 0.274 and a degree of 10.97. The most densely connected nodes in each module were considered “hubs”. Among the 31 ARGs that were detected in at least half of the samples and presented significant co-occurrence with at least another ARG, 15 corresponded to multidrug resistance. Module I (48%) comprised 15 ARGs and *efrA* was its hub gene with direct connections to 19 ARGs. The *efrA* was detected in 56% of all the individual samples collected from each environmental compartment (Supplementary Table

8, see online). The *efrA* gene was not detected in slurry-amended pasture soil, vegetables or grazed soil. In Module I, the *tet(W/N/W)* gene, with 16 connections, was detected in 56% of all the individual samples collected from each environmental compartment (it was not observed in aged manure-amended orchard soil, vegetables, grazed soils and unamended control soil). We also included *tet(W/N/W)* gene in our screening because it was one of the hubs in the MGE-associated ARGs network (see below) (Fig. 4.6B). Module II (32%) comprised 10 ARGs and *mexK* (the inner membrane resistance-nodulation-cell division transporter in the MexJK multidrug efflux protein) was its hub gene with 18 ARGs connected. This gene was detected in 56% of all the individual samples collected from each environmental compartment (it was not found in slurry-amended pasture soil and vegetables). Module III (13%) included 4 ARGs and *MexF* (the multidrug inner membrane transporter of the MexEF-OprN complex) was its hub gene with connections to 6 ARGs. The *MexF* gene was detected in 56% of all the individual samples collected from each environmental compartment (it was not detected in fresh slurry). Module IV (7%) was composed of only two ARGs, *rpoB* (according to CARD, *rpoB* mutant conferring resistance to rifampicin) and *rpoB2* (they co-occurred exclusively with each other). Both genes were detected in 97% of all the individual samples collected from each environmental compartment and in all the environmental compartments studied here.

A second network analysis was performed to identify potential co-occurrence patterns among annotated MGE-associated ARGs (Fig. 4.6B). The constructed network consists of 49 nodes, 567 edges, a modularity of 0.317 and a degree of 23.143. Among the 49 ARGs that were detected in at least half of the samples and presented a significant co-occurrence with at least another ARG, 25 corresponded to aminoglycoside resistance (Supplementary Table 9, see online). Module I (61%) included 30 ARGs related to aminoglycoside, phenicol and sulfonamide resistance. The *aad(6)* and *ANT(6)-Ib* genes (its hub genes with 30 ARGs connected) were detected in 83% of all the individual samples collected from each environmental compartment. The *aad(6)* and *ANT(6)-Ib* genes were only detected in animal dejections. Module II (39%) consisted of 19 ARGs. The hub genes of Module II were *tet(W/N/W)*, *tet32*, *tet44*, *tetM*, *tetO*, *tetS* and *tetW*. These ARGs showed 18 significant connections and were detected in 83% of all the individual samples collected from each environmental compartment. Again, these hub genes were only observed in animal dejections.

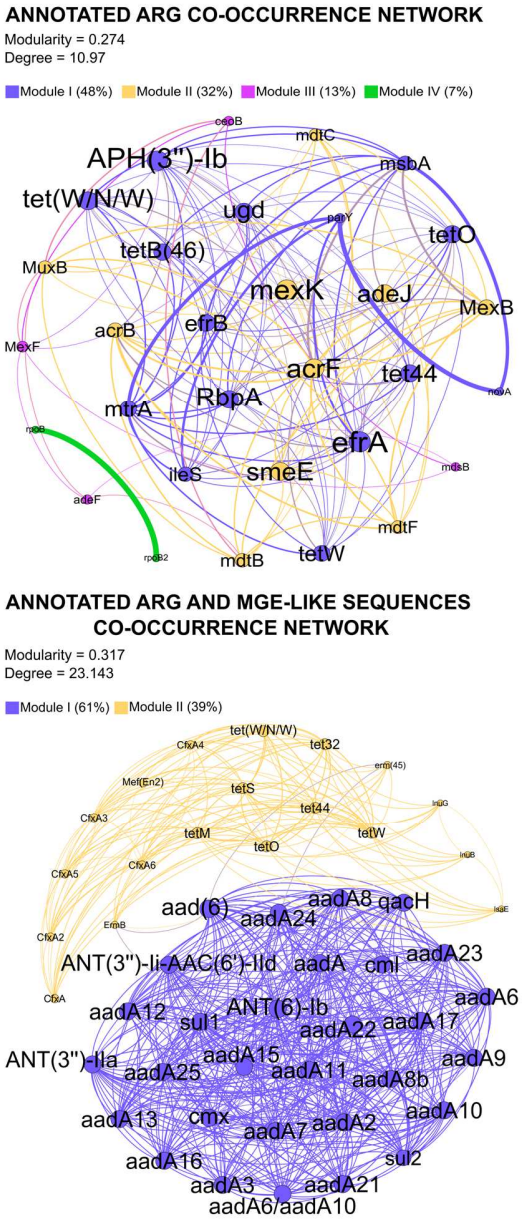


Figure 4.6. Network analysis based on (A) ARGs co-occurrence and (B) MGE-associated ARGs. Node size is proportional to the number of connections. An edge represents a significant correlation, where edge thickness is proportional to the Spearman's correlation coefficient.

In addition to hub ARGs, we searched for high dissemination potential ARGs. Supplementary Table 4.10 shows the detection percentages of ARGs that met our high dissemination potential criteria (see below): one related to aminoglycoside (*aad(6)*) resistance, two to phenicol (*cmlA*, *floR*) resistance (*e.g.*, chloramphenicol), three to tetracycline (*tetM*, *tetO*, *tetW*) resistance, and six to diaminopyrimidine (*dfrA1*, *dfrA14*, *dfrA15*, *dfrA17*, *dfrA25*, *dfrA5*) resistance (*e.g.*, trimethoprim). In

at least one of the subsamples collected from stored slurry and fresh manure, all these 12 ARGs were detected, except for *cmlA* in fresh manure.

Based on these results, we propose a list of ARGs with potential as resistome risk markers (based on their presence in hubs of co-occurrence networks and high dissemination potential) for them to be closely monitored in future studies (Table 4.1).

Table 4.1. Antibiotic resistance genes (ARGs) with potential as resistome risk markers, based on their presence in hubs of co-occurrence networks and high dissemination potential.

ARG	Antibiotic	Mechanism of resistance	Reason for selection
aad(6)	Aminoglycoside	Antibiotic inactivation	Co-occurrence among MGE-associated ARGs, high dissemination potential
ANT(6)-Ib	Aminoglycoside	Antibiotic inactivation	Co-occurrence among MGE-associated ARGs
cmlA	Phenicol	Antibiotic efflux	High dissemination potential
dfrA1	Diaminopyrimidine	Antibiotic target replacement	High dissemination potential
dfrA14	Diaminopyrimidine	Antibiotic target replacement	High dissemination potential
dfrA15	Diaminopyrimidine	Antibiotic target replacement	High dissemination potential
dfrA17	Diaminopyrimidine	Antibiotic target replacement	High dissemination potential
dfrA25	Diaminopyrimidine	Antibiotic target replacement	High dissemination potential
dfrA5	Diaminopyrimidine	Antibiotic target replacement	High dissemination potential
efrA	Multidrug	Antibiotic efflux	Co-occurrence
floR	Phenicol	Antibiotic efflux	High dissemination potential
MexF	Multidrug	Antibiotic efflux	Co-occurrence
mexK	Multidrug	Antibiotic efflux	Co-occurrence
rpoB	Ansamycin	Antibiotic target alteration, target replacement	Co-occurrence
rpoB2	Ansamycin	Antibiotic target alteration, target replacement	Co-occurrence
tet(W/N/W)	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs
tet32	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs
tet44	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs
tetM	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs, high dissemination potential
tetO	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs
tetS	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs
tetW	Tetracycline	Antibiotic target protection	Co-occurrence among MGE-associated ARGs, high dissemination potential

4.4. Discussion

4.4.1 Which is the resistome risk in the studied environmental compartments from the dairy cow farms?

In this study, we characterized the resistome, mobilome and pathogenome of ten environmental compartments from five dairy cow farms using a metagenomic approach. Since a great percentage of

the antibiotics used worldwide are administered to livestock, it is critical to better understand the pathways of dissemination of antibiotic resistance within and from livestock and agricultural settings. In our dairy cow farms, the dejections from productive cows generate slurry, while those from non-productive heifers and dry cows are mixed with wheat straw and then generate manure. After being stored (*e.g.*, stored slurry and aged manure), these animal subproducts are often used as organic amendments due to their potential benefits for soil quality and crop yield (Epelde et al., 2018). Here, the stored slurry was applied to pasture soil used to produce fodder for feeding dairy cows. Six-month aged manure was applied to orchard soil to produce vegetables (lettuce plants) for human consumption. As indicated above, in general, the following resistome risk gradient was detected, by means of using the MetaCompare computational pipeline, along the environmental compartments studied here: animal dejections > soils > vegetables.

It is well-known fact that cow slurry and manure can be reservoirs of antibiotic resistance (Fahrenfeld et al., 2014; Udikovic-Kolic et al., 2014; Jauregi et al., 2021a). In our study, in both exposure pathways (*i.e.*, those associated to productive and non-productive cows), fresh dejections (*i.e.*, fresh slurry, faeces and fresh manure) tended to present a higher resistome risk than stored dejections (*i.e.*, stored slurry and aged manure). The tendency of ARG abundances to increase or decrease during the storage and/or composting of these animal subproducts appears to be highly gene-specific (Xu et al., 2018). The reduction of ARG abundances observed in some composting processes may be the result of the elimination of ARG-bearing bacteria during the aerobic thermophilic stage (Wang et al., 2015a).

Our resistome risk score for stored slurry (31.1) was higher than the one reported by Oh et al. (2018) for dairy lagoons (between 22.7 and 29.0). The aged manure-amended orchard soil risk (24.7) was similar to that reported for antibiotic-manure-amended soils after 120 of amendment application (around 25) by Chen et al. (2019b). Our unamended control soil presented a slightly higher risk score (26.3) compared to that reported by Chen et al. (2019b) (20-25). In any case, it is important to emphasize that, to properly interpret these values, it is key to establish background and baseline antibiotic resistance levels for agroecosystems (Rothrock et al., 2016).

Vegetables were the last environmental compartment in the non-productive cows' pathway and the expected decrease in the resistome risk was observed. Still, previous studies indicate that soils and

organic amendments are important sources of vegetable resistome (Chen et al., 2017a; Zhu et al., 2017b). There are many potential pathways through which ARGs and ARB can be transferred from amended soils to plants: surviving as phyllosphere microorganisms, colonizing leaf tissues as foliar endophytes, or transferring to vegetable roots as root endophytes (Zhang et al., 2019a).

4.4.2 Which are the main types of ARGs and associated MGE-genes and priority human pathogens in the studied environmental compartments?

Farmers reported that the most commonly used antibiotics were β-lactams, fluoroquinolones and tetracyclines (the latter only on Farm 4). Remarkably, although farmers did not report the use of rifamycin to treat common diseases, in fresh slurry, stored slurry, faeces and all the studied soils, ansamycin was the main resistance profile in terms of total annotated ARGs. As an example of the different mechanisms associated to ansamycin resistance, mutations in *rpoB* prevent rifamycin from binding to *rpoB* and then the bactericidal activity of rifamycin is lost (rifamycin is an ansamycin subclass antibiotic that binds to the β subunit of RNA polymerase -RNAP, *rpoB*- and blocks RNA synthesis) (Ning et al., 2021). Module IV in our first network analysis was composed of only two ARGs, *rpoB* and *rpoB2*. On the other hand, in accordance with farmers' reports, the most abundant unique ARG profile detected in fresh slurry, faeces, fresh manure and aged manure was β-lactam resistance. However, here it must be taken into consideration that existing ARG databases (*e.g.*, the CARD database used in this study) are biased towards β-lactam resistances, mainly those related to human infections (Gil-Gil et al., 2021).

The main MGE-associated ARGs found in our animal dejections were related to aminoglycoside and β-lactam resistance. The use of β-lactams is very common in the studied dairy cow farms. By contrast, during the interviews, the farmers did not report the common use of aminoglycosides. In general, we can say that the use of antibiotics on the different farms did not impact on the observed ARG patterns. Nonetheless, an enrichment in the abundance of specific ARGs is often observed in the absence of the corresponding antibiotics through co-selection of resistance genes and/or selection of a microbiome portion containing those resistance genes (Rovira et al., 2019).

In terms of unique ARGs, the resistomes observed here for soils and vegetables (lettuces) were mostly constituted of multidrug ARGs, in accordance with previous metagenomic studies (Chen et al., 2019b; Wind et al., 2021). Multidrug efflux pump mechanisms can be involved in multiple bacterial

functions (Piddock, 2006), not just antibiotic efflux. In our study, the multidrug ARGs from soil and vegetable samples were not associated with MGE-genes, suggesting that such genes might correspond to intrinsic resistances or play other primary functions (Berglund, 2015). Interestingly, it is a well-known fact that soil microbial communities from environments that have been completely isolated from the impact of anthropogenic activity can harbor multiple ARGs (D'Costa et al., 2006).

Horizontal gene transfer is most often responsible for the enrichment of ARGs in environmental compartments and, hence, for the spread of antibiotic resistance. Plasmid conjugation is thought to be prevalent in complex microbial communities, such as animal gut and soil microbiomes (Ogilvie et al., 2012). Although harboring a plasmid can reduce bacterial host fitness, compensatory mutations may alleviate plasmid fitness costs (Andersson & Hughes, 2010). In our study, the vast majority of MGE-associated ARGs were found in animal dejections. These MGE-associated ARGs were not transferred to soils or vegetables, which is certainly a positive finding from a human exposure point of view. Among all the soil and vegetable samples analyzed in our study, only two MGE-associated ARGs were detected in aged manure-amended orchard soil. Soils might alleviate the mobility of animal dejection-derived antibiotic resistance (Rieke et al., 2018). Importantly, several bottlenecks have been proposed for the ARG transfer from environmental bacteria to human pathogens, such as ecological connectivity, founder effect, fitness costs, and maintenance of the resistance in the absence of selection pressure (Martínez, 2012). On the other hand, the risk that an ARG can pose for human health depends, among other factors (including its genetic context) (Zhang et al., 2021), on the specific bacterial strain in which it is present. Although we have only broadly estimated the occurrence of pathogens (from information on the most likely hosts of the identified MGE-associated ARGs), interestingly, all the pathogens included in the WHO priority list were detected (most of them showed aminoglycoside resistance).

Mobile genetic elements associated with priority pathogens were classified here in three categories according to their transmissibility: conjugative, mobilizable and non-mobilizable (Smillie et al., 2010). The most common MGEs detected in *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *Enterobater* sp., *Serratia* sp., *Proteus* sp., *Campylobacter* and *Shigella* sp. were conjugative plasmids. Conjugative plasmids encode a set of mobility genes as well as a mating pair formation (MPF) complex (Smillie et al., 2010), while type IV secretion systems provide a mating channel for plasmid transfer from one bacterium to another (Cascales & Christie, 2003; Álvarez-Rodríguez et al., 2020). On the contrary, mobilizable plasmids were the most common MGEs found in *Acinetobacter* sp. and *E. faecium*.

Mobilizable plasmids are not self-transmissible, but they use the MPF complex of another genetic element (Smillie et al., 2010). Both conjugative and mobilizable plasmids present a broader host range (BHR) than non-transmissible plasmids (Redondo-Salvo et al., 2020). Since some BHR plasmids can be transferred between different bacterial phyla and even domains of life, the possible dissemination of BHR plasmids is of special concern (Waters, 2001).

The Chao1 index has been recommended, based on studies on large metagenomic datasets from multiple environments, for estimating ARG diversity (Lal Gupta et al., 2020). We found higher resistome diversity in animal dejections compared to soils and vegetables, as previously reported (Li et al., 2015b; Jauregi et al., 2021a; Macedo et al., 2021). Prior to fecal excretion, ARGs can be horizontally transfer between different bacterial species within the animal gut. In fact, animal guts (and in particular, livestock guts) are considered important reservoirs of ARGs and MGE-genes (Thanner et al., 2016).

4.4.3 Which are the links, if any, between antibiotic resistance and prokaryotic community composition?

The PCA spatial distribution of ARGs and prokaryotic orders was very similar, suggesting their close relatedness. Some environmental compartments, such as fresh manure, were spatially more dispersed because they presented a high diversity of both ARGs and prokaryotic orders. Instead, the studied soils showed a low diversity of ARGs but a relatively high prokaryotic diversity at order level.

Clostridiales was the most abundant taxon in fresh slurry, stored slurry and faeces. Clostridiales is a common colonizer of the intestinal tract of animals and, then, often found in faeces (Lima et al., 2020). In fact, Clostridiales is very commonly detected in animal manure (Peng et al., 2018; Gao et al., 2020). On the other hand, soil prokaryotic communities were dominated by Actinobacteria, which are dominant in soil ecosystems worldwide (Hill et al., 2011; Fierer et al., 2013). Actinobacteria are known for their capacity to produce multiple secondary metabolites, such as antibiotics, which can play an important role in regulating the fate of microbial invading species in the soil ecosystem (van Elsas et al., 2012; Forsberg et al., 2014). Rhizobiales, well-studied associates of plants (Erlacher et al., 2015), were also abundant in our soils.

4.4.4 Which genes, if any, could be used as resistome risk markers, based on their presence in hubs of co-occurrence networks and high dissemination potential?

A significant co-occurrence between multiple ARGs was observed in the studied environmental compartments. From the information obtained in our network analyses, we estimate that the hub genes from each module can act as ARG proxies, thus being useful as representatives of other co-occurring ARGs. Regarding the ARG co-occurrence network (first network analysis), the following hub genes were identified: *efrA*, *mexK*, *MexF* and *rpoB* and *rpoB2*. Using a similar metagenomic approach, the multidrug gene *mexK* was also detected in faeces and soil samples from another dairy production system (Rovira et al., 2019). Likewise, Li et al. (2020a) reported the multidrug encoding *MexF* ARG as an indicator of 283 co-occurring ARGs across 72 black soils in China. When genes that make up the Mex superfamily are overexpressed in pathogenic bacteria such as *C. jejuni*, *E. coli*, *P. aeruginosa*, and *S. typhimurium*, they could export multiple antibiotics and become resistance genes (Piddock, 2006). Interestingly, *rpoB* and *rpoB2* genes encode the β subunit of bacterial RNAP. Given the high degree of conservation of RNAP, resistance-determining mutations in these genes are also expected to be conserved between bacterial species (Goldstein, 2014). In fact, in our study, both *rpoB* and *rpoB2* genes were detected in all the environmental compartments. Module IV was exclusively composed of *rpoB* and *rpoB2* genes. All these housekeeping genes (*i.e.*, *MexF*, *mexK*, *rpoB* and *rpoB2*) could confer resistance when they are "decontextualized" by mobilization (Dantas & Sommer, 2012; Martínez et al., 2015; Blanco et al., 2016).

Instead, the hub genes from the network analysis carried out with MGE-associated ARGs (second network analysis) can be used as representatives of potentially mobile ARGs. In this second co-occurrence network, *aad(6)* and *ANT(6)-Ib* genes showed the same indicator potential for Module I. The plasmid-encoded aminoglycoside nucleotidyltransferase, *aad(6)*, gene was identified as the top 50% ARG found in swine wastewater (He et al., 2019). The *ANT(6)-Ib* gene has frequently been found to be involved in streptomycin resistance in *Campylobacter* strains (Hormeño et al., 2018; Ocejo et al., 2021). The *tet(W/N/W)*, *tet32*, *tet44*, *tetM*, *tetO*, *tetS* and *tetW* tetracycline resistance genes were hubs of Module II. In 2018, tetracyclines accounted for 31% of the total sales of antimicrobial agents by 31 European countries for food-producing animals (EMA/24309/2020). Moreover, the distribution of tetracycline resistance in dairy cow dejections is well documented (Pitta et al., 2016; Nobrega et al., 2018).

Van Hoek et al. (2011) reported an extensive summary of ARGs involved in acquired resistance against several classes of antibiotics. The abundance of acquired resistance genes is related to antimicrobial exposure but it may also be disseminated even in the absence of selective pressure (Argudín et al., 2017). On the other hand, Zhang et al. (2021) applied a risk ranking framework, based on three factors (anthropogenic enrichment, mobility and host pathogenicity) to classify 132 out of a total of 4050 ARGs in Rank I (*i.e.*, ARGs with the highest risk of dissemination among pathogens). Within the MGE-associated ARGs annotated in our study, those associated to acquired resistances by van Hoek et al. (2011) and classified in Rank I by Zhang et al. (2021) were considered as high dissemination potential ARGs (see defined criteria below). These identified high dissemination potential ARGs [*aad(6)*, *cmlA*, *floR*, *tetM*, *tetO*, *tetW*, *dfrA1*, *dfrA14*, *dfrA15*, *dfrA17*, *dfrA25* and *dfrA5*] confer resistance to aminoglysocides, phenicols (*e.g.*, chloramphenicol), tetracyclines and diaminopyrimidines (*e.g.*, trimethoprim). The use of chloramphenicol in food animals was banned in 1994 in the EU. However, its derivative florfenicol is licensed for treating respiratory infections in pigs and cattle. Both *cmlA* and *floR* genes, which encode efflux pumps, have been detected in swine (Li et al., 2013b), broiler (He et al., 2014), and cattle (Karczmarczyk et al., 2011) farms. Roberts et al. (2016) reported that *cmlA* gene is frequently located in multiresistance plasmids in a variety of enteric bacteria. On the other hand, tetracycline-resistance genes are commonly found in cow dejections. Actually, the cow gut microbiome maintains a diverse pool of tetracycline-resistance genes (Kyselkova et al., 2015). Lastly, diaminopyrimidine resistance genes (*dfr*) have also been previously associated with MGEs, in particular with class 1 and class 2 integrons (Deng et al., 2015).

Antibiotic resistant genes identified as hub genes in our network analyses, as well as those with high dissemination potential, should be carefully monitored in similar studies. Interestingly, three genes, *i.e.*, *aad(6)*, *tetM* and *tetW*, fulfill both criteria, suggesting their potential as markers of antibiotic resistance under similar livestock and agricultural settings.

4.5. Conclusion

Our metagenomic study provides further evidence that dairy cow farms can be reservoirs of ARGs, MGE-genes and priority pathogens. Nonetheless, a reduction of the antibiotic resistome risk from cow slurry and manure microbiomes to soil and vegetable microbiomes was observed (*i.e.*, the following resistome risk gradient was detected, by means of using the MetaCompare computational pipeline, in the environmental compartments studied here: animal dejections > soils > vegetables). Almost no

MGE-associated ARGs were detected in soil and vegetable samples. Abundant and co-ocurring ARGs and MGE-genes, together with high dissemination potential ARGs, should be carefully monitored. Three genes, *i.e.*, *aad(6)*, *tetM* and *tetW*, have shown their potential as markers of antibiotic resistance under similar livestock and agricultural settings. Further research is needed to improve the management of livestock dejections (and, in particular, their use as soil amendments for organic fertilization) to minimize as much as possible the antibiotic resistome risk, as reflected by the resistome, mobilome and pathogenome of all the associated environmental compartments.

4.6. Supplementary information

Supplementary Table 4.1. Physicochemical properties of the studied soils. Mean values ± standard errors. No statistically significant differences were detected among the studied four soil types.

Property	Slurry-amended pasture soil	Aged manure-amended orchard soil	Grazed soil	Unamended control soil
Organic matter (OM) (%)	6.8 ± 2.3	11.5 ± 6.3	9.3 ± 1.0	9.6 ± 4.3
Cation exchage capacity (CEC) (mEq 100 g^{-1})	22.2 ± 7.5	30.2 ± 17.5	26.7 ± 9.0	27.4 ± 8.9
Electrical conductivity (EC) (mS cm^{-1})	0.23 ± 0.08	0.33 ± 0.12	0.33 ± 0.12	0.26 ± 0.04
pH	6.8 ± 0.59	6.4 ± 0.61	6.9 ± 0.49	7.1 ± 0.19
Clay (%)	26.4 ± 6.7	17.5 ± 5.7	21.6 ± 7.8	15.7 ± 8.5
Sand (%)	23.5 ± 8.4	39.3 ± 17.4	26.9 ± 9.9	29.6 ± 7.2
Silt (%)	52.0 ± 6.3	44.2 ± 10.3	51.5 ± 5.1	54.7 ± 4.1
NH_4^+ (mg kg^{-1})	6.1 ± 3.2	6.6 ± 2.2	11.1 ± 8.6	6.9 ± 3.5
NO_3^- (mg kg^{-1})	190 ± 157	385 ± 213	212 ± 46	274 ± 106
N (%)	0.39 ± 0.16	0.66 ± 0.37	0.56 ± 0.08	0.50 ± 0.17
Olsen phosphorus (mg kg^{-1})	47.8 ± 19.2	136 ± 65.7	67.6 ± 27.4	64.8 ± 46.3
Potassium (mg kg^{-1})	415 ± 212	871 ± 417	897 ± 348	365 ± 88
C/N	8.0 ± 1.1	7.9 ± 0.72	7.6 ± 1.2	8.3 ± 1.1
Cd (mg kg^{-1} DW)	0.78 ± 0.47	1.3 ± 1.14	0.86 ± 0.42	0.87 ± 0.44
Co (mg kg^{-1} DW)	21.1± 21.0	14.8 ± 15.5	22.8 ± 20.9	19.0 ± 19.4
Cr (mg kg^{-1} DW)	28.3 ± 20.0	23.6 ± 18.4	29.2 ± 21.4	27.3 ± 25.7
Cu (mg kg^{-1} DW)	41.4 ± 33.1	49.0 ± 32.6	48.7 ± 37.2	43.8 ± 40.7
Ni (mg kg^{-1} DW)	30.9 ± 28.8	34.0 ± 30.8	42.2 ± 42.9	37.4 ± 40.9
Pb (mg kg^{-1} DW)	42.4 ± 21.0	22.9 ± 12.1	33.1 ± 14.8	35.4 ± 19.7
Zn (mg kg^{-1}DW)	117 ± 42.0	144 ± 73.9	135 ± 25.0	139 ± 58.2

Supplementary Table 4.2. Physicochemical properties of the animal dejections. Mean values ± standard errors. Values with different letters are significantly ($p < 0.05$) different according to Games-Howell test.

Property	Fresh slurry	Stored slurry	Faeces	Fresh manure	Aged manure
Dry matter (%)	11.8 ± 2.5[b]	12.9 ± 6.5[ab]	12.6 ± 2.4[ab]	23.1 ± 5.8[a]	21.7 ± 12.2[ab]
EC (mS cm^{-1})	0.86 ± 0.27	0.85 ± 0.31	0.72 ± 0.36	1.16 ± 0.38	0.76 ± 0.43
pH	8.4 ± 0.5	8.5 ± 0.4	8.1 ± 0.5	8.6 ± 0.4	8.1 ± 0.9
OM (%)	82.7 ± 3.3	79.4 ± 3.5	84.2 ± 4.6	84.5 ± 2.0	62.2 ± 13.6
Organic C (%)	5.7 ± 1.3[ab]	5.9 ± 2.7[ab]	6.2 ± 1.3[b]	11.3 ± 2.7[a]	7.4 ± 4.4[ab]
N (%)	0.45 ± 0.06	0.42 ± 0.16	0.38 ± 0.12	0.66 ± 0.25	0.61 ± 0.33
C/N	17.8 ± 4.3	22.9 ± 6.2	23.4 ± 8.1	22.0 ± 4.3	16.0 ± 5.4
P_2O_5 (%)	0.82 ± 0.18	0.84 ± 0.22	0.85 ± 0.27	0.74 ± 0.27	0.84 ± 0.17
K_2O (%)	2.6 ± 1.3	2.9 ± 1.3	2.0 ± 1.3	2.9 ± 0.77	2.7 ± 1.3
Cd (mg kg^{-1} DW)	0.13 ± 0.12	0.13 ± 0.05	0.12 ± 0.11	0.07 ± 0.05	0.43 ± 0.27
Co (mg kg^{-1} DW)	1.8 ± 0.29	2.1 ± 0.93	1.6 ± 0.82	1.5 ± 0.37	3.1 ± 1.2
Cr (mg kg^{-1} DW)	7.5 ± 2.4	8.0 ± 3.1	8.9 ± 1.7	10.3 ± 5.2	15.2 ± 8.7
Cu (mg kg^{-1} DW)	53.0 ± 33.2	47.9 ± 39.0	46.0 ± 21.5	31.4 ± 12.2	73.9 ± 43.6
Ni (mg kg^{-1} DW)	5.8 ± 1.8	8.3 ± 3.2	5.4 ± 2.4	5.4 ± 0.77	12.2 ± 3.2
Pb (mg kg^{-1} DW)	2.7 ± 2.2	5.3 ± 4.4	3.3 ± 1.6	2.4 ± 0.99	8.1 ± 5.3
Zn (mg kg^{-1} DW)	297 ± 124	269 ± 121	237 ± 94.3	250 ± 113	295 ± 79

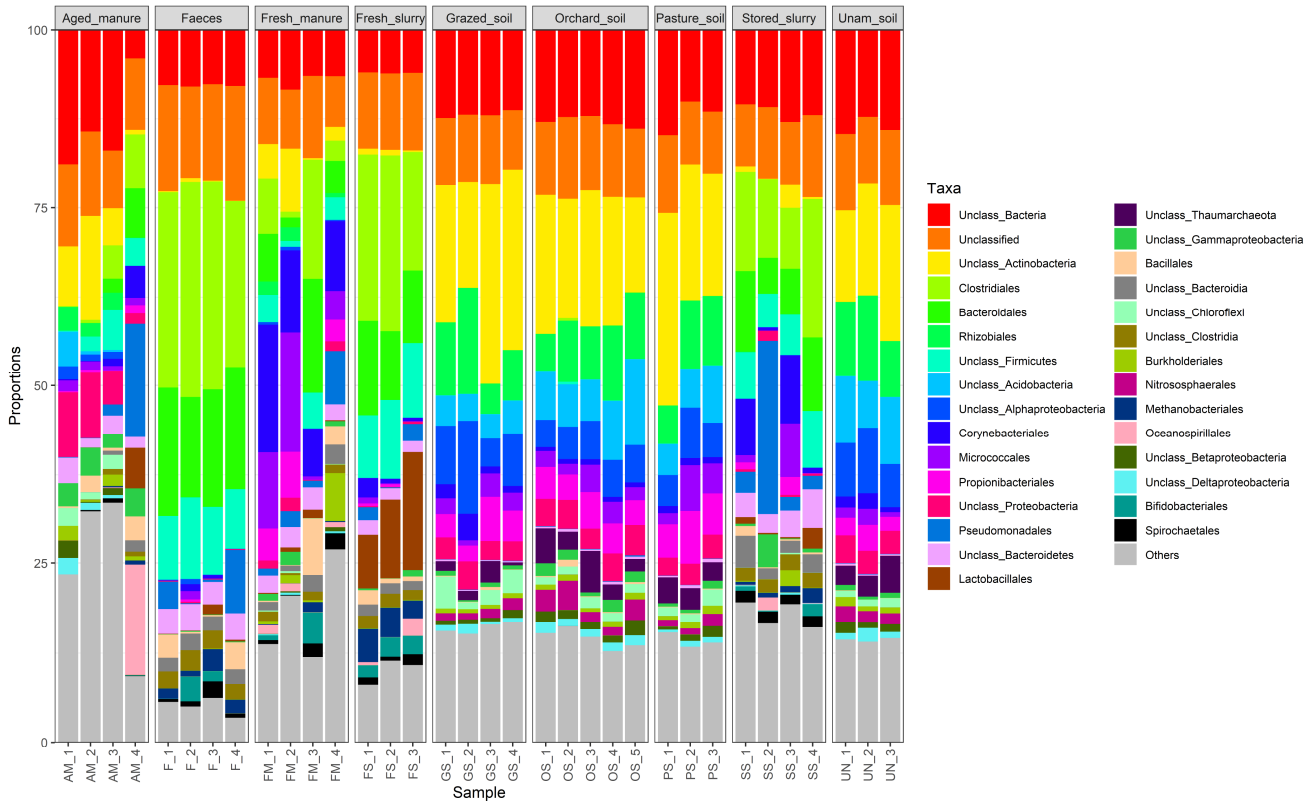
Supplementary Table 4.6. Summary of MetaCompare output.

Environmental _compartment	Farm	nContigs	nARG	nMGE	nPAT	ARG& MGE	ARG& MGE& PAT	Risk_ score
Aged manure	1	77126	151	201	12404	8	8	23.13
Aged manure	2	116983	348	472	24424	14	14	26.51
Aged manure	3	127487	373	588	25505	17	16	26.44
Aged manure	5	49649	265	193	11593	22	22	41.49
Faeces	1	61066	223	62	11919	10	10	29.40
Faeces	2	103739	295	144	16680	13	12	26.07
Faeces	3	80670	235	98	12501	7	7	25.99
Faeces	4	50956	243	223	9615	38	37	44.78
Faeces	5	35569	116	72	6890	7	7	28.31
Fresh manure	1	66789	260	375	14613	23	21	32.48
Fresh manure	2	110235	469	768	25351	35	31	33.64
Fresh manure	3	117698	384	557	20609	37	36	29.59
Fresh manure	4	117653	602	597	26349	44	42	38.78
Fresh manure	5	73151	277	494	18580	21	20	31.37
Fresh slurry	2	56016	229	123	8941	21	21	34.02
Fresh slurry	3	89699	331	234	16224	20	20	30.26
Fresh slurry	4	87179	290	127	12015	12	12	27.92
Fresh slurry	5	67893	295	156	12554	19	18	33.64
Grazed soil	1	26861	64	141	4431	0	0	23.60
Grazed soil	2	23364	72	71	3930	0	0	25.74
Grazed soil	3	24145	78	78	5030	0	0	26.21

Grazed soil	5	21168	70	77	4814	0	0	26.46
Orchard soil	1	18740	51	64	3135	0	0	24.62
Orchard soil	2	17058	54	60	3049	1	1	26.56
Orchard soil	3	24193	66	68	4083	0	0	24.64
Orchard soil	4	10089	26	43	2061	0	0	24.18
Orchard soil	5	12998	31	45	1937	0	0	23.61
Pasture soil	2	34624	103	124	7036	0	0	25.40
Pasture soil	3	11945	33	54	1611	0	0	24.75
Pasture soil	4	10414	40	28	2490	0	0	28.22
Pasture soil	5	9301	37	28	1993	0	0	28.69
Stored slurry	1	89754	357	237	15486	27	27	32.46
Stored slurry	2	82873	335	170	11211	22	21	32.16
Stored slurry	3	99542	347	352	17961	17	16	28.80
Stored slurry	4	55239	220	120	8191	17	17	32.58
Stored slurry	5	78572	268	141	10985	20	20	29.53
Unamended soil	1	16684	47	89	2534	0	0	24.91
Unamended soil	2	21274	61	96	3350	0	0	25.07
Unamended soil	3	28042	70	91	5841	0	0	23.94
Unamended soil	4	4708	26	50	830	0	0	34.10
Unamended soil	5	20994	45	71	2551	0	0	22.91
Vegetable	1	72198	25	24	1214	0	0	18.33
Vegetable	2	70067	2	1	33	0	0	17.63
Vegetable	3	39263	0	2	24	0	0	17.57
Vegetable	4	68409	0	1	33	0	0	17.57
Vegetable	5	17212	0	1	20	0	0	17.57

Supplementary Table 4.10. Distribution of high dissemination potential ARGs in the studied environmental compartments, expressed in percentage.

ARG	Fresh slurry (n=5)	Stored slurry (n=5)	Faeces (n=5)	Fresh manure (n=5)	Aged manure (n=4)
aad(6)	100	100	40	100	100
cmlA	0	20	0	0	0
dfrA1	0	20	20	40	0
dfrA14	0	20	20	40	0
dfrA15	0	20	20	40	0
dfrA17	0	20	0	40	0
dfrA25	0	20	20	40	0
dfrA5	0	20	20	40	0
floR	25	20	0	80	50
tetM	100	100	100	100	25
tetO	100	100	100	100	25
tetW	100	100	100	100	25



Supplementary Figure 4.1. Barplots representing the distribution of the 30 most abundant prokaryotic taxa (order level).

5. CHARACTERIZATION OF COMPOSTED ORGANIC AMENDMENTS FOR AGRICULTURAL USE

Abstract

The application of organic amendments to agricultural soil provides organic matter and valuable nutrients, improves soil structure, increases its water holding capacity and stimulates soil microbial communities. However, when using organic amendments of animal and/or anthropogenic origin, the risk of contamination with organic and/or inorganic compounds, as well as the risk of dissemination of potential human pathogens and antibiotic resistance genes, must be taken into account. Here, we characterized seven amendments used in organic farming, in order to evaluate the agro-environmental consequences of their utilization. Amendments (vermicompost, bokashi, municipal solid waste, compost in pellet form, composted cow manure from intensive farms, composted cow manure from organic farms, composted sheep manure from organic farms) were sampled for the determination of (i) the presence of chemical (metals, aromatics, halogenated hydrocarbons, pesticides, phthalates and total petroleum hydrocarbons) and biological (*Escherichia coli*, *Salmonella*, relative abundance of the integrase *intl1* gene) contaminants; and (ii) their quality, in terms of physicochemical (moisture, organic matter, nutrients, C/N ratio) and microbial (potentially mineralizable nitrogen, microbial biomass carbon, bacterial and fungal abundance by real-time PCR, community-level physiological profiles through Biolog EcoPlates™) properties. Regarding metal concentrations, the only amendments that met the Spanish legal requirements for "Class A" fertilizers were composted intensive and organic cow manure. Zinc was the most limiting metal for the use of these amendments. None of the amendments showed high concentrations of organic contaminants. Bokashi was the only amendment in which *Salmonella* was detected. Besides, bokashi showed a high abundance of the integrase *intl1* gene associated with class 1 integrons. Composted organic sheep manure showed the highest content of organic matter, total nitrogen and extractable humic acids. Composted intensive cow manure showed highest values of microbial activity (potentially mineralizable N) and biomass (microbial biomass C, total bacteria). Owing to its low content of potentially health-threatening contaminants and its highest quality, as reflected by the Amendment Quality Index, composted intensive cow manure was concluded to be the most suitable amendment for agricultural use.

5.1. Introduction

Many agricultural soils have a low organic matter (OM) content and are thus more susceptible to erosion, desertification and climate change (Diacono & Montemurro 2011). The application of organic amendments to agricultural soil can be a suitable option to reverse these negative effects. Besides, organic amendments can (i) improve soil structure and aggregation, (ii) enhance water holding capacity (Bulluck et al., 2002), (iii) increase soil OM and nutrient content (Park et al., 2011), (iv) control soil erosion and degradation (Ros et al., 2003), and (v) stimulate soil microbial activity and plant growth (García et al., 1994; Pascual et al., 1997; Van-Camp et al., 2004).

The application of composted organic amendments has numerous advantages, compared to non-composted ones (Fernández et al., 2008). Besides, composting is considered a suitable waste management strategy, along with anaerobic digestion (Alvarenga et al., 2017). The production of organic wastes is increasing worldwide and then, nowadays, farmers have more access to composted amendments from different origins. Furthermore, the use of on-farm compost has increased in recent years, thus replacing commercial compost (Pane et al., 2015). Therefore, the use of organic compost represents both an appropriate waste management strategy and an interesting agricultural practice (Pérez-Piqueres et al., 2006), in compliance with the ‘‘end-of-waste’’ policy in Europe (Saveyn & Eder, 2014).

However, when using organic amendments of animal and/or anthropogenic origin, the risk of contamination with inorganic and/or organic compounds, as well as the risk of dissemination of potential human pathogens and antibiotic resistance genes, must be taken into account. In recent years, emerging contaminants (*e.g.*, pesticides and their metabolites, pharmaceuticals, personal and house care products, food additives, industrial products and wastes, nanomaterials) have become a matter of much concern (Gomes et al., 2017). For example, the dissemination of antibiotic resistance genes, which can occur via the application of manure- and sewage sludge-derived amendments to agricultural soil (Martinez-Carballo et al., 2007; Udikovic-Kolic et al., 2014), is currently a matter of much concern due to the risk of transfer of antibiotic resistance genes to human pathogens (Marshall & Levy, 2011).

Physicochemical characteristics of the organic amendments are often determined in order to assess their suitability for agricultural use: organic carbon, total and organic nitrogen, C/N relationship, P_2O_5, K_2O, humic substances, etc. (Decree Law No.999/2017). It is much less common to determine

parameters related to the microbial activity, biomass and diversity of the organic amendments. Microbial activity is an essential factor for the successful operation of composting processes. It is then necessary to study microbial parameters in order to effectively control the composting process (Beffa et al., 1996), as well as to evaluate the final quality of the compost obtained.

Farmers, particularly those involved in organic agriculture, need appropriate organic amendments which, apart from meeting the current legal requirements, had been exhaustively characterized in terms of both benefits and risks. The selection of amendments for this study (Table 1) emerged from a deliberation process carried out by a local farmers´ association, which identified locally abundant sources of organic amendments (urban or animal origin, commercial or not) suitable for their use in agriculture. In consequence, the aim of this study was to: (i) carry out an in depth-characterization of seven composted organic amendments, (ii) propose a methodology to select the most suitable amendments, and (iii) to deepen the knowledge of the potential benefits and risks associated with the application of composted organic amendments.

5.2. Materials and Methods

5.2.1. Amendments

Seven composted organic amendments, collected in the provinces of Araba, Gipuzkoa and Navarre (northern Spain), were studied: vermicompost (VC), bokashi (BK), municipal solid waste (MSW), compost in pellet form (P), composted cow manure from intensive farms, (ICM), composted cow manure from organic farms (OCM), and composted sheep manure from organic farms (OSM). The elaboration process of each amendment is described in Table 5.1. Three representative replicates of each amendment were sampled and stored at 4ºC until analysis.

Table 5.1. Amendment origin, composition, composting time and frequency of turning over.

Amendment	Origin	Composition	Composting time	Turning over
Vermicompost (VC)	Hazieko (Aizarnazabal, Gipuzkoa)	50%: manure of different origins (cow, swine, sheep, horse) and animal guts; 50%: straw and dry grass	5.5-7.5 months	Twice
Bokashi (BK)	Ximaur Pila Association (Lasarte-Oria, Gipuzkoa)	Sheep manure, clay soil, rice husks, coal, pulverized rock, molasses, yeast and straw	3-4 weeks	Every 12 hours (first 3 days); then, every 2 days
Municipal solid waste (MSW)	Epele Composting Plant (Bergara, Gipuzkoa)	Organic waste and pruning remains	10 weeks	4-5 times (first 4 weeks)
Compost in pellet form (P)	FERT-IB (Andosilla, Navarre)	Vegetable and animal raw materials	> 12 months	
Composted intensive cow manure (ICM)	A-I Abereak S.L (Ondategi, Araba)	60% intensive cow manure; 40% straw	6-8 months	Twice
Composted organic sheep manure (OSM)	Telleri Zahar farm (Hernani, Gipuzkoa)	40% organic sheep manure; 60% straw	5-6 months	Twice
Composted organic cow manure (OCM)	Sarobe farm (Anoeta, Gipuzkoa)	60% organic cow manure; 40% straw	6-8 months	Twice

5.2.2. Presence of contaminants

For the determination of the concentration of chemical contaminants in the studied amendments, the TerrAttesT® analytic package from Eurofins Scientific was used. This analytical package includes 185 contaminants (metals, aromatics, halogenated hydrocarbons, pesticides, phthalates, total petroleum hydrocarbons) determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES), large volume injection-mass spectrometry (LVI-FC-MS) and gas chromatography with flame ionization detector (GC-FID).

Regarding biological contaminants, the presence (most probable number: MPN) of *Escherichia coli* was quantified following ISO 7251 (2005). The presence of *Salmonella* was determined following UNE-EN-ISO 6579 (2003).

DNA extraction was carried out from 0.25 g of amendment (three replicates per amendment) using PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad CA), following the manufacturer's instructions. Prior to DNA extraction, samples were washed with 20 mM K_2PO_4 to remove extracellular DNA (Kowalchuk et al., 2002). DNA extracts were stored at -20ºC. Real-time PCR was carried out to determine the relative abundance of the class 1 integron-integrase *intl1* gene. The sequence-specific primers were intl1-F (GCCTTGATGTTACCCGAGAG) and intl1-R (GATCGGTCGAATGCGTGT), following Barraud et al. (2010). Each 25 µl reaction contained 2.5 µl of template, 12.5 µl of SYBR Premix Ex Taq (Takara), 2.5 µl of each primer at a concentration of 10 µM, 1.25 µl bovine serum albumin (40 mg ml^{-1}), 0.5 µl of ROX dye and 3.25 µl of sterile Milli-Q water. Each sample was measured in triplicate. PCR conditions were as follows: 95ºC for 15 s; 94ºC for 30 s, 60ºC for 30 s, 72ºC for 1 min (40 cycles); and 95ºC for 15 s, 60ºC for 1 min, 95ºC for 30 s for the melting curve, with a final extension of 60ºC for 15 s. The relative abundance of *intl1* gene was expressed in terms of relative abundance with respect to the structural 16S rRNA gene abundance (see below quantification of the 16S rRNA gene).

5.2.3. Amendment quality

The following physicochemical parameters were measured in the studied amendments: organic matter (UNE-EN 13039:2012), modified Kjeldahl nitrogen (UNE-EN 13654-1:2002), water soluble nutrients (P_2O_5, K_2O, CaO and MgO; UNE-EN 13652:2002), and humic substances (Decree Law No. 1110/1991).

Concerning microbial parameters, potentially mineralizable nitrogen (PMN), an indicator of microbial activity, was determined following Powers (1980). Microbial biomass carbon (MBC) was measured using the fumigation extraction method (Vance et al., 1987). Real-time PCR measurements were performed to determine the abundance of 16S rRNA (Ba519F/Ba907R primers) and 18S rRNA (Fung5F/FF390R primers) gene fragments for total bacteria and fungi, respectively (Lueders et al., 2004a,b). Reaction mixtures and PCR conditions are described in Epelde et al. (2014). Standards were made from plasmids containing the target sequence (Dhanasekaran et al., 2010).

Community-level physiological profiles (CLPPs) of cultivable heterotrophic bacteria were analyzed with Biolog EcoPlates™ (Insam, 1997) according to Epelde et al. (2008). Average well colour development (AWCD) was calculated as the mean of every well´s absorbance value at each

reading time. The highest growth rate was observed at 48 hours of incubation. At this time point, we calculated the Shannon's diversity index (H´), Simpson's diversity index (D), Shannon's evenness (E) and species richness (S). For these calculations, the number of utilized substrates (*i.e.*, the number of substrates with an absorbance value >0.25) was used as proxy for species richness, and absorbance values at each well as equivalent to species abundance. The maximum slope and the area under the absorbance curve were measured following a trapezoidal approximation (Guckert et al., 1996).

5.2.4. Data analysis

An Amendment Quality Index (AQI) was calculated from the values of OM, total N, P_2O_5, K_2O, CaO, MgO, extractable humic acids, PMN, MBC, abundance of 16S and 18S rRNA genes, and area under the curve from Biolog EcoPlates™. For each parameter, the percentage of the maximum value found for that specific parameter in the whole set of samples was used for the calculation of the AQI:

$$AQI = \frac{\Sigma \frac{AQP_i * 100}{Max\ AQP_i}}{n}$$

where AQP_i is the value of each parameter, $Max\ AQP_i$ is the maximum value for each parameter, and n is the number of parameters.

One-way analysis of variance (ANOVA) was conducted to look for statistical differences among the values of all the parameters measured here. Duncan Multiple Range Test post-hoc method was used for multiple comparisons at a 5% significance level. These analyses were conducted in R Core Team (v.3.4.0.).

The relationships between the quality parameters determined here in all the amendments were studied by means of a principal component analysis (PCA), using Canoco 5 (Ter Braak & Ŝmilauer, 2012).

5.3. Results

5.3.1. Presence of contaminants

In relation to metal concentrations (Table 5.2), bokashi showed significantly highest values of 6 out of the 14 metals measured here: arsenic (As), barium (Ba; together with MSW), chromium (Cr), cobalt (Co), nickel (Ni) and vanadium (V). The compost in pellet form, instead, had the highest values of

copper (Cu; together with VC and OSM), mercury (Hg; together with P), tin (Sn) and zinc (Zn; together with all the other amendments, except for OCM). Municipal solid waste showed the highest concentration of cadmium (Cd; together with VC), mercury (Hg; together with P) and lead (Pb). Lastly, composted intensive cow manure showed the highest concentration of molybdenum (Mo). On the other hand, composted organic cow manure showed significantly lowest values of 12 out of the 14 metals measured here, while composted intensive cow manure and composted organic sheep manure showed the lowest concentrations of 9 out of the 14 metals.

Table 5.2. Metal concentrations (mg kg^{-1}) in amendments. Mean values (n=3) and standard errors. Different letters indicate significant differences ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

	VC	BK	MSW	P	ICM	OSM	OCM	***Limit**** *Class A/B/C*
As	3.4 ± 0.3^{d}	6.9 ± 0.5^{a}	5.0 ± 0.3^{b}	4.1 ± 0.2^{c}	3.0 ± 0.0^{d}	3.0 ± 0.0^{d}	3.0 ± 0.0^{d}	
Ba	54.3 ± 0.6^{d}	95.7 ± 4.0^{a}	86.3 ± 11.2^{ab}	82.7 ± 8.3^{bc}	74.7 ± 4.9^{c}	74.7 ± 2.5^{c}	31.7 ± 2.3^{e}	
Be	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.4 ± 0.7^{a}	
Cd	0.34 ± 0.01^{ab}	0.30 ± 0.00^{c}	0.37 ± 0.04^{a}	0.31 ± 0.02^{bc}	0.30 ± 0.00^{c}	0.30 ± 0.00^{c}	0.30 ± 0.00^{c}	*0.7/2/3*
Cr	17.3 ± 2.3^{c}	23.0 ± 1.0^{a}	14.0 ± 1.0^{d}	19.7 ± 1.2^{b}	5.2 ± 0.1^{ef}	3.6 ± 0.1^{f}	6.7 ± 1.6^{ef}	*70/250/300*
Co	3.6 ± 0.2^{bc}	10.7 ± 0.6^{a}	4.8 ± 2.1^{b}	2.7 ± 0.1^{c}	2.2 ± 0.2^{c}	2.0 ± 0.0^{c}	2.1 ± 0.1^{c}	
Cu	57.3 ± 1.5^{ab}	22.0 ± 2.0^{c}	50.3 ± 10.4^{b}	62.7 ± 1.2^{a}	29.3 ± 2.1^{c}	54.0 ± 8.5^{ab}	12.0 ± 1.0^{d}	*70/300/400*
Hg	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	*0.4/1.5/2.5*
Pb	24.0 ± 4.4^{b}	11.7 ± 0.6^{b}	57.3 ± 29.4^{a}	20.0 ± 7.0^{b}	7.3 ± 0.3^{b}	10.1 ± 3.5^{b}	5.5 ± 0.8^{b}	*45/150/200*
Mo	1.6 ± 0.1^{cd}	1.0 ± 0.0^{d}	2.0 ± 0.4^{bc}	2.5 ± 0.2^{b}	4.2 ± 1.0^{a}	1.8 ± 0.1^{bc}	1.7 ± 0.1^{cd}	
Ni	12.7 ± 2.1^{c}	26.7 ± 1.5^{a}	10.3 ± 0.6^{d}	15.0 ± 1.7^{b}	6.1 ± 0.5^{e}	4.5 ± 0.4^{e}	5.3 ± 1.2^{e}	*25/90/100*
Sn	5.0 ± 0.0^{b}	5.0 ± 0.0^{b}	5.0 ± 0.0^{b}	7.8 ± 3.7^{a}	5.0 ± 0.0^{b}	5.0 ± 0.0^{b}	5.0 ± 0.0^{b}	
V	11.0 ± 0.0^{b}	41.0 ± 3.6^{a}	8.9 ± 0.9^{bc}	11.0 ± 0.0^{b}	5.9 ± 0.6^{de}	4.4 ± 0.4^{e}	8.1 ± 1.5^{cd}	
Zn	286.7 ± 5.8^{ab}	75.3 ± 0.6^{ab}	313.3 ± 240.9^{ab}	350.0 ± 303.2^{a}	113.3 ± 5.8^{ab}	223.3 ± 40.4^{ab}	59.7 ± 3.2^{b}	*200/500/1000*

*Spanish legal limits for metal concentrations in amendments (Decree Law No.506/2013, on fertilizer products).

According to Spanish Law No.506/2013, only two amendments, *i.e.* composted intensive cow manure and composted organic cow manure, met the requirements for "Class A" fertilizers. The rest of the amendments fitted in "Class B" fertilizers, due to the high concentrations of Zn (VC, MSW, P, OSM), Ni (BK) and Pb (MSW).

The concentrations of organic contaminants present in the amendments are shown in Supplementary Table 1 (see online). Bokashi showed significantly highest concentrations of some

phenols. Regarding polycyclic aromatic hydrocarbons (PAHs), all amendments, apart from composted organic cow manure, showed highest values in one or another PAH fraction. Composted organic sheep manure showed the highest concentration of monochlorobenzene. In relation to chlorophenols, bokashi and municipal solid waste had the highest concentrations of 2,4,6-trichlorophenol and pentachlorophenol, respectively. Municipal solid waste also showed the highest concentration of some polychlorinated biphenyls. Regarding total petroleum hydrocarbons (TPHs), municipal solid waste, compost in pellet form and composted organic sheep manure showed highest values in two or three TPH fractions. Finally, bokashi and compost in pellet form showed the highest concentration of biphenyl and dibenzofuran, respectively.

In respect to the analyses performed here to quantify the presence of *E. coli,* the Spanish Decree Law No.506/2013 indicates that MPN values must be lower than 1000 MPN g^{-1}. Regarding the presence of *Salmonella* spp., it is compulsory (Decree Law No.506/2013) not to detect its presence in 25 g of the sample. Highest values of MPN for *E.coli* were detected in composted organic sheep manure, followed by vermicompost, bokashi and composted organic cow manure (Table 5.3). The abundance of *E. coli* was below the quantification limit for municipal solid waste, compost in pellet form, and composted intensive cow manure. None of the amendments exceeded the legal requirement for *E. coli*. Instead, one of the replicates of bokashi was positive for *Salmonella* spp. (Table 5.3), exceeding the legal limit. The *Salmonella* serotype found in our bokashi amendment did not correspond to Enteritidis, Typhimurium, Hadar, Infantis or Virchow (the serovars most often monitored in Europe).

Table 5.3. Presence of pathogens in the amendments. Mean values (n=3) and standard errors. Different letters indicate significant differences ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

	E. coli **(MPN g^{-1})**	***Salmonella*** **spp (presence or absence)**
VC	47 ± 26^{a}	Absence
BK	30 ± 12^{ab}	Presence
MSW	$< 3^{b}$	Absence
P	$< 3^{b}$	Absence
ICM	$< 3^{b}$	Absence
OSM	53 ± 36^{a}	Absence
OCM	18 ± 22^{ab}	Absence
Limit*	***<1000***	***Absence***

*Spanish legal limits for pathogen content in amendments (Decree Law No.506/2013, on fertilizer products).

Finally, the quantification of the class 1 integron-integrase *intl*1 gene abundance showed highest values in bokashi and compost in pellet form, followed by composted intensive cow manure and composted organic sheep manure (Figure 5.1). Vermicompost, municipal solid waste and composted organic cow manure showed the lowest abundances of *intl*1 gene.

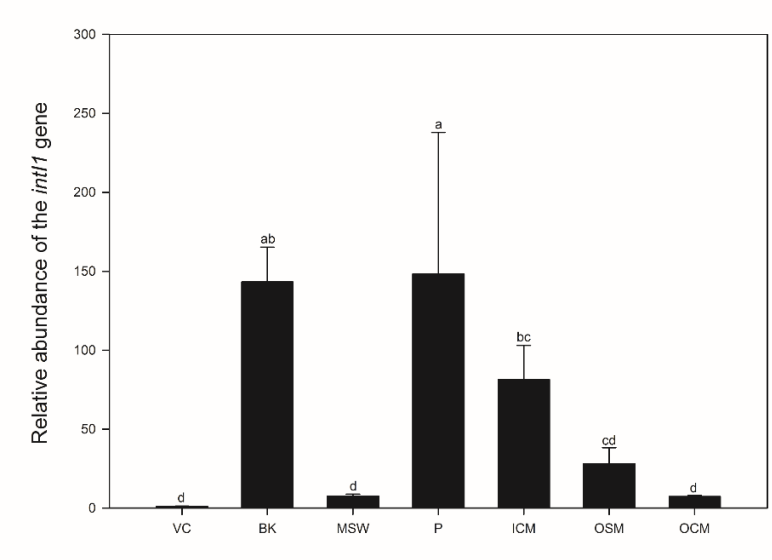


Figure 5.1. Relative abundance of the *intl1* gene. Bars represent mean values (n=3) and error bars represent standard errors. Bars labeled with different letters are significantly different ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

5.3.2. Amendment quality

Table 5.4 shows the results of the physicochemical properties measured here. Bokashi showed the highest clay percentage, and the compost in pellet form the lowest. Composted organic sheep manure showed the highest moisture content (an average of 77.6%), while bokashi had the lowest. All the amendments but bokashi, municipal solid waste and compost in pellet form surpassed the maximum limit (40% moisture) established by Spanish Decree Law No.999/2017.

Table 5.4. Physicochemical properties of the amendments. Mean values (n=3) and standard errors. Different letters indicate significant differences ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

	VC	BK	MSW	P	ICM	OSM	OCM
Clay (%)	8.5 ± 2.1^{d}	23.0 ± 2.6^{a}	5.3 ± 2.2^{d}	6.5 ± 1.1^{d}	13.1 ± 4.4^{bc}	15.7 ± 2.2^{b}	8.8 ± 2.6^{cd}
Moisture (%)	43.9 ± 0.2^{d}	15.6 ± 0.6^{f}	31.1 ± 0.6^{e}	31.1 ± 0.7^{e}	68.9 ± 2.3^{b}	77.6 ± 0.3^{a}	65.9 ± 2.5^{c}
Organic matter (%)	54.8 ± 0.3^{b}	16.9 ± 0.6^{e}	54.6 ± 1.9^{b}	32.7 ± 0.3^{d}	55.9 ± 2.2^{b}	70.7 ± 0.9^{a}	47.9 ± 0.9^{c}
Total nitrogen (%)	2.8 ± 0.0^{b}	0.62 ± 0.0^{e}	2.6 ± 0.0^{b}	1.8 ± 0.3^{d}	2.8 ± 0.1^{b}	3.6 ± 0.1^{a}	2.0 ± 0.0^{c}
Extractable humic acids (%)	17.5 ± 2.5^{b}	8.4 ± 1.1^{d}	22.7 ± 1.8^{a}	17.3 ± 0.4^{b}	15.6 ± 2.9^{bc}	22.9 ± 2.6^{a}	13.3 ± 0.4^{c}
Humic acids (%)	11.5 ± 4.0^{bc}	5.7 ± 1.2^{d}	15.4 ± 4.1^{ab}	9.6 ± 0.3^{cd}	8.9 ± 2.2^{cd}	16.7 ± 0.9^{a}	6.6 ± 0.8^{d}
Fulvic acids (%)	5.9 ± 1.6^{a}	2.7 ± 0.9^{b}	7.4 ± 2.4^{a}	7.8 ± 0.6^{a}	6.7 ± 0.8^{a}	6.3 ± 1.9^{a}	6.7 ± 1.1^{a}
C/N	9.8 ± 0.1^{c}	13.7 ± 0.6^{a}	10.3 ± 0.6^{c}	8.4 ± 0.1^{d}	9.8 ± 0.3^{c}	9.8 ± 0.3^{c}	12.0 ± 0.0^{b}
P_2O_5 (%)	1.1 ± 0.1^{b}	0.26 ± 0.0^{f}	0.85 ± 0.1^{c}	1.3 ± 0.2^{a}	0.47 ± 0.0^{d}	0.40 ± 0.0^{de}	0.34 ± 0.0^{ef}
K_2O (%)	0.25 ± 0.0^{f}	1.1 ± 0.0^{c}	1.0 ± 0.0^{c}	1.3 ± 0.1^{b}	1.6 ± 0.2^{a}	0.64 ± 0.0^{e}	0.83 ± 0.1^{d}
CaO (%)	5.2 ± 0.4^{c}	5.1 ± 0.3^{c}	7.3 ± 0.7^{b}	10.0 ± 1.2^{a}	4.2 ± 0.5^{cd}	3.5 ± 0.2^{d}	10.3 ± 0.2^{a}
MgO (%)	0.30 ± 0.0^{f}	1.0 ± 0.0^{a}	0.41 ± 0.0^{de}	0.45 ± 0.0^{d}	0.88 ± 0.1^{b}	0.65 ± 0.1^{c}	0.38 ± 0.0^{e}

Composted organic sheep manure had the highest content of OM (an average of 70.7%) and total N, while bokashi had the lowest. According to Spanish Decree Law No.999/2017, this type of amendments must have an OM content higher than 35% (30% for vermicomposts); thus, bokashi and compost in pellet form did not fulfill this requirement. Composted organic sheep manure, together with municipal solid waste, had the highest content of extractable humic acids. The highest C/N ratio was found in bokashi (13.7) and the lowest in compost in pellet form (8.4). In any case, all C/N values were below the maximum limit (C/N = 20) established by Decree Law No.999/2017. Finally, compost in pellet form showed the highest content of phosphorus and calcium (the latter together with composted organic cow manure), while composted intensive cow manure and bokashi had the highest content of potassium and magnesium, respectively.

Regarding microbial properties, composted intensive cow manure had significantly highest values of PMN (an average of 774 mg $N\text{-}NH_4\ kg^{-1}$) (Fig. 5.2) By contrast, the lowest PMN values were

found in vermicompost, together with municipal solid waste, compost in pellet form, composted organic sheep manure and composted organic cow manure.

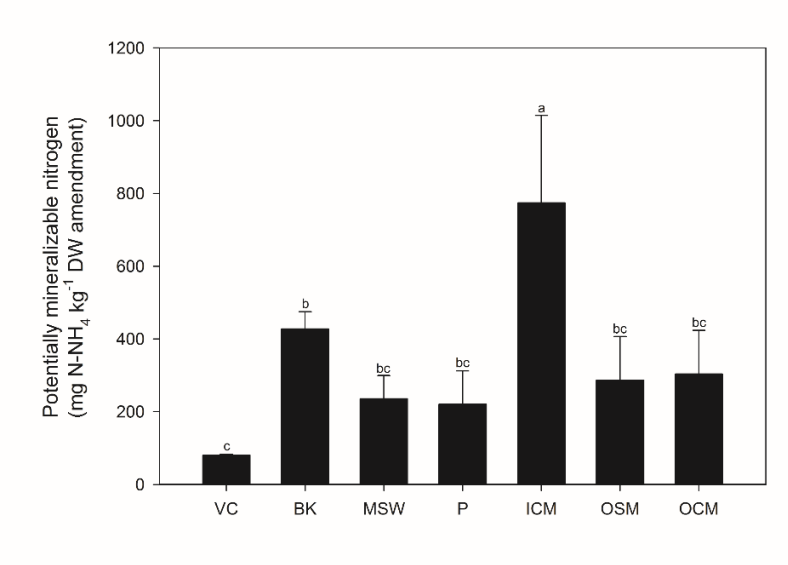


Figure 5.2. Potentially mineralizable nitrogen (PMN). Bars represent mean values (n=3) and error bars represent standard errors. Bars labeled with different letters are significantly different ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

In respect to microbial biomass, composted intensive cow manure showed the highest value of MBC (an average of 12,476 mg C kg^{-1}) (Fig. 5.3), followed by composted organic sheep and cow manure. Instead, vermicompost showed the lowest MBC value (<2000 mg C kg^{-1}), together with compost in pellet form. Bokashi showed the highest fungal gene abundance (Table 5.5). Composted intensive cow manure showed the highest bacterial gene abundance, together with vermicompost, bokashi and municipal solid waste. Finally, bokashi showed the highest F:B (fungi:bacteria) ratio.

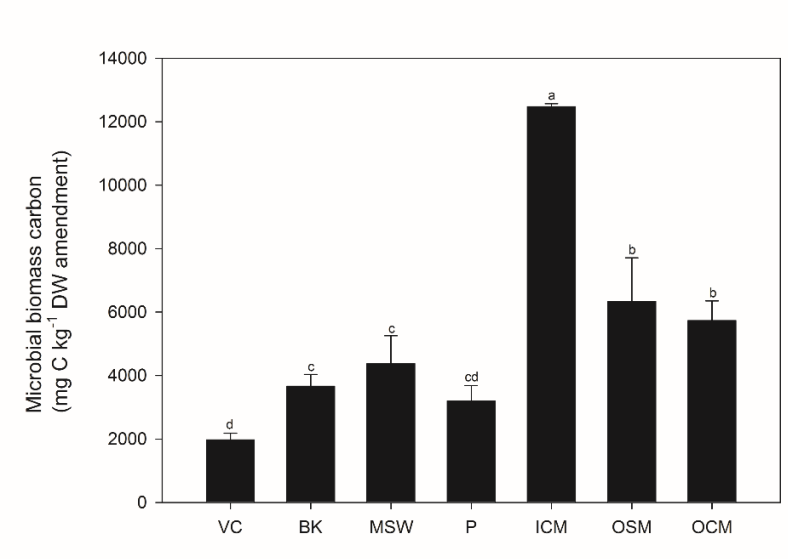


Figure 5.3. Microbial biomass carbon (MBC). Bars represent mean values (n=3) and error bars represent standard errors. Bars labeled with different letters are significantly different ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

Table 5.5. Total bacterial and fungal gene abundance, and fungi-to-bacteria ratios. Mean values (n=3) and standard errors. Different letters indicate significant differences ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

	18S rRNA abundance (10^8 copies kg^{-1} DW)	**16S rRNA abundance (10^{13} copies kg^{-1} DW)**	**F:B ratio (10^{-5})**
VC	24.6 ± 8.6^{c}	20.4 ± 11.5^{ab}	1.5 ± 0.8^{c}
BK	224 ± 63^{a}	18.3 ± 6.9^{ab}	12.7 ± 1.9^{a}
MSW	94.4 ± 9.4^{b}	20.3 ± 6.8^{ab}	5.0 ± 1.7^{b}
P	0.86 ± 0.72^{c}	9.2 ± 2.7^{b}	0.008 ± 0.007^{c}
ICM	12.8 ± 2.5^{c}	30.2 ± 6.7^{a}	0.040 ± 0.004^{c}
OSM	35.5 ± 61.4^{bc}	6.5 ± 9.2^{b}	2.1 ± 3.6^{bc}
OCM	24.1 ± 31.2^{c}	9.0 ± 10.5^{b}	2.2 ± 1.9^{bc}

Figure 5.4 shows the AWCD curves obtained with Biolog EcoPlates™. As reflected by the value of the area under the curve, vermicompost showed the highest overall microbial growth for the different C substrates (Table 5.6). Instead, composted organic sheep manure had the highest Shannon´s diversity, Simpson´s diversity and species richness, closely followed by vermicompost and composted organic cow manure. On the other hand, the lowest values obtained in these CLPPs (H´, D, S, maximum

slope and area under the curve; the last two, together with composted intensive cow manure) corresponded to municipal solid waste and compost in pellet form.

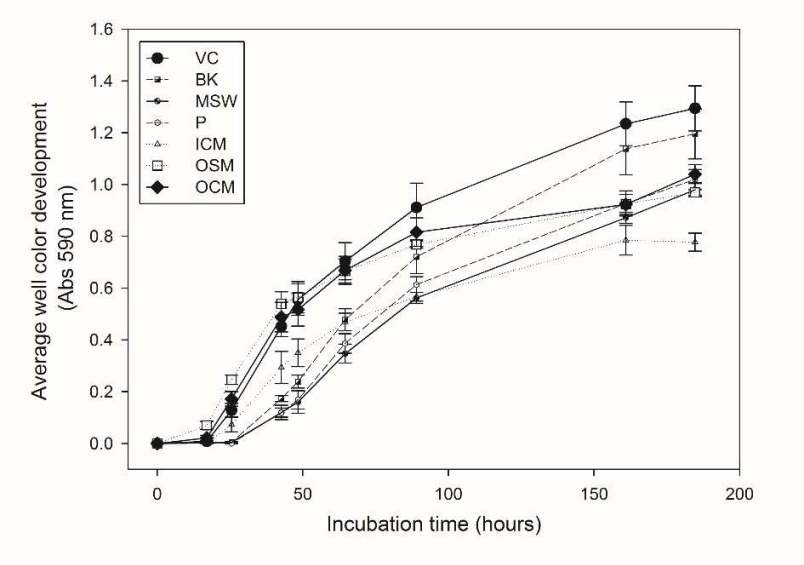


Figure 5.4. Average well colour development (AWCD) curves from Biolog Ecoplates™. Simbols at different time points represent mean values (n=3) and error bars represent standard errors. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

Table 5.6. Diversity indexes, maximum slope and area under the curve from Biolog EcoPlates™. Mean values (n=3) and standard errors. Different letters indicate significant differences ($p < 0.05$) according to Duncan Multiple Range Test. AU: absorbance unit. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

	Shannon's diversity	Simpson's diversity	Shannon's evenness	Richness	Maximum slope (100 AU h^{-1})	Area under the curve (AU h)
VC	4.06 ± 0.16^{ab}	0.93 ± 0.01^{ab}	0.96 ± 0.01^{b}	20.3 ± 1.2^{a}	2.03 ± 0.21^{a}	145.7 ± 12.5^{a}
BK	2.98 ± 0.13^{c}	0.87 ± 0.01^{bc}	0.98 ± 0.01^{ab}	12.0 ± 2.7^{c}	1.48 ± 0.13^{b}	117.7 ± 10.0^{b}
MSW	2.22 ± 0.56^{d}	0.77 ± 0.09^{d}	0.99 ± 0.01^{a}	7.3 ± 2.1^{d}	1.16 ± 0.07^{c}	90.7 ± 3.0^{c}
P	2.55 ± 0.51^{cd}	0.82 ± 0.06^{cd}	0.98 ± 0.01^{ab}	8.0 ± 1.7^{d}	1.35 ± 0.08^{bc}	97.1 ± 5.3^{c}
ICM	3.60 ± 0.33^{b}	0.91 ± 0.02^{ab}	0.97 ± 0.00^{ab}	15.3 ± 1.2^{b}	1.29 ± 0.21^{bc}	91.8 ± 2.4^{c}
OSM	4.42 ± 0.05^{a}	0.95 ± 0.00^{a}	0.98 ± 0.00^{ab}	23.0 ± 2.0^{a}	2.07 ± 0.11^{a}	122.7 ± 2.7^{b}
OCM	4.14 ± 0.07^{ab}	0.94 ± 0.00^{ab}	0.98 ± 0.00^{ab}	19.7 ± 1.5^{a}	1.87 ± 0.21^{a}	123.2 ± 6.5^{b}

The PCA performed from all the amendment quality parameters distributed the amendments in several groups (Fig. 5.5). Composted organic sheep manure samples appeared associated with Biolog EcoPlates™ parameters (except for Shannon's evenness), while vermicompost appeared more related to the content of OM and total N towards the positive region of PC1 (which accounted for 31% of the

variance). Along the positive region of PC2 (which accounted for 24% of the variance), bokashi samples showed high values of magnesium, C/N ratio and 18S rRNA gene abundance, and low values of fulvic acids. Instead, towards the negative region of PC2, municipal solid waste and compost in pellet form showed high contents of calcium.

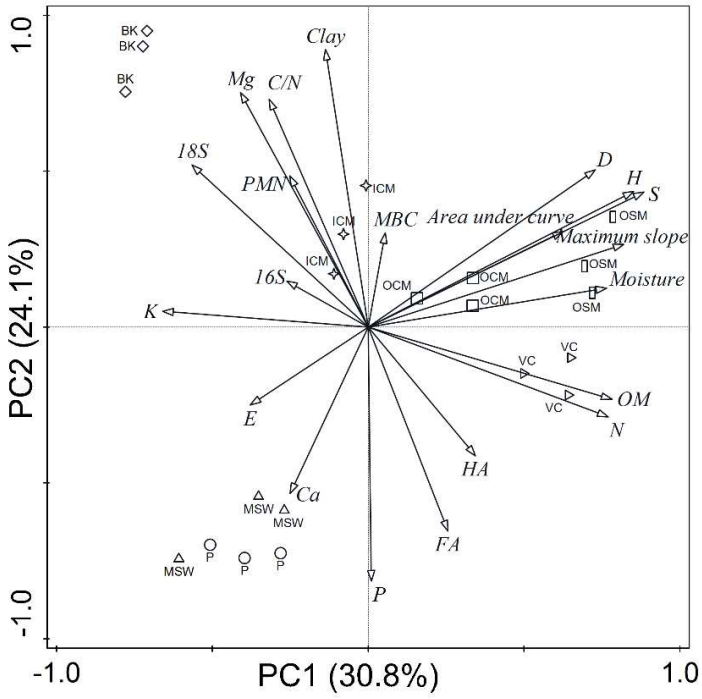


Figure 5.5. Principal component analysis obtained from all the amendment quality parameters. 16S: 16S rRNA gene copy abundance, 18S: 18S rRNA gene copy abundance, EHA: extractable humic acids, FA: fulvic acid, HA: humic acid, MBC: microbial biomass carbon, OM: organic matter, N: total nitrogen, PMN: potentially mineralizable nitrogen, H: Shannon's diversity, D: Simpson's diversity, E: Shannon's evenness, S: richness. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: composted intensive cow manure, OSM: composted organic sheep manure, OCM: composted organic cow manure.

Finally, composted intensive cow manure showed significantly higher values of the AQI, compared to the other amendments (Fig. 5.6).

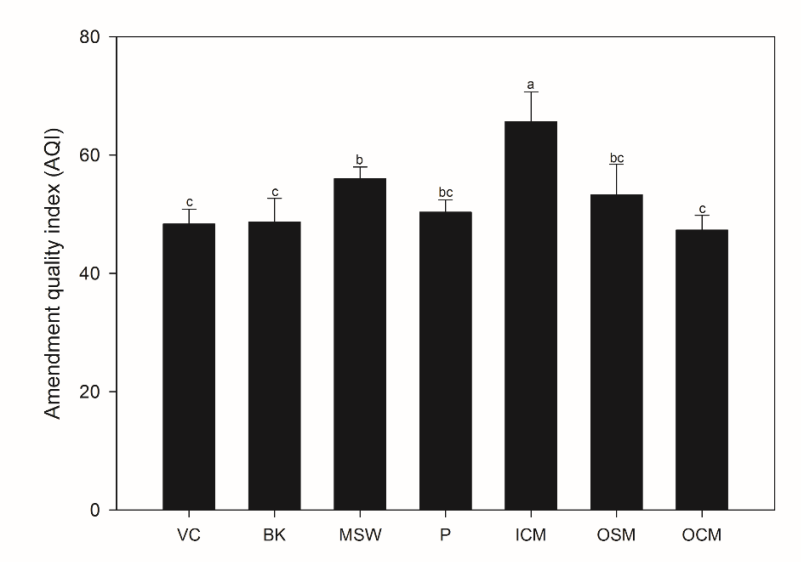


Figure 5.6. Amendment Quality Index (AQI). Bars represent mean values (n=3) and error bars represent standard errors. Bars labeled with different letters are significantly different ($p < 0.05$) according to Duncan Multiple Range Test. VC: vermicompost, BK: bokashi, MSW: municipal solid waste, P: compost in pellet form, ICM: intensive cow manure, OSM: organic sheep manure, OCM: organic cow manure.

5.4. Discussion

5.4.1. Presence of contaminants

The application of composted organic amendments to agricultural soil can enhance soil quality and fertility (Scotti et al., 2015). However, organic amendments represent a potential source of contaminants (*e.g.*, heavy metals, organic compounds, human pathogens, mobile genetic elements, antibiotic resistance genes, etc.) and, hence, a potential risk for the environment and food security, since those contaminants can be distributed in the soil ecosystem and then become available for plant uptake (Smith, 2009).

Regarding metal concentrations, the only two amendments that met the Spanish legislation were composted intensive and organic cow manure. Zinc was the most limiting metal for the use of the studied amendments. Alvarenga et al. (2017) emphasized the risk of metal accumulation in soil and plants when organic amendments are regularly applied to agricultural land.

Compost quality standards differ considerably across European Member States (Cesaro et al., 2015). Unfortunately, there are no legal limits for organic and emerging (compounds previously

unknown or undetected; the term includes a large variety of products such as pharmaceuticals, veterinary products, pesticides, nanomaterials, etc.) contaminants (Petrie et al., 2015). In this respect, highest abundance values for the *intl1* gene, related to class 1 integrons, were found in compost in pellet form and bokashi. The presence of this mobile genetic element has been reported to be closely associated with the emergence and spread of antibiotic resistance genes in soil (Binh et al., 2008). The *intl1* gene was proposed as a proxy for anthropogenic contamination, due to its relationship with antibiotic resistance genes, disinfectants and heavy metals (Gillings et al., 2015). According to Sun et al. (2016), anaerobic digestion generally reduces integron abundance, eliminating the aerobic hosts of such integrons.

Bokashi was the only amendment in which *Salmonella* was detected. The term bokashi refers to an anaerobic fermentation process that allows the generation of an amendment in just 2-3 weeks. Then, our bokashi amendment should be further hygienized by other means, in order to kill *Salmonella* strains.

5.4.2. Amendment quality

Most of the amendments surpassed the maximum legal limit of 40% moisture content. This criterion is mainly intended to ease the spreading of the amendment. In any event, non-composted or immature compost amendments are known to present potential adverse effects on crops (*e.g.*, inhibition of seed germination, weed problems, etc.) (García et al., 1992).

The OM content of bokashi and compost in pellet form was too low for them to be considered organic amendments by the Spanish legislation. Instead, composted organic sheep manure showed the highest content of OM, total nitrogen and extractable humic acids. Extractable humic acids, which include humic and fulvic acids, improve soil fertility, permeability, aeration and aggregation when applied to soil (Salman et al., 2005). Extractable humic acids and C/N ratios are normally measured to determine the stability and maturity of composted amendments: in this respect, maturity is related with plant-growth potential and phytotoxicity, while stability is associated with the microbial activity of the amendment (Iannottil et al., 1993).

Microbial parameters which reflect the activity, biomass and diversity of microbial communities can provide useful information regarding (i) the properties of organic amendments, and

(ii) the impact of contaminants and agricultural practices on soil quality (Epelde et al., 2010; Mijangos et al., 2010; Muñoz-Leoz et al., 2012; Pardo et al., 2014). Highest values of microbial activity (PMN) and biomass (MBC, 16S rRNA gene abundance) were found in composted intensive cow manure, suggesting that, when applied to agricultural soil, this amendment could stimulate soil microbial processes, such as, for instance, nutrient cycling. Ginting et al. (2003) reported that, 4 years after the last application, compost and manure application resulted in 20-40% higher soil MBC and 42-74% higher PMN, compared with the synthetic N fertilizer treatment.

Regarding the abundance of fungi and bacteria, bokashi showed the highest fungi-to-bacteria ratio. In general, fungi have longer lives than bacteria (Rousk & Bååth, 2007), and may be distinctive of mature and stable amendments. This seems to be contradictory with the faster elaboration time of bokashi, in comparison with the other amendments studied here. In any case, we could speculate that the anaerobic fermentation process carried out to obtain bokashi might have favoured fungal communities, compared to the composting process used with the other amendments.

In relation with CLPPs, Biolog EcoPlates^{TM} contain 31 carbon sources to measure the potential of the cultivable fraction of the heterotrophic bacterial community to metabolize them. Although vermicompost showed low values of microbial activity and biomass, it did show the highest values of area under the curve. This might be related to the presence of a more diverse bacterial community in vermicompost, capable of using a higher number of C sources, as a result of the higher variety of ingredients used during its production.

Finally, apart from the origin and nature of the ingredients, the composting process itself is a key determinant for the physicochemical and biological properties of the organic amendments. In fact, there is much interest in the temporal variability of the characteristics of the organic amendments during composting. In this respect, it is very important to perform analyses along the composting process in order to assess the maturity and stability of the amendment. In a recent study of the succession of bacterial community function during cow manure composting, Wang et al. (2018a) found that the AWCD of carboxylic acids and amino acids in Biolog EcoPlates^{TM} displayed a steady downtrend along the composting process. In a similar way, a significant change in bacterial DGGE pattern was detected during garbage composting (Takaku et al., 2006). Gomez et al. (2006) found higher values of AWCD, richness and H' diversity from Biolog EcoPlates^{TM} in soil amended with

household solid waste compost, horse and rabbit manure, and chicken manure, compared to unamended soil. An in-depth study of the effects of the application of amendments on soil quality and crop performance is certainly essential to determine their suitability.

5.5. Conclusion

Recommendations for the use of composted organic amendments must be based on the benefits and risks related to their characteristics. When assessing the suitability of this type of amendments for agricultural use, we propose that the first criterion must be the possibility of their containing potentially human health and environment-threatening contaminants. Both composted cow manures (from intensive and organic farms) were chosen according to this criterion. Secondly, the amendment with the highest value of the AQI should be considered. The AQI value could be further optimized by assigning numerical values to weigh the importance of each quality parameter and giving more value to key parameters such as macronutrient levels. Here, the highest AQI value corresponded to composted intensive cow manure. However, for organic agriculture, composted organic cow manure could be selected as the most suitable amendment.

Regulatory standards on the quality of organic amendments for agricultural use must be improved. At the moment, despite increasing awarenes of their well-known key role in nutrient cycling and mineralization, microbial properties are not considered, on a routine basis, when analysing amendment quality (except for some potential human pathogens, such as, for instance, *Escherichia coli* and *Salmonella* spp.). Besides, only measurements of heavy metal concentrations are usually required, without paying attention to many organic and emergent contaminants, due to, among other reasons, cost restrictions. There is a strong demand for reliable and simple diagnostic markers (a minimum set of variables) that integrate the most relevant information regarding the benefits and risks associated with the use of organic amendments in agriculture. In this sense, the abundance of the intergrase *intl1* gene appears a suitable biological diagnostic marker. Much efforts are still needed in this regard.

6. POLLUTION-INDUCED TOLERANCE OF SOIL BACTERIAL COMMUNITIES TO OXYTETRACYCLINE-SPIKED MANURE

Abstract

The use of manure as a fertilizer is a common agricultural practice that can improve soil physicochemical and biological properties while improving crop yields. However, antibiotics and their metabolites are often present in both manure and manure amended soil, leading to the adaptation of soil bacterial communities to their presence. The aim of this study was to assess the effects of the extensively used, broad-spectrum antibiotic oxytetracycline on soil microbial community adaptation using a pollution-induced community tolerance assay. Manure-amended soil was spiked with oxytetracycline (0, 2, 20, 60, 150, and 500 mg kg^{-1}) three times every ten days in the selection phase. The detection phase was conducted in Biolog EcoPlates with a second oxytetracycline exposure (0, 5, 20, 40, 60, and 100 mg L^{-1}). All treatments demonstrated decreased metabolic activity after exposure to ≥ 5 mg L^{-1} oxytetracycline during the detection phase. Meanwhile, a significant increase in tolerance was observed following exposure to ≥ 20 mg oxytetracycline per kg soil during the selection phase. Therefore, the pollution-induced community tolerance approach with Biolog EcoPlates was a useful system for the detection of antibiotic selection pressures on soil bacterial communities. It is important to properly manage animal waste before their application to the soil to reduce the antibiotic induced tolerance in the environment.

6.1. Introduction

Livestock production has increased to meet the rising demand for food, with animal biomass for feed far exceeding human biomass (Van Boeckel et al., 2015). Meat production worldwide has increased by 400% from 65 to 279 million tons in the last 50 years (Marques et al., 2018). Under these circumstances, 70% of the antibiotics used worldwide are administered to animals for veterinary or food production (Van Boeckel et al., 2017). In 2018, tetracyclines (31%), penicillins (29%), and sulfonamides (8%) accounted for 68% of the total antimicrobial sales for use in food-producing animals in 31 European countries (EMA/24309/2020). Antibiotics are poorly metabolized with approximately 30% to 90% excreted in urine and feces (Sarmah et al., 2006). For example, Winckler & Grafe (2001) recovered 72% of orally administered tetracyclines in swine manure within two days of application. Additionally, the following oxytetracycline (OTC) concentrations were found in various animal

manures: between 0.41 and 354 mg kg^{-1} in swine manure (Campagnolo et al., 2002; Chen et al., 2012), 871 mg kg^{-1} in calf manure (De Liguoro et al., 2003), and between 0.5 and 200 mg kg^{-1} in cow manure (Ince et al., 2013).

Antibiotics may be subjected to various processes in the soil environment, including sorption by soil components, transformation, photodegradation, and plant uptake and transport (Kumar et al., 2005; Sarmah et al., 2006; Kuchta et al., 2009; Kong et al., 2012; Reichel et al., 2013). Tetracyclines are particularly susceptible to adsorption by the soil matrix (Loke et al., 2002; Schmitt et al., 2005; Sarmah et al., 2006), resulting in low bioavailability. Antibiotic half-lives range from hours to several months depending on their molecular structure and soil physicochemical properties (Sarmah et al., 2006; Walters et al., 2010; Braschi et al., 2013). OTC is a broad-spectrum antibiotic that inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (Brodersen et al., 2000). However, microorganisms can also use antibiotics as a carbon source, resulting in increased microbial biomass (Thiele-Bruhn & Beck 2005) and activity (Liu et al., 2015). For example, Jiang et al. (2018) reported that the bacterial population increased in soil polluted with 5 mg kg^{-1} of OTC.

The application of animal organic wastes to agricultural soils can enhance soil quality and crop yield (Epelde et al. 2018; Urra et al. 2019a; Urra et al. 2020). However, this common agricultural practice can also lead to the spread of antibiotics, antibiotic-resistant bacteria, and/or antibiotic resistance genes in the soil (Chee-Sanford et al. 2009; Berendonk et al. 2015; Jauregi et al., 2021b).

Assessment of the potential risks of pollutants released into the environment is of utmost importance, particularly emerging contaminants such as antibiotics. Pollution-induced community tolerance (PICT) is an ecotoxicological tool that is used to determine the selective pressure of a pollutant and exerts a direct effect on the community (Blanck et al., 1988; Schmitt et al., 2005). A PICT assay comprises of the following two phases: (i) a selection phase where the soil bacterial community is exposed to a concentration gradient of the studied pollutant, and (ii) a detection phase where the soils are subjected to a second pollutant gradient for the evaluation of tolerance levels. The increase in tolerance could be due to: (i) the replacement of sensitive species by more tolerant species, (ii) physiological changes that make the organisms less sensitive, or (iii) genetic changes through horizontal gene transfer (HGT) to acquire mobile genetic elements encoding for enhanced resistance (Schmitt et al., 2005). Cause-effect relationships between several pollutants and microbial communities

using PICT are established for antibiotics (Schmitt et al., 2004; Brandt et al., 2009; Fang et al., 2018), herbicides (Zabaloy et al., 2010), metals (Stefanowicz et al., 2009; Aliasgharzad et al., 2011), and metals that confer co- or cross-tolerance to other pollutants (Wakelin et al., 2014; Li et al., 2015c; Santás-Miguel et al., 2020).

The main objective of this study was to evaluate the effects of OTC exposure on soil bacterial communities in a PICT assay using Biolog EcoPlates. The effects of OTC exposure on soil bacterial metabolic activity, the number of utilized substrates, and the Shannon diversity index were analyzed. The OTC concentrations at which the metabolic activity decreased by half (EC_{50}) and the tolerance of soil bacterial communities was also assessed. The novel aspect of this study could be highlighted by the fact that we performed simulation of a repeated application of antibiotics into soils through manure application under controlled conditions. The acquired tolerance to successive applications of OTC is of interest from the point of view of bacterial community evolution. It is hypothesized that soils exposed to higher OTC concentrations demonstrate increased tolerance to OTC.

6.2. Material and methods

6.2.1. Collection and characterization of soil and manure

Soil sample for the experiment was collected from the upper 30-cm layer of a semi-natural grassland field located in Derio, northern Spain, which has never been amended with inorganic or organic fertilizer to our knowledge. The soil was air-dried at 30 °C for 48 h, following which it was sieved to < 4 mm and was subjected to physicochemical characterization according to standard methods (MAPA, 1994). It was characterized as a clay loam with a pH of 6.2, 6.3% organic matter (OM), 0.32% total N content, with Olsen P and K^+ content at 3.4 and 395 mg kg^{-1} dry weight (DW) soil, respectively.

The aged manure (~two years old) from heifers was kindly provided by an organic dairy farm located in the province of Biscay, Spain, without a known history of antibiotic treatment. It was collected in polyethylene bags, following which it was immediately transferred to the laboratory and stored at 4 °C; then, it was sieved to < 4 mm and subjected to physicochemical characterization (MAPA 1994). It had a pH of 8.7, 66% OM, 0.77% total N content, with Olsen P and K^+ content of 9.9 and 25 mg kg^{-1} DW soil, respectively. The following metal concentrations were determined following aqua

regia digestion (McGrath & Cunliffe 1985): 0.93, 24, 25, 13, 61, and 303 mg kg^{-1} DW soil for Cd, Cr, Cu, Ni, Pb, and Zn, respectively.

6.2.2. Experimental design of the selection phase

Aged manure was manually incorporated into pots with one kg of soil and was thoroughly mixed to obtain an equivalent of 100 kg N ha^{-1} (to simulate the amount of required N for the lettuce crop) except for the control treatment (OTC0-M, no OTC and no manure). The pots were incubated at room temperature in the dark and allowed to exchange air for 55 days. During incubation, distilled water was added every week to maintain constant soil moisture.

Soil communities were exposed to a gradient concentration of OTC (CAS 2058-46-0, ≥95% purity, Merck) in the selection phase of the PICT assay. Each pot was contaminated by spiking with 60 mL OTC solution to obtain final concentrations of 2, 20, 60, 150, and 500 mg OTC per kg soil (OTC2, OTC20, OTC60, OTC150, and OTC500, respectively).

This spiking procedure was repeated three times on days 0, 10, and 20. OTC0-M and the other control pot containing manure with no OTC (OTC0) received an equivalent amount of distilled water. Each treatment was performed in triplicate.

6.2.3. Detection phase with Biolog EcoPlates

A second OTC gradient was established in the detection phase to reveal differences in community tolerance using 96-well Biolog EcoPlates. The plates contained a triplicate set of 31 relevant carbon sources for environmental samples (Insam, 1997). Fresh soil equivalent to 5 g DW was added to 50 mL of autoclaved Milli-Q water, following which the mixture was agitated for 1 h in an orbital shaker (220 rpm) and then allowed to settle for 5 min. Subsequently, 450 µL of the liquid was mixed with 30 mL of Milli-Q water. This solution (100 µL) was mixed with OTC (20 µL), and then the mixture was aliquoted into each well to obtain the following six final OTC concentrations: 0, 5, 20, 40, 60, and 100 mg L^{-1}. The plates were incubated at 30 °C for 14 days (336 h), followed by color development and absorbance measurement at 595 nm using a microplate reader (Anthos Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria).

6.2.4. Data processing

Average well color development (AWCD) was determined by calculating the mean absorbance values of each treatment at each time point. The absorbance value for each well was corrected by subtracting the zero hour time point and the blank control for each reading time (Epelde et al., 2008). The values corresponding to the incubation time of the midpoint of the exponential portion of the curve representing the highest microbial growth rate were selected for further calculations. The number of utilized substrates (NUS) was calculated when the absorbance value was > 0.1 (Epelde et al., 2008). Similarly, Shannon's diversity (H') index was determined by considering the absorbance values at each well as equivalent to species abundance. Nonlinear curve fitting based on the Gompertz function was performed to yield the kinetic parameters (Lindstrom et al., 1998):

$$y = OD_{595\,nm} = \frac{K}{(1 + e^{-r(t-s)})}$$

where *K* represents the asymptote that the absorbance curve approaches, *r* represents the exponential rate of absorbance changes, *t* represents the time following microplate inoculation, and *s* represents the time to the midpoint of the exponential portion of the curve when y = K/2. OTC concentrations that reduced the color formation to 50% of the maximum (EC_{50} values) were determined. Bacterial community tolerance was quantified using an adapted tolerance index (TI) (Brandt et al., 2009):

$$TI = \frac{AUC_{OTC}}{AUC_{Control}} - 1$$

where AUC_{OTC} equals the average area under the curve of AWCD in triplicate samples amended with OTC, and $AUC_{Control}$ represents the corresponding average area in triplicate control treatments (OTC0-M). The integral of the Gompertz function based on the trapezoidal method was used to calculate the area under the curve using the pracma R package (Borchers, 2021).

Statistically significant differences between treatments ($p < 0.05$) in AWCD, NUS, H', EC_{50}, and TI were established using ANOVA and Tukey's post-hoc test using the agricolae R package (de Mendiburu, 2020).

6.3. Results

Dose-response curves showed that the application of ≥ 5 mg L^{-1} OTC in the detection phase inhibited the average metabolic activity of the soil bacterial communities in all treatments (Figure 6.1). The most considerable reduction in activity was observed in the OTC0-M, OTC0, OTC2, OTC20, and OTC60 treatments when the OTC concentration was between 5 and 20 mg L^{-1}. The most substantial reduction in metabolic activity with the OTC150 and OTC500 treatments was observed when the OTC concentration was between 20 and 40 mg L^{-1}. Both treatments presented significantly higher AWCD values than the OTC0-M, OTC0, and OTC2 treatments. Overall, the dose-response curves of the treatments with a lower OTC concentration in the selection phase showed a faster reduction in the AWCD values compared to higher OTC concentration treatments.

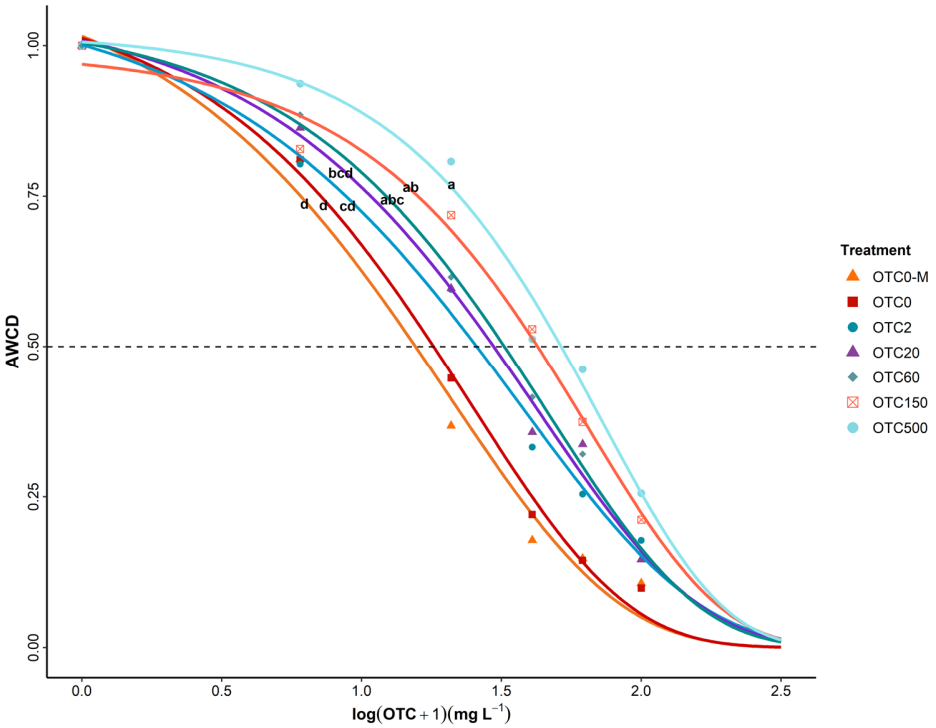


Figure 6.1. Dose-response curves showing OTC inhibition of the metabolic activity of soil bacterial communities in the detection phase fitted with the Gompertz function ($R^2 > 0.9$). Data have been expressed as average AWCD values (n=3). Different letters represent significant differences ($p < 0.05$) according to Tukey's *post hoc* test.

Dose-response curves for the NUS by the bacterial communities were obtained at different OTC concentrations in the detection phase (Figure 6.2). In the OTC0-M, OTC0, and OTC2 treatments, the NUS decreased by approximately half at 20 mg L^{-1} OTC. Meanwhile, the NUS decreased by

approximately half in the OTC150 and OTC500 treatments at 60 mg L^{-1} OTC. OTC150 and OTC500 exhibited a significantly higher NUS than the OTC0-M, OTC0, and OTC2 treatments. Overall, the NUS decreased as the OTC concentration in the detection phase increased.

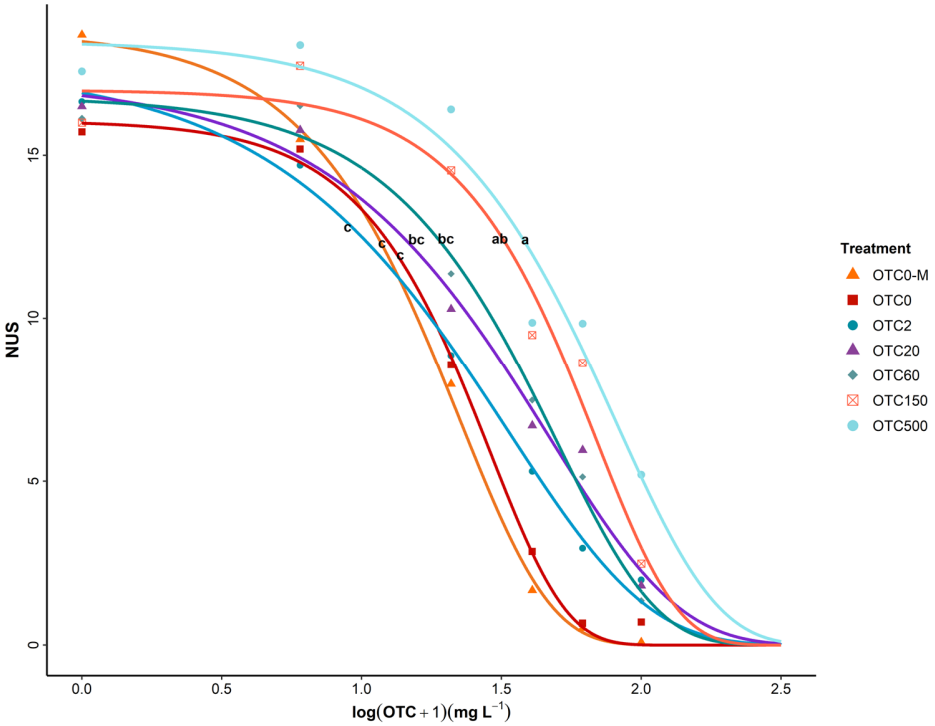


Figure 6.2. Dose-response curves showing OTC inhibition on the average number of utilized substrates (NUS) in the detection phase fitted with the Gompertz function (n=3). Different letters represent significant differences ($p < 0.05$) according to Tukey's *post hoc* test.

Dose-response curves of each treatment with respect to the Shannon diversity index against different antibiotic concentrations in the detection phase are shown in Figure 6.3. When both OTC0-M and OTC0 treatments were exposed to 20 mg OTC L^{-1}, a notable decrease in H' was observed. The H' curve was similar in the OTC20, OTC60, OTC150, and OTC500 treatments, with each of these treatments having a significantly higher H' than the OTC0-M and OTC0 treatments. In summary, OTC exerted an inhibitory effect on H' and decreased as the antibiotic concentration in the detection phase increased.

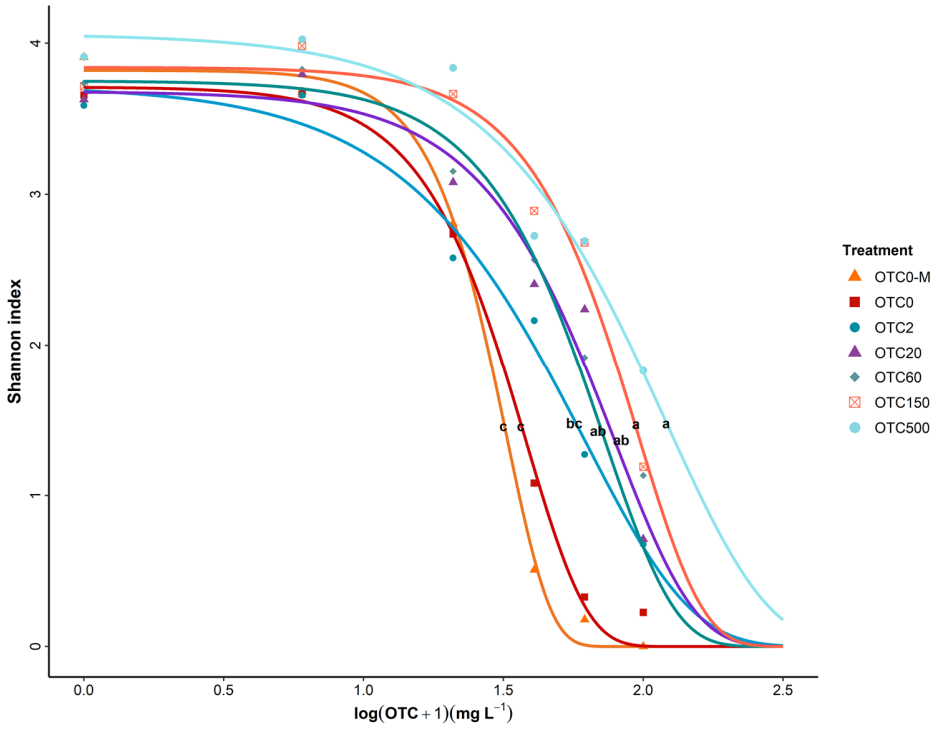


Figure 6.3. Dose-response curves of OTC inhibition on the average Shannon diversity index value (n=3) in the detection phase fitted with the Gompertz function. Different letters represent significant differences ($p < 0.05$) according to Tukey's *post hoc* test.

The following average EC_{50} values represent the OTC concentrations required to reduce the microbial activity by half in the tested treatments: 15.6, 17.1, 24.5, 30.2, 32.1, 38.9 and 49.0 mg L^{-1} for OTC0-M, OTC0, OTC2, OTC20, OTC60, OTC150, and OTC500, respectively (Figure 6.4). OTC150 and OTC500 treatments presented significantly higher EC_{50} values than the OTC0-M treatment. In summary, higher OTC concentration in the selection phase resulted in the requirement of greater OTC concentrations (higher EC_{50}) in the detection phase to reduce the activity of soil bacterial communities by half.

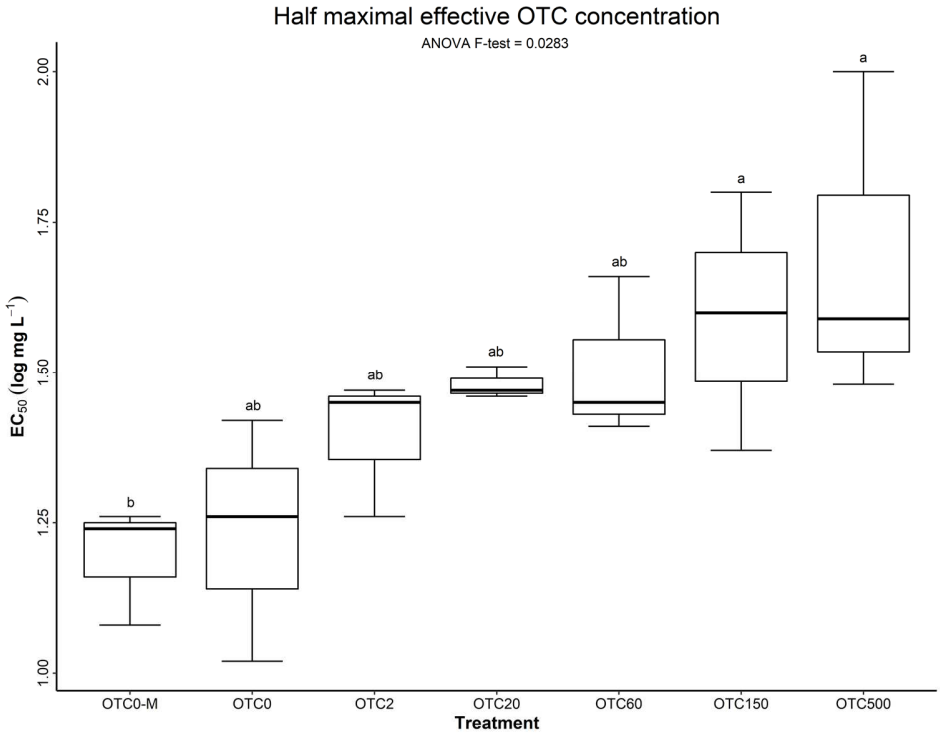


Figure 6.4. OTC concentrations that reduced the AWCD to 50% of the maximum AWCD (EC50 values) have been fitted with the Gompertz function (n =3). Different letters represent significant differences ($p < 0.05$) according to Tukey's *post hoc* test.

The following average TI values were found: 0, 0.04, 0.13, 0.18, 0.19, 0.29, and 0.38 for OTC0-M, OTC0, OTC2, OTC20, OTC60, OTC150, and OTC500, respectively (Figure 6.5). Furthermore, OTC150 and OTC500 presented significantly higher TIs than OTC0-M, OTC0, and OTC2 treatments. Therefore, the TI values increased as the OTC concentration in the selection phase increased.

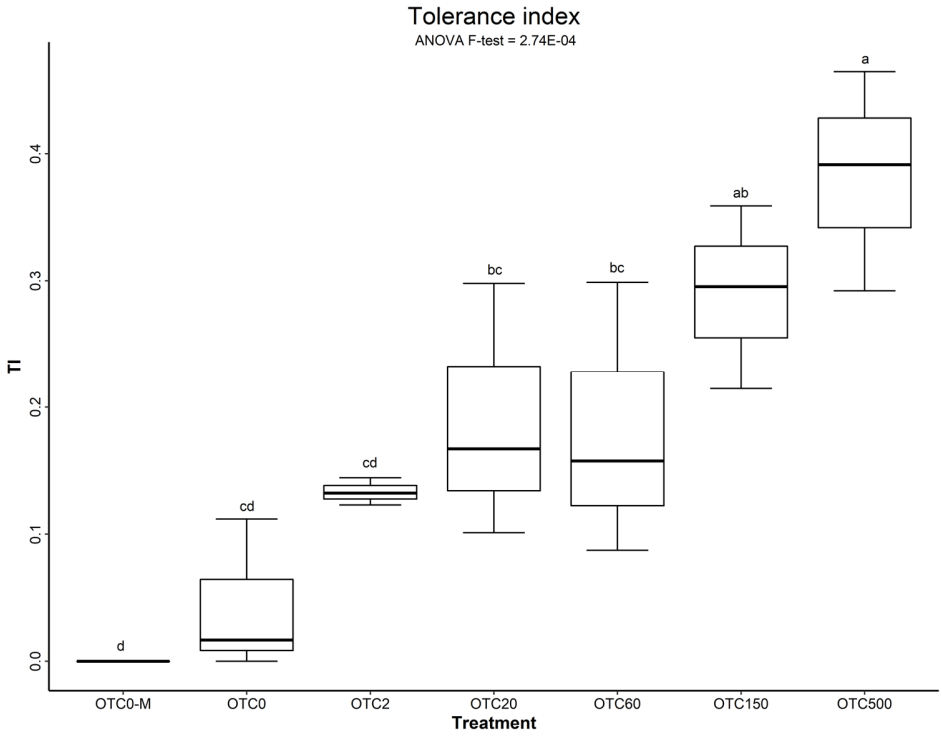


Figure 6.5. Bacterial community tolerance index (TI) to OTC (n=3). Different letters indicate statistically significant differences ($p < 0.05$) according to Tukey's *post hoc* test.

6.4. Discussion

Soil microorganisms play essential roles in soil function, such as organic matter decomposition, nutrient cycling, and formation of soil structure (Loreau, 2001; Nannipieri et al., 2003; Bronick & Lal, 2005), and are extremely sensitive to slight environmental fluctuations. Thus, soil microbial parameters are deemed suitable indicators for determining disturbances in soil quality (Epelde et al., 2010; Garbisu et al., 2011). Changes in the absorbance values of Biolog EcoPlates are indicators of the overall metabolic activity of culturable heterotrophic bacteria possessing the ability to utilize different carbon substrates (Ma et al., 2016). Although only a limited proportion of the total community exhibits responses to this growth-dependent method (Smalla et al., 1998), it can help detect toxicant effects on soil communities (Epelde et al., 2008). In this study, the PICT assay and EcoBiolog approach were considered to assess whether OTC addition to soils exerted impact on microbial communities. In fact, the assessment of tolerance to a chemical helps infer causal relationships between exposure and effects (Tlili et al., 2015).

In this study, OTC inhibited metabolic activity in all treatments. However, treatments exposed to increasing OTC in the selection phase required higher OTC concentrations in the detection phase to reduce their metabolic activity. The same response was observed with the NUS and the H' index. A concentration of 5 mg L^{-1} of OTC resulted in an overall reduction of metabolic activity, while 20 mg L^{-1} of OTC was necessary for reductions in the NUS and the H' index. This indicated that although the metabolic activity of certain microorganisms was affected by exposure to 5 mg L^{-1} of OTC, the soil microbial community utilized substrates in a similar way until 20 mg L^{-1} of OTC. The soils in this study presented higher H' values than those reported in soils amended with pig manure contaminated with up to 200 mg kg^{-1} of OTC (Liu et al., 2012).

The EC_{50} helps estimate the concentration of a toxicant that causes a 50% reduction of test populations against a specific endpoint under particular conditions (Rozman et al., 2010). An EC_{50} of 79 mg sulfachloropyridazine kg^{-1} soil amended with pig slurry was found in a PICT assay with Biolog EcoPlates (Schmitt et al., 2005). The EC_{50} values in this study ranged from 15.6 to 49.0 mg L^{-1} for the non-OTC-exposed soil in the selection phase to the soil treated with the highest OTC concentration (500 mg kg^{-1}), respectively. This indicated that soil exposed to higher OTC concentration in the selection phase required higher OTC concentrations in the detection phase to reduce their microbial metabolic activity by half.

In this study, the tolerance of soil microbial communities to OTC increased with higher OTC concentrations in the selection phase which correlated with findings reported in previous studies conducted using antibiotics such as sulfachloropyridazine, tylosin, sulfadiazine, carbendazim and ciprofloxacin (Schmitt et al., 2004; Demoling & Bååth, 2008; Fang et al., 2014; Fang et al., 2016; Han et al., 2019; Santás-Miguel et al., 2020). Thus, repeated OTC spiking of a microbial community that is sensitive to antibiotic results in increased tolerance. In culture-based studies, antibiotic resistance is considered when soil bacteria grow at 20 mg L^{-1} (D'Costa et al., 2006; Bhullar et al., 2012). In our case, a significant increase in tolerance was observed, beginning from 20 mg kg^{-1} OTC exposure during the selection phase. These results indicated that repeated OTC application over time in the selection phase promoted the adaptation of soil microbial communities due to selective pressure (Fang et al. 2014). Ppb levels of tetracycline concentrations statistically increase the prevalence of HGT from *E. coli* to activated sludge (Kim et al., 2014). Similarly, pretreatment of sewage effluent populations with a very low 10 µg L^{-1} of tetracycline increases the prevalence of HGT by four-fold compared with

untreated populations (Jutkina et al., 2016). Therefore, exposure to non-lethal antibiotic concentrations reported in this study and previous studies may have induced the survival of tolerant bacteria through HGT (Andersson & Hughes, 2012; Liu et al., 2017).

In another respect, differences between the treatment amended with aged manure and the unamended treatment were expected since the presence of metals in aged manure might promote the spread of antibiotic resistance. This occurs via the following co-selection mechanisms: (i) co-resistance, when different resistance systems are present in the same genetic element, and (ii) cross-resistance, when one resistance system confers resistance to both a metal and an antibiotic (Baker-Austin et al., 2006). However, statistically significant differences were not found between the treatments.

6.5. Conclusion

Repeated short-term contamination of soils by manure amended with OTC promoted antibiotic tolerance in microbial communities with possible adverse effects exerted on the environment. The repeated application of organic amendments containing antibiotics to soils is a common agricultural practice. Its use should be limited well below 20 mg OTC per kg soil to avoid an increase in tolerance. To this end, it is important to effectively manage animal waste and to decrease the amount of antibiotics present in manure before applying them to the soil.

Although PICT is a sensitive method used for detecting changes in soil microbial communities due to OTC exposure, it is difficult to determine the cause of this tolerance. A metagenomic analysis may provide insights into the structure of microbial communities and their adaptation processes along the OTC gradient.

7. ANTIBIOTIC RESISTANCE IN AGRICULTURAL SOIL AND CROPS ASSOCIATED TO THE APPLICATION OF COW MANURE-DERIVED AMENDMENTS FROM CONVENTIONAL AND ORGANIC LIVESTOCK FARMS

Abstract

The application of organic amendments to agricultural soil can enhance crop yield, while improving the physicochemical and biological properties of the recipient soils. However, the use of manure-derived amendments as fertilizers entails environmental risks, such as the contamination of soil and crops with antibiotic residues, antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). In order to delve into these risks, we applied dairy cow manure-derived amendments (slurry, fresh manure, aged manure), obtained from a conventional and an organic farm, to soil. Subsequently, lettuce and wheat plants were grown in the amended soils. After harvest, the abundance of 95 ARGs and MGE-genes from the amended soils and plants were determined by high-throughput qPCR. The structure of soil prokaryotic communities was determined by 16S rRNA amplicon sequencing and qPCR. The absolute abundance of ARGs and MGE-genes differed between treatments (amended *vs.* unamended), origins of amendment (conventional *vs.* organic) and types of amendment (slurry *vs.* fresh manure *vs.* aged manure). Regarding ARG-absolute abundances in the amendments themselves, higher values were usually found in slurry *vs.* fresh or aged manure. These abundances were generally higher in soil than in plant samples, and higher in wheat grain than in lettuce plants. Lettuce plants fertilized with conventional amendments showed higher absolute abundances of tetracycline resistance genes, compared to those amended with organic amendments. No single treatment could be identified as the best or worst treatment regarding the risk of antibiotic resistance in soil and plant samples. Within the same treatment, the resistome risk differed between the amendment, the amended soil and, finally, the crop. In other words, according to our data, the resistome risk in manure-amended crops cannot be directly inferred from the analysis of the amendments themselves. We concluded that, depending on the specific question under study, the analysis of the resistome risk should specifically focus on the amendment, the amended soil or the crop.

7.1. Introduction

Antibiotics are indispensable tools for the treatment of bacterial infections in human medicine and veterinary medicine. Antibiotics are mainly used for the curative and, to a lesser extent, preventive treatment of bacterial infectious diseases. Besides, they are also used in many countries as growth promoters in animal production farms (Chowdhury et al., 2014). However, the use of antibiotics for disease prevention is not recommended by the Word Health Organization (2017) and the European Union banned the use of antibiotics for animal growth promotion in 2006 [Regulation (EC) No. 1831/2003]. The use, abuse and inappropriate use of antibiotics (i) in livestock farms for animal production purposes, (ii) in human medicine for the treatment of bacterial infections, and (iii) in agriculture for crop production purposes is gradually causing the emergence and dissemination of antibiotic resistant bacteria (some of them show simultaneous resistance to many - *multiresistant* - or even all - *panresistant* – known antibiotics), due to the selective pressure exerted by antibiotics on exposed bacterial populations. Many antibiotics used in veterinary practice are the same used to treat bacterial infections in humans or have the same mode of action or belong to the same antibiotic family (Moulin et al., 2008), leading to the alarming intensification and augmentation of the well-known huge problem of multiresistant bacterial strains currently putting at risk, at a global scale, our capacity to fight and control bacterial human pathogens (WHO, 2014).

Most antibiotics administered to livestock are not fully metabolized and, hence, are released, together with their transformation products, into the environment along with the faeces and urine (Kumar et al., 2005). In fact, a considerable percentage (30-90%) of the antibiotic administered to a given animal for veterinary purposes can be directly excreted in the urine and faeces (Kumar et al., 2005). Animal manure is therefore a source of antibiotic contamination (antibiotics are nowadays considered emerging contaminants) and a reservoir of antibiotic resistant bacteria (ARB) harbouring and potentially spreading antibiotic resistance genes (ARGs) (Zhu et al., 2013). Animal manure is commonly applied to agricultural soil as organic fertilizer. Apart from providing valuable plant nutrients that can enhance crop yield, the application of manure can simultaneously improve soil physicochemical and biological properties, *i.e.* soil quality (Fließbach et al., 2007; Epelde et al., 2018; Urra et al., 2019a). Regrettably, the agronomic application of manure can also lead to the emergence and dissemination of ARB and ARGs in the amended agricultural soil and, subsequently, in the food crops grown for human consumption (Marti et al., 2013; Wang et al., 2015b). To make matters worse,

ARB can disseminate ARGs to other bacteria through horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs), such as integrons, phages, plasmids, integrative conjugative elements, transposons, etc. (Heuer et al., 2011; Jechalke et al., 2014).

Understandably, most of the attention given to the problem of antibiotic resistance (AR) has been directed to hospital settings. Nonetheless, in the last years, more and more awareness is being developed concerning the vastly complex environmental dimension of AR and its central role in the emergence, maintenance and spread of AR at a global scale (Larsson et al., 2018). Undeniably, the emergence and dissemination of AR in agroecosystems, resulting from the application of animal manure as organic fertilizer, begets a potential risk for human health and the environment, being currently an issue of much global concern that, urgently, requires the development and implementation of practices and management measures that mitigate (or, better, eliminate), such a risk (Pruden et al., 2013). Among other measures aimed at enhancing the sustainability of animal production practices, organic livestock farming promotes a considerable reduction of the use of antibiotics for veterinary purposes, compared to conventional livestock systems. In principle, this reduction in antibiotic use implies concomitantly a lower level of selective pressure for bacterial populations to acquire and maintain AR by evolutionary adaptation mechanisms (Heinemann et al., 2000). In addition, the composting of animal manure has recurrently been reported as an effective option for the reduction of antibiotic concentrations in animal manure and, to a lesser extent, for the decrease in the abundance of ARGs in these animal-derived organic amendments (Gou et al., 2018; Qian et al., 2018).

On the other hand, the presence of antibiotics and their transformation products (some of these are also bioactive compounds) in animal manure may significantly alter the composition of soil microbial communities when applied to agricultural soil. These antibiotic-induced changes in soil microbial composition frequently have important consequences for the soil resistome and mobilome (Gillings, 2013; Nesme & Simonet, 2015). Relevantly, soil microbial diversity (in terms of richness, evenness, composition, etc.) is regularly used as a biological indicator of the impact of disturbances (*e.g.*, contamination) on soil health (Epelde et al., 2010; Garbisu et al., 2011; Burges et al., 2017).

Our objective was to study, under controlled microcosm conditions, the emergence and dissemination of AR in agricultural soil and food crops (lettuce and wheat) derived from the application of dairy cow wastes as organic fertilizer. In order to delve into possible management practices that

could minimize the resistome risk, we compared the effects of the application of: (i) three types of commonly used amendments: slurry *vs.* fresh manure *vs.* aged manure; and (ii) amendments from a conventional livestock farm *vs.* an organic livestock farm. To quantify the magnitude of the resistome risk in agricultural soil and food crops, we used the following end-points: (i) antibiotic concentrations; (ii) abundance of ARGs and MGE-genes in soils and plants (lettuce and wheat grain); and (iii) observed relationships between the structural diversity of soil prokaryotic communities (from 16S rRNA amplicon sequencing data) and the abundance of ARGs and MGE-genes. We hypothesized that the resistome risk will be higher in soils and plants: (i) amended with dairy cow wastes, compared to non-amended controls; (ii) amended with dairy cow wastes from the conventional livestock farm *vs.* the organic livestock farm; and (iii) amended with slurry wastes *vs.* fresh and aged wastes. We also hypothesized that lettuce samples will show a higher resistome risk than wheat grain samples.

7.2. Materials and methods

7.2.1. Experimental design

The amendments used in this study were kindly provided by two dairy cow farms located in the province of Biscay (Spain): a conventional livestock farm and an organic livestock farm. Three types of amendments (*i.e.*, slurry, fresh manure, aged manure) from these two different origins (*i.e.*, conventional livestock farm and organic livestock farm) were studied. In both farms, representative samples of cow slurry were taken from a pool where the faeces and urine from the cows in production were deposited. In contrast, fresh manure samples were taken from the cow bedding (made from faeces, urine and wheat straw) of the non-producing cows: heifers, dry cows and cows undergoing treatment (the latter only in the conventional farm). As for the aged manure, a composite sample was taken from a manure pile that had been stored for approximately 6 months. All samplings were carried out on the same day. Fresh and aged manure samples were collected in polyethylene bags, while slurry samples were contained in plastic barrels. All samples were immediately transferred to the laboratory and stored at 4ºC until use. The experimental soil was collected from the upper 30 cm layer of a semi-natural grassland field which, to our knowledge, has never been amended with any kind of inorganic or organic fertilizer. Immediately after collection, the soil was sieved to <4 mm. For our microcosm study, experimental pots containing 2 and 4 kg of dry weight (DW) soil were used for lettuce and wheat plants, respectively. The dose of amendment was carefully adjusted in order to provide an equivalent of 100 and 180 kg N ha^{-1} for lettuce and wheat plants, respectively. The amendments were manually

incorporated into the soil and thoroughly mixed for homogenization purposes. A 2-week stabilization period was allowed before crop planting (lettuce seedlings) or sowing (wheat seeds). Lettuce (*Lactuca sativa* L. var. Batavia) and hard winter wheat (*Triticum aestivum* L. var. Qualidu) plants were used in this study, since they are most commonly grown in our region for agricultural purposes. Our experiment was carried out in a growth chamber under the following controlled conditions: 14/10 h light/dark cycle, 20/16ºC day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 µmol photon m^{-2} s^{-1}. Throughout the experimental period, plants were bottom watered every 2-3 days. Each treatment was replicated four times. Lettuce plants were harvested after 44 days of growth, while wheat plants were harvested after 171 days. For the determination of crop production, lettuce plants (aerial part = shoot biomass) were cut from the base with a scalpel and then freshly weighed. Similarly, in wheat plants, spikes were husked, and wheat grains freshly weighed. Dry weight of lettuce plants and wheat grains was determined by drying in an oven at 70ºC until reaching a constant mass. On the other hand, soil samples were collected from the pots at crop harvest time (see below section 7.2.3).

7.2.2. Amendment and soil physicochemical characterization

Before the beginning of the experiment, the dairy cow manure-derived amendments and the experimental semi-natural grassland soil were physicochemically characterized (MAPA, 1994) according to the following parameters: pH, organic matter (OM) content, total nitrogen (N), potassium (K^+) and Olsen phosphorus (P). Dry weight of soils was determined by drying in an oven at 30ºC until reaching a constant mass. Mineral and pseudo-total metal concentrations were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) following aqua regia digestion (McGrath & Cunliffe, 1985). Antibiotic concentrations were determined by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) in SAILab Instrumental Analytical Solutions (Barcelona, Spain). In particular, the concentration of 57 antibiotics belonging to 9 families (aminoglycosides, cephalosporins, macrolides, nitrofurans, penicillins, polypeptides, quinolones, sulfonamides and tetracyclines) was quantified. For confirmation purposes and in order to assess the rate of degradation of the antibiotics present in the manure and soil samples, a second analysis of antibiotic concentrations was carried out two months later. In this second analysis, the antibiotic families that, in the first analysis, exceeded the detection limit of the technique in at least one of the studied antibiotics (*i.e.*, polypeptides and quinolones) were again analysed.

7.2.3. Effect of treatments on biological parameters related to the resistome risk

For the assessment of the effect of treatments on biological parameters that provide information on resistome risk, at crop harvest time, soil samples were collected from the experimental pots and then sieved to <2 mm. Prior to DNA extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk et al., 1997). DNA was extracted from soil samples (0.25 g DW soil) using the Power Soil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Similarly, DNA was extracted from plant samples using the innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). The concentration of soil and plant DNA was quantified with a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). Soil and plant DNA was stored at -20ºC until use.

For the quantification of ARG and MGE-gene abundances, high-throughput real-time PCR (HT-qPCR) reactions were performed using the nanofluidic qPCR BioMark™ HD system, with 48.48 and 96.96 Dynamic Array Integrated Fluidic Circuits (ICFs) (Fluidigm Corporation) following Urra et al. (2019b). A total of 96 validated primer sets (Hu et al., 2016) were used, including 85 primer sets targeting ARGs conferring resistance against all major classes of antibiotics [10 aminoglycosides, 14 β-lactams, 5 FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol), 13 MLSB (macrolide, lincosamide, streptogramin B), 5 multidrugs (*i.e.*, those conferring resistance to more than one antibiotic), 4 sulfonamides, 24 tetracyclines and 10 vancomycines], 10 primers sets targeting MGE-genes (8 genes encoding transposases and 2 genes encoding integrases), and the 16S rRNA as reference gene. DNA samples were pre-amplified with a pool of primers (final concentration for each primer pair = 50 nM; 16 PCR cycles) and then treated with exonuclease I. Subsequently, 1:10 dilutions of specific target amplification reactions were loaded onto the Dynamic Array IFCs, following the Fluidigm's Fast Gene Expression Analysis - EvaGreen® Protocol. The SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, Redmond, WA) was used for amplification (with a final primer concentration, both forward and reverse, of 500 nM). The cycling program consisted of 1 min at 95°C, followed by 30 cycles at 95°C for 5 seconds and 60°C for 20 seconds, followed by a melting curve. Four replicates were included for each sample. Measurements were conducted in the Gene Expression Unit of The Genomics Facility of SGIker – University of the Basque Country, Spain. Raw data obtained from the analysis were processed with the Fluidigm Real-Time PCR Analysis Software (v.3.1.3) with linear baseline correction and manual threshold settings.

A threshold cycle, C_T value, of 31 was chosen since the highest C_T value obtained in our study was 30.53. A detection of an ARG or MGE-gene was considered positive when 3 out of the 4 technical replicates for each sample were above the detection limit. The value of the detection limit was used for non-amplified genes. Furthermore, a comparative C_T method was used to calculate ARG and MGE-gene relative abundances, normalized to the abundance of the 16S rRNA control gene, expressed as fold-change (FC) (Livak & Schmittgen, 2001):

$$\Delta C_T = C_{T(target\ gene)} - C_{T(16S\ rRNA\ gene)}$$

$$\Delta\Delta C_T = \Delta C_{T(amended\ sample)} - \Delta C_{T(unamended\ sample)}$$

$$FC = 2^{-\Delta\Delta C_T}$$

Real-time PCR measurements of the abundance of the 16S rRNA gene were performed to estimate total bacterial biomass, following the reaction mixtures and PCR conditions described in Epelde et al. (2014). The relative copy number (GR) was calculated as the proportion of the abundance of the ARG or MGE-gene to the abundance of the 16S rRNA gene (Looft et al., 2012). Absolute ARG and MGE-gene abundances ($GA_{ARG,MGE}$) were calculated as follows (Zhu et al., 2017a):

$$GR = 10^{\frac{(31-C_T)}{(10/3)}}$$

$$GA_{ARG,MGE} = \frac{GA_{16S} \times GR_{ARG,MGE}}{GR_{16S}}$$

In order to assess the impact of amendments on soil prokaryotic community composition, the preparation of amplicon libraries was carried out using a dual indexing approach with sequence-specific primers (Lanzén et al., 2016) targeting the V4 region of the 16S rRNA gene: primers 519F (CAGCMGCCGCGGTAA) adapted from Øvreås et al. (1997) and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso et al. (2012). Sequencing was performed with an Illumina MiSeq V2 platform and paired-end sequencing strategy (2 × 250 nt) at Tecnalia, Spain. Read paired ends were merged, quality filtered and clustered into operational taxonomic units (OTUs) as described in Lanzén et al. (2016). The taxonomic classification was performed using CREST (Lanzén et al., 2012).

7.2.4. Statistical analysis

One-way ANOVA with Duncan's multiple-range tests was performed to compare absolute abundance values of ARGs and MGE-genes among treatments: *type of amendment* = slurry *vs.* fresh manure *vs.* aged manure, and *origin of amendment* = conventional livestock farm *vs.* organic livestock farm. Identical analyses were performed for crop production data. The effect of the experimental factors (*type* x *origin*) was tested by two-way ANOVA using package *agricolae* of R software (v.3.6.3). R package *vegan* (Oksanen et al., 2015) was used to calculate α-diversity indices (*i.e.*, richness, Shannon´s, Simpson´s, Pielou´s) for soil prokaryotic diversity data and 16S rRNA amplicon sequencing data visualization. Principal component analysis (PCA) of ARG and MGE-gene absolute abundances was performed using Canoco 5 (Ter Braak & Ŝmilauer, 2012). Venn diagram was performed to examine the overlapping, in terms of the presence of ARGs and MGE-genes, between soil and plant samples with *venn* package in R. Kendall's rank correlation coefficients, followed by Bonferroni's multiple comparisons test, between soil prokaryotic taxa (at order level) and absolute abundances of ARGs and MGE-genes (grouped by antibiotic family and MGE category) were obtained using R software.

7.3. Results

7.3.1. Amendment and soil physicochemical characterization

The soil was characterized as a clay loam, with a pH of 6.2, an OM of 6.3%, a total N content of 0.32%, an Olsen P content of 3.4 mg kg^{-1} DW soil, and a K^+ content of 395 mg kg^{-1} DW soil. Regarding the physicochemical properties of the dairy cow manure-derived amendments (Table 7.1), we observed that: (i) amendments from the conventional farm showed higher OM content than those from the organic farm; (ii) all pH values ranged between 8.2 and 9.4; (iii) slurry samples from both the conventional and organic farm showed higher N content, compared to fresh and aged manure; (iv) Pb, Cr and Ni concentrations were higher in fresh and aged manure from the organic *vs.* the conventional farm; and (v) the following metal concentration gradient for Pb, Cr and Ni was observed in the amendments from both the conventional and organic farm: aged manure > fresh manure > slurry.

Table 7.1. Physicochemical properties of the amendments.

	ORGANIC FARM			CONVENTIONAL FARM		
	Aged manure	Fresh manure	Slurry	Aged manure	Fresh manure	Slurry
Dry matter (%)	29.87	21.74	7.30	17.51	18.51	12.12
OM (%)	41.33	67.48	74.62	78.30	80.20	83.41
pH (1:25)	9.41	9.29	8.40	8.48	9.16	8.25
N (%)	2.78	3.45	3.97	1.94	2.32	3.38
Olsen phosphorus (g kg^{-1})	5.61	8.12	6.36	3.65	4.83	6.66
Potassium (g kg^{-1})	37.21	33.56	43.43	16.67	22.06	25.03
Cd (mg kg^{-1} DW)	0.62	0.71	0.40	0.50	0.36	0.15
Pb (mg kg^{-1} DW)	150.88	36.95	17.59	4.14	3.31	1.55
Cr (mg kg^{-1} DW)	51.80	22.32	11.58	17.58	15.23	13.54
Ni (mg kg^{-1} DW)	27.16	11.05	6.07	8.93	8.25	7.69

Concerning antibiotic concentrations in the amendments and the semi-natural grassland soil (Table 7.2), in the first analysis, colistin was detected in fresh and aged manure from both the conventional and organic farm. Furthermore, marbofloxacin was detected in all the amendments from the conventional farm, as well as in the slurry from the organic farm. In the second analysis carried out two months later, only colistin was detected in the fresh manure from the conventional farm (Table 7.2), indicating a possible degradation of marbofloxacin.

Table 7.2. Antibiotic concentrations in the amendments and the semi-natural grassland soil. Antibiotics whose concentration did not exceed the detection limit are not included.

	Antibiotic (µg kg^{-1})	ORGANIC FARM			CONVENTIONAL FARM			SOIL
		Aged manure	Fresh manure	Slurry	Aged manure	Fresh manure	Slurry	
First analysis	Colistin	470	230	<50	223	147	<50	<50
	Marbofloxacin	<5	<5	139	81	245	41.6	<5
Second analysis	Colistin	<50	<50	<50	<50	117	<50	<50

7.3.2. Effect of treatments on crop production

Pertaining to lettuce shoot biomass, higher values were found when the soil was amended with aged manure from both the conventional and organic farm, as well as with slurry from the conventional

farm, compared to slurry from the organic farm, fresh manure from the conventional farm and the unamended control (Table 7.3).

Table 7.3. Effect of treatments on lettuce (shoot biomass) and wheat production (grain weight). Means (n=4) and standard errors. Errors with different letters are significantly different ($p<0.05$) according to Duncan`s multiple range test. ns: non-significant.

	ORGANIC FARM			CONVENTIONAL FARM			UNAMENDED CONTROL
	Aged manure	Fresh manure	Slurry	Aged manure	Fresh manure	Slurry	
LETTUCE							
Shoot biomass (g)	138.1±12.7 ^{ab}	122.0±3.9 ^{bc}	107.4±15.7 ^{c}	142.2±6.9 ^{a}	118.3±14.5 ^{c}	142.7±11.6 ^{a}	103.8±14.4^{c}
WHEAT							
Grain weight (g)	6.4±2.9^{ns}	5.6±1.8^{ns}	6.2±3.0^{ns}	5.9±1.6^{ns}	4.3±2.6^{ns}	6.0±1.7^{ns}	4.3±1.2^{ns}

As far as wheat production is concerned, no statistically significant ($p<0.05$) differences among treatments were observed. In any case, the highest value of wheat grain weight was found in pots amended with aged manure from the organic farm.

7.3.3. Effect of treatments on biological parameters related to the resistome risk

Regarding the absolute abundances of ARGs and MGE-genes in the amendments collected from the livestock farms (Supplementary Table 7.1), higher values were detected for aminoglycoside resistance genes, compared to all the other genes. In turn, lower values were observed for β-lactam, vancomycin and multidrug resistance genes. No single amendment could be identified as the best or worst amendment according to the absolute abundances of ARGs and MGE-genes (Supplementary Table 7.1).

Out of the 95 ARGs and MGE-genes quantified here, 44 and 64 genes were detected in lettuce plants and lettuce soils, respectively (Figure 7.1). In addition, 5 and 25 genes were exclusively detected in lettuce plants and lettuce soils, respectively (*i.e.*, lettuce plants and lettuce soils shared 39 genes) (Figure 7.1). Those five genes that were only detected in lettuce plants (and not in lettuce soils) encoded resistance to β-lactam (one gene), MLSB (one gene), vancomycin (one gene) and tetracycline (2 genes). In turn, the 25 genes that were found only in lettuce soils (and not in lettuce plants) encoded resistance to FCA (one gene), tetracycline (3 genes), multidrug (3 genes), β-lactam (4 genes), MLSB (4 genes), aminoglycosides (5 genes) and vancomycin (5 genes).

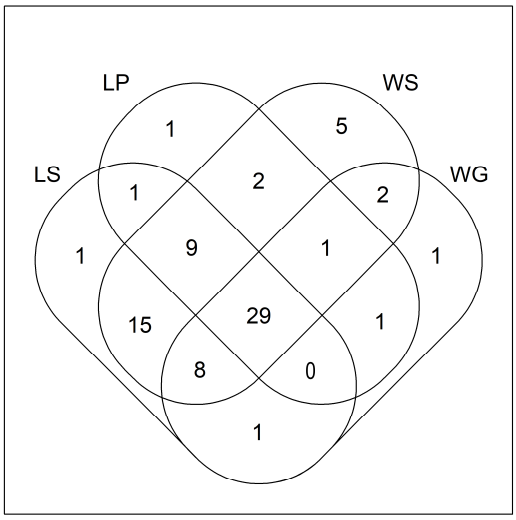


Figure 7.1. Venn diagram showing the number of ARGs and MGE-genes for lettuce and wheat samples. LS: lettuce soil; LP: lettuce plant; WS: wheat soil; WG: wheat grain.

Values of ARG absolute abundances in lettuce soils ranged from 3.25×10^{8} (for soil amended with slurry from the conventional farm) to 1.81×10^{9} (for the unamended control soil) copies g^{-1} DW soil (Supplementary Table 7.1). In these lettuce soils, the absolute abundance of MGE-genes was higher than that of ARGs: from 1.27×10^{10} (for soil amended with fresh manure from the organic farm) to 8.76×10^{10} (for the unamended control soil) copies g^{-1} DW soil. In lettuce soils, integrase-related genes showed the highest absolute abundance values. By contrast, multidrug resistance genes presented the lowest absolute abundance values in lettuce soils (however, differences were not statistically significant). Furthermore, the lettuce unamended (control) soil showed higher absolute abundance values for vancomycin resistance genes, compared to all the other lettuce soils. In relation to the effect of the experimental variables (type and origin of amendment) on absolute abundance values in lettuce soils, the application of aged manure led to significantly higher absolute abundances of aminoglycoside resistance genes, compared to the application of slurry (Supplementary Table 7.1). Moreover, lettuce soils amended with aged manure showed higher absolute abundance values for tetracycline resistance genes, compared to lettuce soils amended with fresh manure or slurry.

In lettuce plants, the absolute abundance of ARGs ranged from 1.08×10^{8} (for plants fertilized with slurry from the conventional farm) to 2.56×10^{9} (for plants fertilized with fresh manure from the conventional farm) copies g^{-1} DW plant tissue (Supplementary Table 7). The absolute abundance of

MGE-genes in lettuce plants ranged from 3.53×10^{8} (for plants fertilized with slurry from the conventional farm) to 5.59×10^{9} (for plants fertilized with fresh manure from the conventional farm) copies g^{-1} DW plant tissue. In lettuce plants, the absolute abundance of ARGs was higher in plants fertilized with fresh manure from the conventional farm, compared to all the other lettuce plants, except for the unamended control (Supplementary Table 7.1). Genes encoding resistance to β-lactam, FCA, multidrug, tetracycline and vancomycin showed lower absolute abundance values than genes encoding sulfonamide and transposase in lettuce plants. Lettuce plants fertilized with fresh manure from the conventional farm showed higher absolute abundance values for aminoglycoside resistance, tetracycline resistance and transposase-related genes than lettuce plants from the other treatments (except for aged manure from the conventional farm and the unamended control). Similarly, lettuce plants fertilized with amendments from the conventional farm exhibited higher absolute abundance values of tetracycline resistance and transposase related genes than those fertilized with amendments from the organic farm.

Regarding wheat, out of the 95 ARGs and MGE-genes quantified here, 43 and 71 genes were detected in wheat grains and wheat soils, respectively (Figure 7.1). In addition, 3 and 31 genes were exclusively detected in wheat grains and wheat soils, respectively (*i.e.*, wheat grains and wheat soils shared 40 genes) (Figure 7.1). Specifically, three tetracycline-resistance genes were only detected in wheat grain (and not in wheat soil). In turn, the 31 genes that were found only in wheat soil (and not in wheat grain) encoded resistance to multidrug (2 genes), aminoglycosides (3 genes), MLSB (3 genes), vancomycin (7 genes), β-lactam (8 genes) and tetracycline (8 genes). The absolute abundance of ARGs in wheat soils ranged from 1.50×10^{10} (for wheat soil amended with slurry from the conventional farm) to 7.64×10^{10} (for wheat soil amended with fresh manure from the organic farm) copies g^{-1} DW soil (Supplementary Table 7.1). In these wheat soils, the absolute abundance of MGE-genes ranged between 3.03×10^{11} (for wheat soils amended with slurry from the conventional farm) to 1.17×10^{12} (for wheat soils amended with fresh manure from the organic farm) copies g^{-1} DW soil. On the other hand, the absolute abundance of ARGs in wheat grains ranged from 4.04×10^{9} (for wheat fertilized with fresh manure from the conventional farm) to 1.47×10^{10} (for wheat fertilized with slurry from the organic farm) copies g^{-1} DW grain. The absolute abundance of MGE-genes in wheat grains ranged from 8.74×10^{10} (for wheat fertilized with aged manure from the organic farm) to 2.33×10^{11} (for control unamended pots) copies g^{-1} DW grain. Wheat soils amended with fresh manure from both livestock farms showed higher absolute abundance values of aminoglycoside resistance genes, compared to

wheat soils amended with slurry (Supplementary Table 7.1). Likewise, higher absolute abundance values of aminoglycoside, MLSB and vancomycin resistance genes were detected in wheat soils supplemented with amendments from the organic *vs.* conventional farm. Wheat grains grown with amendments from the organic farm exhibited higher absolute abundance values of FCA resistance genes, compared to those from pots treated with amendments from the conventional farm.

Figure 7.2 represents ARG and MGE-gene absolute abundances grouped by antibiotic family and MGE category for all soil and plant samples. The PCA clearly separated three clusters: (i) wheat soils; (ii) wheat grains; and (iii) lettuce soils and plants. The first axis (PC1) accounted for 75.2% of the total variance and showed negative loadings for the following absolute abundances: aminoglycoside, β-lactam, FCA, integrase, MLSB, sulfonamide, tetracycline and transposase genes. In addition, PC2 accounted for 16.1% of the total variance and showed positive loading for multidrug and negative loading for vancomycin genes.

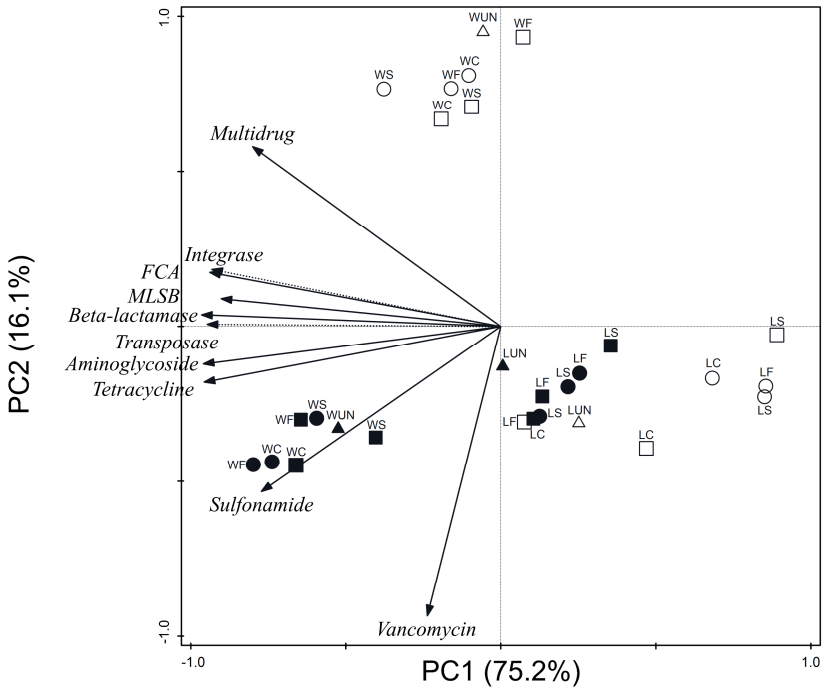


Figure 7.2. Principal component analysis of absolute abundances of ARGs and MGE-genes grouped by antibiotic family or MGE category. W: wheat samples; L: lettuce samples; AG: aged manure; F: fresh manure; S: slurry; UN: unamended. Circle: organic farm. Square: conventional farm. Empty symbol: plant. Full symbol: soil.

No statistically significant differences were found among treatments for both lettuce and wheat data (soil and plant data) in relation to the relative abundances of ARGs and MGE-genes grouped by antibiotic family and MGE category (Supplementary Figures 7.1 and 7.2).

Regarding the impact of treatments on soil prokaryotic diversity in lettuce soils, as reflected by Illumina MiSeq sequencing data, 73.1, 53.4 and 22.0% of the reads were taxonomically classified to order, family and genus rank, respectively. Concerning wheat soils, 67.6, 51.1 and 20.4% of the reads were classified to order, family and genus rank, respectively. Statistically significant differences were found in 15 and 3 orders in lettuce and wheat soils, respectively (Supplementary Table 7.2). For lettuce soils, out of these 15 orders, the following belong to the 30 most abundant orders detected in those soils: *Cytophagales*, *SC-I-84*, *Pseudonocardiales*, *Solirubrobacterales*, *C0119*, *KD4-96* and *Nitrososphaerales* (Supplementary Figure 7.3). Similarly, out of the abovementioned three orders in wheat soils, the following two belong to the 30 most abundant orders: *Rhodospirillales* and *Desulfurellales* (Supplementary Figure 7.4).

Data on the impact of treatments on soil prokaryotic α-diversity are shown in Table 7.4. Lettuce soils amended with slurry from the conventional farm showed higher richness than those amended with slurry from the organic farm (and also higher richness, compared to the untreated control soil). Moreover, Shannon's diversity was lower in soils amended with aged manure for the organic farm and the unamended control soil, compared to all the other soils. In wheat soils, higher richness values were observed in soil amended with aged manure from the conventional farm, compared to soil amended with fresh manure and slurry from the organic farm (Table 7.4).

Table 7.4. Effect of treatments on soil prokaryotic diversity. Means (n=4) and standard errors. Errors with different letters are significantly different ($p<0.05$) according to Duncan`s multiple range test. ORG_AG: aged manure form organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAMEN: unamended control. ns: non-significant.

Lettuce soil	**Richness**	**Shannon´s**	**Simpson´s**	**Pielou´s**
ORG_AG	3626 ± 200^{cd}	6.78 ± 0.04^{b}	$0.997 \pm 5.2E\text{-}04^{ns}$	0.811 ± 0.015^{ns}
ORG_FRES	3894 ± 48^{ab}	7.03 ± 0.04^{a}	$0.998 \pm 2.9E\text{-}04^{ns}$	0.815 ± 0.005^{ns}
ORG_SLU	3722 ± 65^{bc}	6.99 ± 0.03^{a}	$0.998 \pm 5.2E\text{-}05^{ns}$	0.814 ± 0.003^{ns}
CONV_AG	3774 ± 73^{abc}	6.98 ± 0.02^{a}	$0.998 \pm 9.2E\text{-}05^{ns}$	0.825 ± 0.015^{ns}
CONV_FRES	3784 ± 33^{abc}	7.00 ± 0.03^{a}	$0.998 \pm 1.4E\text{-}04^{ns}$	0.813 ± 0.009^{ns}
CONV_SLU	3941 ± 149^{a}	7.05 ± 0.06^{a}	$0.998 \pm 2.9E\text{-}04^{ns}$	0.815 ± 0.007^{ns}
UNAMEN	3550 ± 95^{d}	6.89 ± 0.04^{bc}	$0.997 \pm 1.1E\text{-}04^{ns}$	0.808 ± 0.002^{ns}
Wheat soil	**Richness**	**Shannon´s**	**Simpson´s**	**Pielou´s**
ORG_AG	4517 ± 85^{ab}	7.04 ± 0.04^{ns}	$0.998 \pm 2.0E\text{-}04^{ns}$	0.817 ± 0.010^{ns}
ORG_FRES	4285 ± 176^{b}	6.95 ± 0.07^{ns}	$0.997 \pm 3.1E\text{-}04^{ns}$	0.810 ± 0.005^{ns}
ORG_SLU	4268 ± 259^{b}	6.91 ± 0.12^{ns}	$0.997 \pm 3.6E\text{-}04^{ns}$	0.811 ± 0.014^{ns}
CONV_AG	4710 ± 53^{a}	7.08 ± 0.03^{ns}	$0.998 \pm 1.8E\text{-}04^{ns}$	0.817 ± 0.005^{ns}
CONV_FRES	4530 ± 98^{ab}	7.00 ± 0.04^{ns}	$0.997 \pm 2.1E\text{-}04^{ns}$	0.818 ± 0.010^{ns}
CONV_SLU	4510 ± 289^{ab}	7.01 ± 0.14^{ns}	$0.998 \pm 4.3E\text{-}04^{ns}$	0.814 ± 0.010^{ns}
UNAMEN	4441 ± 47^{ab}	6.95 ± 0.04^{ns}	$0.997 \pm 3.0E\text{-}04^{ns}$	0.805 ± 0.008^{ns}

In lettuce soils, Kendall's rank correlation coefficients showed significant correlations (positive and negative) among 43 orders and 7 ARG and 2 MGE-gene absolute abundances grouped by antibiotic family and MGE category (Supplementary Table 7.2). Among these 43 orders, the following five presented multiresistance: *Micrococcales*, *Pseudonocardiales*, *Rhizobiales, Rubrobacterales* and *Solirubrobacterales* (Supplementary Table 7.2). The orders *Micrococcales*, *Pseudonocardiales, Rhizobiales* and *Solirubrobacterales* appeared in the list of the 30 most abundant orders in lettuce soils (Supplementary Figure 7.3). The order *Pseudonocardiales* was positively correlated with genes encoding resistance to MLSB, tetracycline and vancomycin (Supplementary Table 7.2). The lettuce unamended soil showed higher abundance of *Pseudonocardiales* than the other soils (Supplementary Table 7.3). Fifteen orders showed, at least, two negative correlations with ARG and MGE-gene absolute abundances (Supplementary Table 7.2).

In wheat soils, Kendall's rank correlation coefficients showed significant correlations (positive and negative) among 14 orders and 6 ARG and 2 MGE-gene absolute abundances grouped by antibiotic family and MGE category (Supplementary Table 7.2). Among these 14 orders, the following three presented multiresistance: *Limnochordales*, *Tepidisphaerales* and *WN-HWB-116* (Supplementary Table 7.2).

As far as differences between lettuce and wheat pots, wheat soil and grain samples showed higher absolute abundances of ARGs and MGE-genes than lettuce soil and plant samples (Supplementary Table 7.4). In terms of absolute abundances, the highest number of statistically significant differences between lettuce and wheat soils was observed in soils amended with fresh manure from the organic farm.

7.4. Discussion

The incorporation of organic amendments into agricultural soil as fertilizers often increases soil OM content (Ghosh et al., 2012) and fertility, and results in an overall improvement of soil quality (Epelde et al., 2018). In particular, organic farming practices promote the maintenance and enhancement of soil OM and fertility by means of the application of farmyard manure and similar organic amendments. In Europe, the area under organic farming increased from 10.0 million hectares in 2012 to 13.4 million hectares in 2018 (Eurostat Statistics for Organic Farming). Despite the abovementioned well-recognized benefits, there is increasing concern about the use of manure-derived amendments as organic fertilizers since their application entails a variety of environmental risks such as, for instance, the emergence, maintenance and dissemination of AR in agricultural soils and crops (Zhu et al., 2013; Gou et al., 2018; Udikovic-Kolic et al., 2014). The application of manure-derived amendments to agricultural soil can also lead to pronounced changes in the diversity and composition of soil microbial communities (Ding et al., 2014), with potential concomitant alterations of soil functioning. We hypothesized that the resistome risk would be higher in soils and plants amended with animal wastes from conventional livestock farming *vs.* organic livestock farming (after all, the administration of antibiotics to animals raised under organic farming is limited by regulations). Nonetheless, such hypothesis is not supported by the results of our study. Actually, even regarding the concentration of antibiotics in the amendments collected from the organic *vs.* conventional farm, no clear differences were observed, which could be due to the fact that organic farms do apply antibiotics in some specific cases, *e.g*. during a long-term mastitis.

As described above, a large proportion (30-90%) of the antibiotics administered to livestock are not fully metabolized and are then excreted, together with their transformation products, into the environment along with the faeces and urine (Kumar et al., 2005). The amount and rate of antibiotic excretion varies greatly among animal species and age (Sarmah et al., 2006; Motoyama et al., 2011), type and dosage of antibiotic, form of administration, etc. (Berendsen et al., 2018). As an example, the

following concentrations (mg kg^{-1}) have been reported for dairy cow manure: 0.43-2.69 for tetracycline, 0.21-10.37 for oxytetracycline, 0.61-1.94 for chlortetracycline, 0.22-1.02 for sulfamethoxazole, 0.43-1.76 for norfloxacin and 0.46-4.17 for enrofloxacin (Li et al., 2013c; Perron et al., 2004). On the other hand, once introduced into the soil matrix, antibiotics are susceptible to a variety of processes, such as adsorption, microbial transformation, photodegradation, plant uptake, sequestration, transport (leaching, runoff), etc. (Sarmah et al., 2006; Blackwell et al., 2007; Du & Liu, 2012; Jechalke et al., 2014). In contrast with other studies (Zhou et al., 2013; Li et al., 2017b; Wallace et al., 2018), macrolides, sulfonamides and tetracyclines were not detected in any of the amendments studied here. Actually, in the first analysis, out of the 57 antibiotics analysed here, only colistin and marbofloxacin were detected. In the second analysis, only colistin (117 μg kg^{-1}) was detected in one of the amendments, *i.e.*, fresh manure from the conventional farm. Nonetheless, we did find genes encoding resistance to those antibiotics in the amendments, which could be due to the fact that: (i) the antibiotics were already completely degraded but the ARGs persisted in the amendments despite the absence of the antibiotics; (ii) antibiotic transformation products, still capable of bioactive effect, are responsible for the induction of the emergence of ARGs in the amendments (Sarmah et al., 2006); and/or (iii) although antibiotic concentrations in the amendments were below the detection limit of the technique, sub-inhibitory concentrations result in an enough level of selective pressure to induce AR (Sandegren, 2014). Furthermore, antibiotic sub-inhibitory concentrations are known to induce horizontal gene transfer (Aminov, 2011), which could spread ARGs among different bacterial populations. Interestingly, some studies (Jechalke et al., 2013; Udikovic-Kolic et al., 2014) have reported an increase in AR in soils amended with manure from animals that had not been subjected to any antibiotic treatment.

In our study, the amendment that showed the highest absolute abundances of ARGs and MGE-genes was the slurry from the conventional farm, but this highest resistome risk was then not reflected, as one would expect, in those soils and crops amended with such slurry. Actually, despite the fact that the slurry from both livestock farms presented greater values of absolute abundance for transposase, aminoglycoside, MLSB, tetracycline and multidrug resistance genes (compared to the other amendments), lettuce soils amended with such slurry showed a lower resistome risk than when fertilized with the other amendments. Also, the absolute abundances for aminoglycoside resistance genes were lower in wheat soils amended with slurry *vs.* fresh and aged manure. Remarkably, within the same treatment, the resistome risk differed between the amendment, the amended soil and, finally,

the crop. In other words, according to our data, the resistome risk in manure-amended crops cannot be directly inferred from the analysis of the amendments themselves. Although ageing and composting are both effective processes (composting is certainly more effective than ageing in this respect) for reducing the concentration of antibiotics and the total abundance of ARGs, the trend in some ARGs is highly gene-specific (Xu et al., 2018). In our case, the manure was not composted following a controlled procedure, but simply aged for approximately 6 months. In any case, dairy fresh manure from both the conventional and the organic farm presented higher absolute abundances of *intI1*, *sul2* and 7 tetracycline-resistance than those reported in previous studies (Sandberg & LaPara, 2016; Sun et al., 2016; Peng et al., 2017).

On the other hand, slurry samples from both livestock farms showed the lowest metal concentrations, compared to aged and fresh manure. The *co-selection* of antibiotic and metal resistance in bacteria, due to *co-resistance* (when two or more different resistance genes are located on the same genetic element, *e.g.* a plasmid or a transposon) or *cross-resistance* (when a single mechanism confers resistance to both antibiotics and metals, *e.g.* an efflux pump) mechanisms, is widely known (Chapman, 2003; Baker-Austin et al., 2006; Seiler & Berendonk, 2012; Pal et al., 2017). Moreover, *co-regulatory mechanisms* (when genes that confer resistance to different compounds are controlled by a single regulatory gene) can promote antibiotic-metal co-selection processes. In this respect, Perron et al. (2004) reported that the regulatory protein CzcR regulates (i) the expression of the CzcCBA efflux pump, which confers resistance to Zn, Cd and Co; and (ii) the expression of the OprD porin, the route of entry of carbapenems in bacteria. This co-selection phenomenon is of the utmost importance as it can be responsible for the maintenance and dissemination of AR in the absence of antibiotics. In our study, as abovementioned, the values of ARG and MGE-gene absolute abundances were lower in aged and fresh manure than in slurry, but it is possible that the higher metal concentrations detected in aged and fresh manure (*vs.* slurry) could induce the spread of ARGs once applied to the agricultural soil.

Overall, the values of absolute abundance of ARGs and MGE-genes were higher in soil *vs.* plant samples, in agreement with previous studies (Cerqueira et al., 2019; Zhang et al., 2019a). Soils are important reservoir of ARGs (Heinemann et al., 2012; Cytryn, 2013). In any event, the typology of ARGs found in lettuce and wheat grains was robustly dependent on the typology of ARGs observed in the corresponding soil. Relevantly, much higher absolute abundances of MGE-genes *vs.* ARGs were detected in both soil and plant samples, pointing out to a high potential risk of dissemination of AR in

the studied soils and crops. In addition to the physical contact and interactions among the plants, the soil and the amendments, in some cases, the water used for irrigation is another factor to be considered, as it might be contaminated with ARGs (Su et al., 2018). However, in our study, this is not a relevant factor since the same tap water was used to irrigate all the treatments.

Furthermore, we found higher ARG and MGE-gene absolute abundances in wheat *vs.* lettuce soils. Plants are known to regulate rhizosphere microbial communities through the excretion of root exudates (Jones et al., 2004). The composition and quantity of root exudates greatly vary depending on the specific plant species and its physiological status (Grayston et al., 1997; Neumann et al., 2000). The type of crop (lettuce *vs.* wheat), dose of amendment (here adjusted to 100 *vs.* 180 kg N ha^{-1} for lettuce and wheat plants, respectively), duration of plant growth until harvest (44 *vs.* 171 days for lettuce and wheat plants, respectively), type of root system (pivotant *vs.* fasciculate for lettuce and wheat plants, respectively), and the amount and composition of the rhizodeposition are all factors that can affect the composition of soil microbial communities and the fate and distribution of ARGs and MGE-genes in agricultural soils. No significant differences were observed, in terms of the absolute abundances of ARGs and MGE-genes, between unamended lettuce soils and unamended wheat soils (neither between lettuce and wheat grain samples), which indicates that the amendment application was responsible for the observed differences among treatments.

Although Zhang et al. (2019a) observed higher ARG abundances in manure-amended lettuce soils (the abundance of ARGs ranged from 4.37×10^{9} to 2.02×10^{10} in soils), compared to ours, the transfer of those ARGs from the lettuce soil to the lettuce was approximately between one and two orders of magnitude higher in our study (the abundance of ARGs ranged from 7.45×10^{6} to 8.24×10^{7} in plant samples). In lettuce soils, the unamended control showed the highest abundance of vancomycin resistance genes. Antibiotic resistance genes have not only been found in antibiotic-free soil (Allen et al., 2010) but also in environments (*e.g.*, permafrost, isolated caves) that have remained isolated from the impact of anthropic activity much before the beginning of the use of antibiotics for the preventive and curative treatment of bacterial infectious diseases in medicine and veterinary (D'Costa et al., 2006; Bhullar et al., 2012).

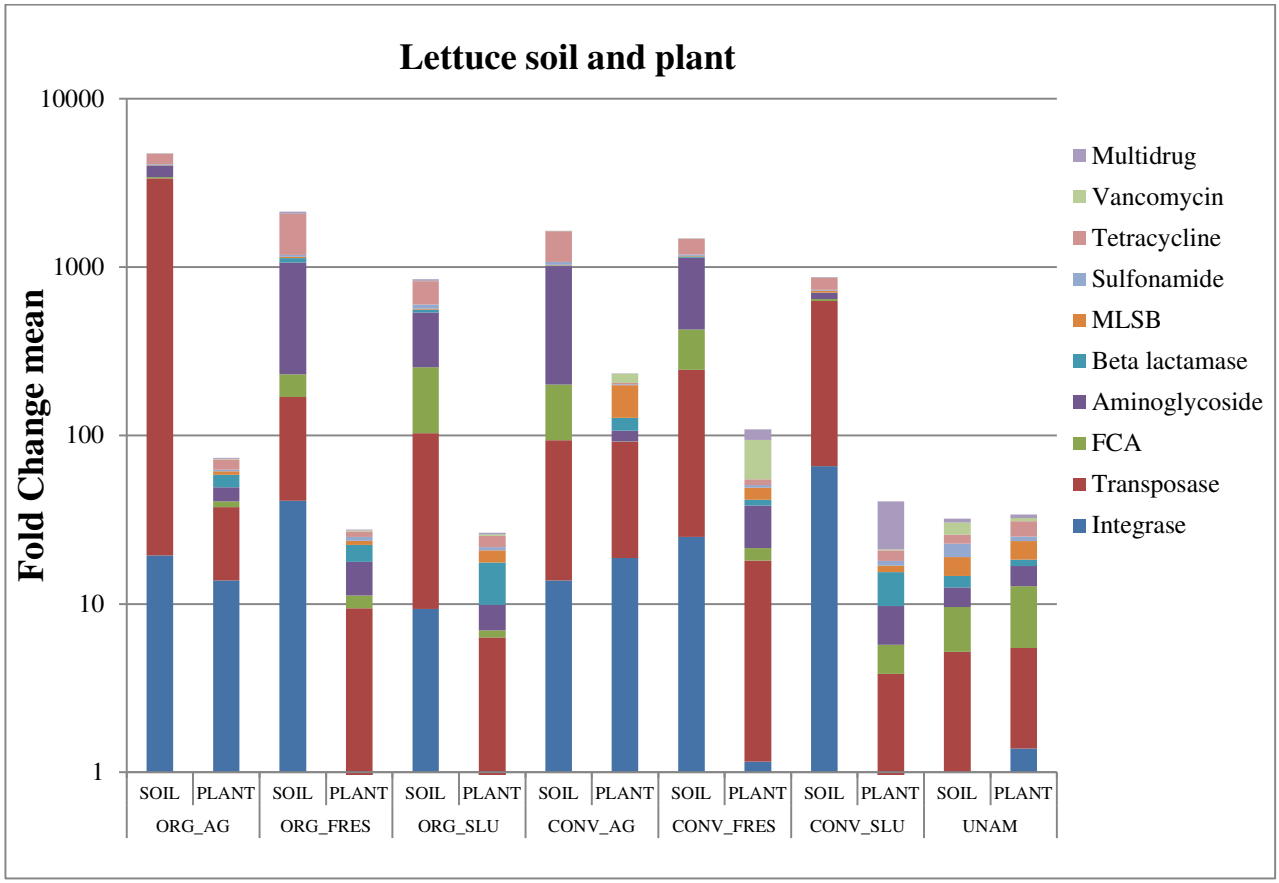
Regarding the possible links between the presence of certain prokaryotic taxa and AR profiles, the order *Pseudonocardiales* presented a positive correlation with vancomycin resistance genes.

Several strains belonging to *Pseudonocardiales* are known to produce biologically active products, such as erythromycin, rifamycin and vancomycin (Platas et al., 1998). The unamended control soil showed significantly higher abundance of *Pseudonocardiales* than the other treated soils (and, as already mentioned, the unamended lettuce soil showed the highest abundance of vancomycin resistance genes). In general, the unamended lettuce soil showed lower abundances of those orders negatively correlated with vancomycin resistance genes (*Cytophagales*, *Obscuribacterales* and *SAR324*), compared to the other treated soils. Many authors (Forsberg et al., 2014; Xiang et al., 2018) have reported that changes in the composition of prokaryotic communities appear to be the key drivers for the magnitude and profile of the antibiotic resistome. The values of bacterial richness and Shannon´s diversity detected in the unamended control soil were significantly lower than those observed in the other soils (except for soils amended with aged manure from the organic farm). These data suggest that vancomycin resistance genes most likely did not enter the soil matrix through the application of the amendments, but that they existed previously in such soil. Chaudhry et al. (2012) found that the application of amendments to soil could lead to an increase of (i) overall bacterial diversity; and (ii) the dominance of certain bacterial taxa which could then play important roles in a variety of soil processes. Highly diverse soil microbial communities can, for instance, act as a biological barrier against biological invasion (Vivant et al., 2013). The decline in microbial diversity has often been related to a loss of ecosystem multi-functionality (Delgado-Baquerizo et al., 2016).

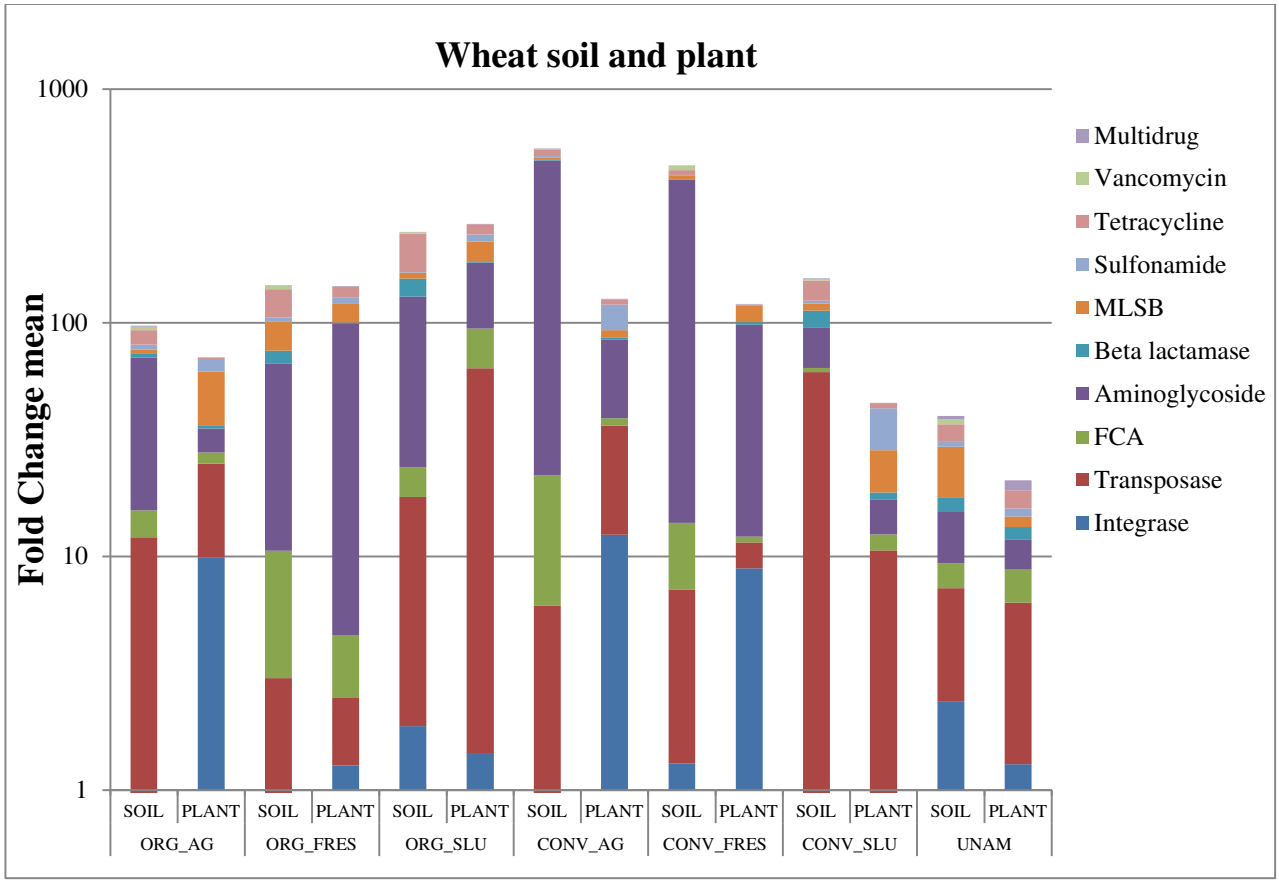
7.5. Conclusion

Despite our initial hypotheses, no single treatment could be identified as the best or worst treatment regarding the risk of antibiotic resistance in soil and plant samples. Interestingly, within the same treatment, the resistome risk differed between the amendment, the amended soil and, finally, the crop. In other words, according to our data, the resistome risk in manure-amended crops cannot be directly inferred from the analysis of the amendments themselves. Then, we concluded that, depending on the specific question under study, the analysis of the resistome risk should specifically focus on the amendment, the amended soil or the crop. In any case, our results confirm the risk of AR dissemination in agricultural settings where dairy cow manure-derived amendments are used as fertilizers. In this respect, much higher absolute abundances of MGE-genes *vs.* ARGs were detected in both soil and plant samples, pointing out to a high potential risk of dissemination of AR in the studied soils and crops.

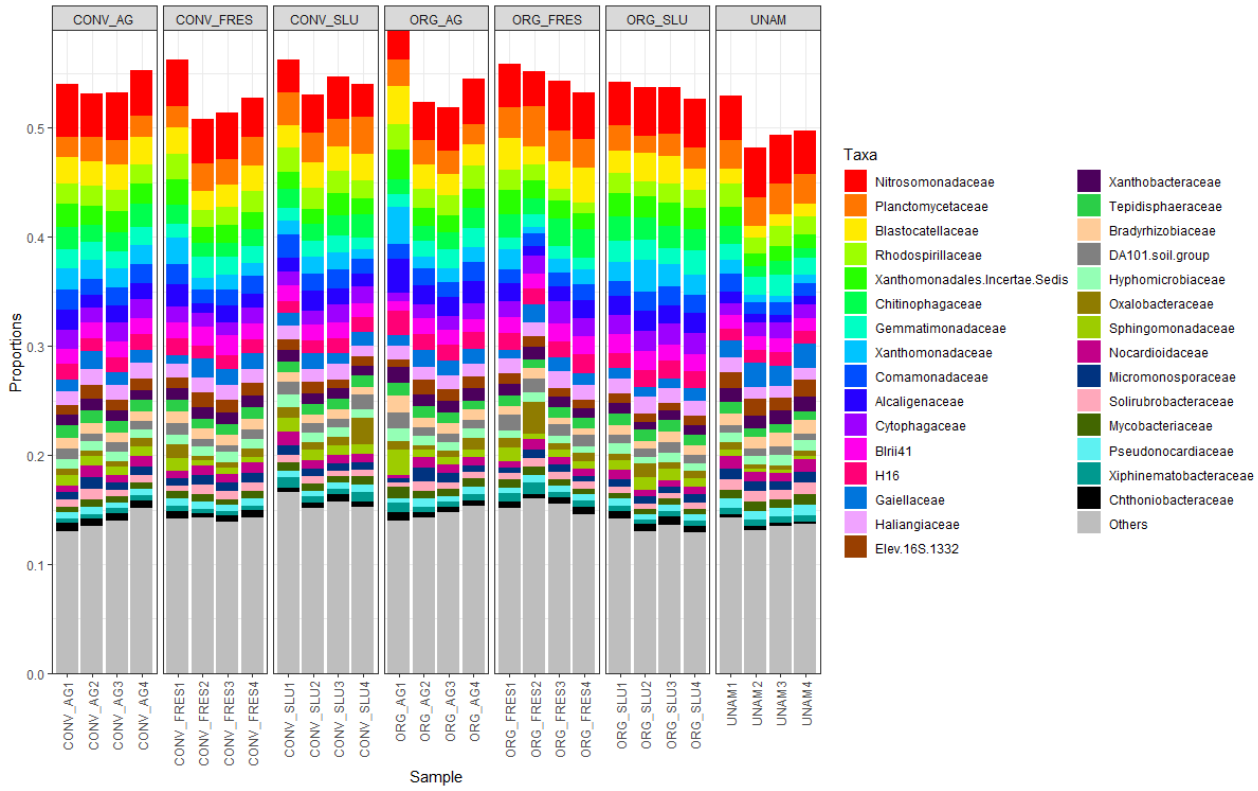
7.6. Supplementary information



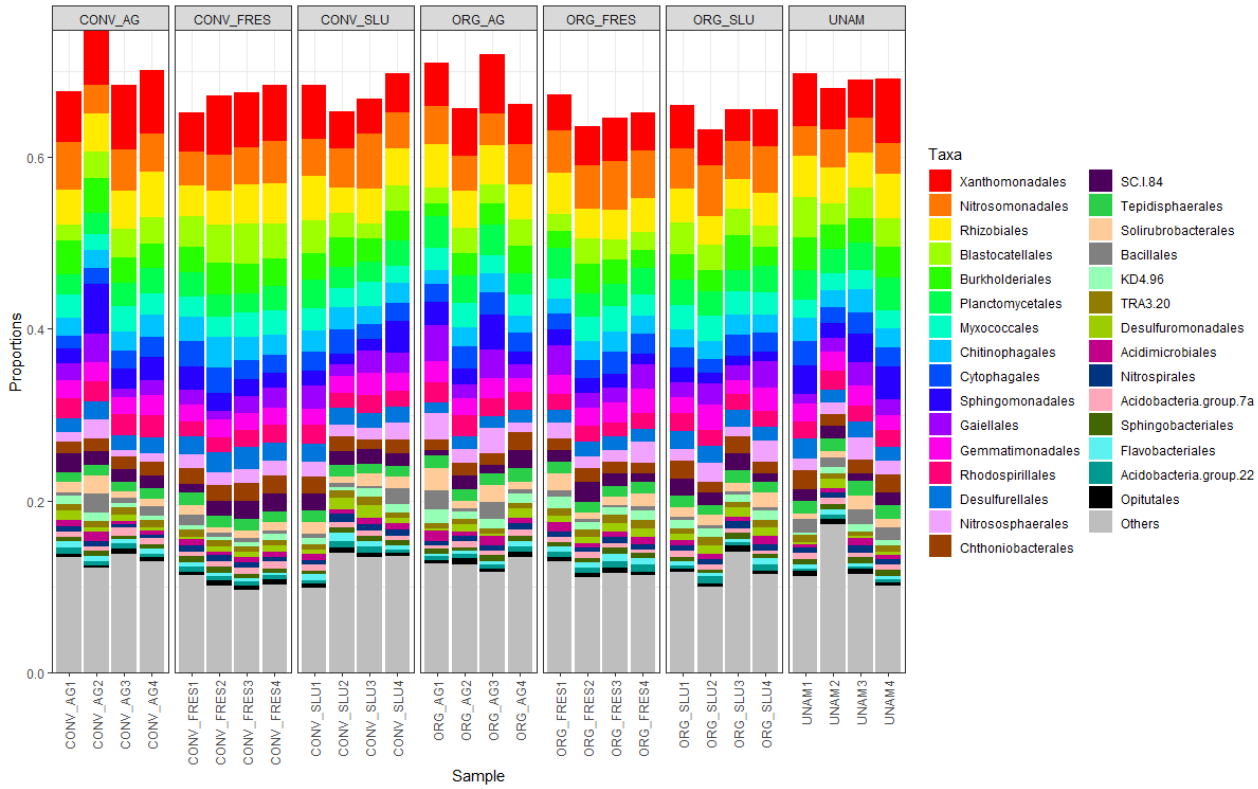
Supplementary Figure 7.1. Relative abundance of ARGs and MGE-genes (grouped by antibiotic family or MGE category) for lettuce soil and plant samples. Data are expressed as fold-change and plotted on a log scale. ORG_AG: aged manure from organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAM: unamended.



Supplementary Figure 7.2. Relative abundances of ARGs and MGE-genes (grouped by antibiotic family or MGE category) for wheat soil and plant (grain) samples. Data are expressed as fold-change and plotted on a log scale. ORG_AG: aged manure from organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAM: unamended.



Supplementary Figure 7.3. The 30 most abundant bacterial orders in lettuce soils. ORG_AG: aged manure from organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAM: unamended.



Supplementary Figure 7.4. The 30 most abundant bacterial orders in wheat soils. ORG_AG: aged manure from organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAM: unamended.

Supplementary Table 7.1. Absolute abundances of ARGs and MGE-genes in the amendments, lettuce soils, lettuce plants, wheat soils and wheat (grain) plants. Different letters indicate significant ($p<0.05$) differences according to one-way and two-way ANOVA and Duncan`s multiple range test. ORG_AG: aged manure from organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAMEN: unamended.TYPE: aged manure *vs.* fresh manure *vs*. slurry. ORIGIN: organic *vs.* conventional. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: non-significant.

AMENDMENT	Integrase	Transposase	Aminoglycoside	β-lactam	FCA	MLSB	Sulfonamide	Tetracycline	Vancomycin	Multidrug	Total MGE (average)	Total ARG (average)
ORG_AG	2.44E+10^{b}	1.49E+11^{b}	1.36E+12^{bc}	8.37E+08^{ns}	2.36E+10^{ns}	2.91E+10^{b}	5.11E+11^{ns}	5.88E+11^{abc}	4.15E+08^{ns}	1.43E+08^{b}	8.68E+10^{ns}	3.14E+11^{ns}
ORG_FRES	1.13E+10^{b}	2.80E+11^{b}	8.35E+11^{bc}	2.29E+07^{ns}	5.82E+09^{ns}	1.18E+10^{b}	4.91E+11^{ns}	2.62E+11^{bc}	5.80E+07^{ns}	8.39E+06^{b}	1.46E+11^{ns}	2.01E+11^{ns}
ORG_SLU	1.73E+10^{b}	1.19E+12^{a}	3.57E+12^{ab}	4.57E+07^{ns}	8.57E+09^{ns}	1.07E+11^{ab}	5.49E+11^{ns}	8.27E+11^{ab}	6.40E+07^{ns}	9.83E+07^{b}	6.04E+11^{ns}	6.33E+11^{ns}
CONV_AG	9.14E+09^{b}	1.66E+11^{b}	5.11E+11^{c}	1.42E+08^{ns}	2.28E+10^{ns}	4.54E+09^{b}	1.70E+11^{ns}	9.81E+10^{c}	4.13E+07^{ns}	7.87E+06^{b}	8.74E+10^{ns}	1.01E+11^{ns}
CONV_FRES	3.42E+11^{a}	2.85E+11^{b}	1.86E+12^{bc}	1.01E+08^{ns}	3.02E+10^{ns}	4.44E+10^{b}	3.82E+11^{ns}	4.55E+11^{abc}	2.85E+07^{ns}	1.03E+06^{b}	3.14E+11^{ns}	3.46E+11^{ns}
CONV_SLU	1.11E+11^{ab}	1.59E+12^{a}	4.84E+12^{a}	4.64E+08^{ns}	1.07E+10^{ns}	1.51E+11^{a}	9.32E+11^{ns}	9.72E+11^{a}	2.25E+08^{ns}	1.01E+09^{a}	8.49E+11^{ns}	8.63E+11^{ns}
TYPE	ns	***	***	ns	ns	**	ns	*	ns	*	ns	ns
ORIGIN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

LETTUCE SOIL	Integrase	Transposase	Aminoglycoside	β-lactam	FCA	MLSB	Sulfonamide	Tetracycline	Vancomycin	Multidrug	Total MGE (average)	Total ARG (average)
ORG_AG	3.10E+10^{ns}	9.04E+09^{ns}	5.46E+09^{ns}	4.58E+08^{ns}	2.53E+08^{ns}	9.95E+08^{ns}	3.62E+09^{ns}	9.61E+08^{ns}	5.23E+08^{b}	2.41E+07^{ns}	2.00E+10^{ns}	1.54E+09^{ns}
ORG_FRES	1.91E+10^{ns}	6.32E+09^{ns}	2.87E+09^{ns}	2.66E+08^{ns}	1.56E+08^{ns}	4.79E+08^{ns}	1.12E+09^{ns}	6.52E+08^{ns}	2.77E+08^{b}	4.31E+07^{ns}	1.27E+10^{ns}	7.33E+08^{ns}
ORG_SLU	3.53E+10^{ns}	2.95E+09^{ns}	2.69E+09^{ns}	4.73E+08^{ns}	2.72E+08^{ns}	5.80E+08^{ns}	2.20E+09^{ns}	6.95E+08^{ns}	4.34E+08^{b}	4.43E+07^{ns}	1.91E+10^{ns}	9.24E+08^{ns}
CONV_AG	4.15E+10^{ns}	1.28E+10^{ns}	5.14E+09^{ns}	5.45E+08^{ns}	2.40E+08^{ns}	4.95E+08^{ns}	4.30E+09^{ns}	1.22E+09^{ns}	6.47E+08^{b}	3.95E+07^{ns}	2.72E+10^{ns}	1.58E+09^{ns}
CONV_FRES	4.17E+10^{ns}	8.07E+09^{ns}	4.40E+09^{ns}	5.19E+08^{ns}	3.50E+08^{ns}	1.04E+09^{ns}	1.97E+09^{ns}	8.85E+08^{ns}	7.61E+08^{b}	2.64E+07^{ns}	2.49E+10^{ns}	1.24E+09^{ns}
CONV_SLU	1.76E+10^{ns}	1.11E+10^{ns}	5.69E+08^{ns}	2.40E+08^{ns}	6.96E+07^{ns}	3.67E+08^{ns}	3.24E+08^{ns}	6.84E+08^{ns}	3.19E+08^{b}	2.80E+07^{ns}	1.43E+10^{ns}	3.25E+08^{ns}
UNAMEN	1.65E+11^{ns}	1.01E+10^{ns}	1.63E+09^{ns}	2.59E+09^{ns}	4.33E+08^{ns}	2.93E+09^{ns}	1.44E+09^{ns}	2.37E+09^{ns}	2.99E+09^{a}	1.12E+08^{ns}	8.76E+10^{ns}	1.81E+09^{ns}
TYPE	ns	ns	*	ns	ns	ns	ns	*	ns	ns	ns	ns
ORIGIN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TYPE X ORIGIN	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns

LETTUCE PLANT	Integrase	Transposase	Aminoglycoside	β-lactam	FCA	MLSB	Sulfonamide	Tetracycline	Vancomycin	Multidrug	Total MGE (average)	Total ARG (average)
ORG_AG	1.89E+09^{ns}	5.82E+08^{b}	9.43E+08^{b}	1.18E+08^{ns}	1.59E+07^{ns}	1.04E+08^{ns}	8.81E+08^{ns}	8.51E+07^{b}	1.15E+06^{ns}	1.15E+06^{ns}	1.24E+09^{ns}	2.69E+08^{b}
ORG_FRES	4.02E+08^{ns}	4.61E+08^{b}	5.17E+08^{b}	5.44E+07^{ns}	8.17E+06^{ns}	4.42E+07^{ns}	6.68E+08^{ns}	4.27E+07^{b}	2.94E+05^{ns}	2.94E+05^{ns}	4.31E+08^{ns}	1.67E+08^{b}
ORG_SLU	2.64E+08^{ns}	5.30E+08^{b}	4.34E+08^{b}	7.02E+07^{ns}	1.03E+07^{ns}	2.13E+07^{ns}	9.20E+08^{ns}	5.25E+07^{b}	4.47E+05^{ns}	4.47E+05^{ns}	3.97E+08^{ns}	1.89E+08^{b}
CONV_AG	7.17E+08^{ns}	2.89E+09^{ab}	1.26E+09^{b}	1.42E+08^{ns}	4.71E+06^{ns}	3.97E+09^{ns}	1.90E+09^{ns}	5.46E+08^{ab}	3.38E+07^{ns}	1.14E+06^{ns}	1.80E+09^{ns}	9.38E+08^{ab}
CONV_FRES	2.53E+09^{ns}	8.65E+09^{a}	7.13E+09^{a}	9.28E+08^{ns}	2.88E+08^{ns}	1.65E+09^{ns}	8.66E+09^{ns}	1.05E+09^{a}	5.75E+08^{ns}	2.08E+08^{ns}	5.59E+09^{ns}	2.56E+09^{a}
CONV_SLU	4.23E+08^{ns}	2.83E+08^{b}	2.75E+08^{b}	3.04E+07^{ns}	2.43E+07^{ns}	3.58E+07^{ns}	4.70E+08^{ns}	2.40E+07^{b}	2.14E+05^{ns}	1.59E+06^{ns}	3.53E+08^{ns}	1.08E+08^{b}
UNAMEN	1.02E+09^{ns}	5.66E+09^{ab}	4.18E+09^{ab}	5.51E+08^{ns}	3.15E+08^{ns}	4.91E+08^{ns}	6.80E+09^{ns}	6.41E+08^{ab}	7.51E+06^{ns}	7.51E+06^{ns}	3.34E+09^{ns}	1.62E+09^{ab}
TYPE	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
ORIGIN	ns	*	ns	ns	ns	ns	ns	*	ns	ns	ns	*
TYPE X ORIGIN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

WHEAT SOIL	Integrase	Transposase	Aminoglycoside	β-lactam	FCA	MLSB	Sulfonamide	Tetracycline	Vancomycin	Multidrug	Total MGE (average)	Total ARG (average)
ORG_AG	1.95E+12^{ns}	6.66E+10^{ns}	1.83E+11^{ns}	5.16E+10^{ns}	1.40E+10^{ns}	1.37E+10^{ns}	1.34E+11^{ns}	3.91E+10^{ns}	2.29E+10^{ns}	2.73E+09^{ns}	1.01E+12^{ns}	5.77E+10^{ns}
ORG_FRES	2.19E+12^{ns}	1.42E+11^{ns}	3.27E+11^{ns}	5.70E+10^{ns}	1.09E+10^{ns}	2.17E+10^{ns}	1.19E+11^{ns}	4.38E+10^{ns}	2.91E+10^{ns}	2.65E+09^{ns}	1.17E+12^{ns}	7.64E+10^{ns}
ORG_SLU	1.62E+12^{ns}	4.11E+10^{ns}	8.40E+10^{ns}	2.87E+10^{ns}	7.42E+09^{ns}	1.68E+10^{ns}	1.93E+10^{ns}	2.73E+10^{ns}	2.72E+10^{ns}	1.59E+09^{ns}	8.33E+11^{ns}	2.66E+10^{ns}
CONV_AG	1.19E+12^{ns}	6.39E+10^{ns}	1.16E+11^{ns}	3.37E+10^{ns}	9.76E+09^{ns}	8.23E+09^{ns}	1.17E+11^{ns}	3.50E+10^{ns}	1.40E+10^{ns}	1.65E+09^{ns}	6.27E+11^{ns}	4.19E+10^{ns}
CONV_FRES	1.89E+12^{ns}	4.62E+10^{ns}	9.73E+10^{ns}	3.16E+10^{ns}	1.68E+10^{ns}	1.32E+10^{ns}	3.86E+10^{ns}	2.49E+10^{ns}	1.83E+10^{ns}	2.66E+09^{ns}	9.66E+11^{ns}	3.04E+10^{ns}
CONV_SLU	5.89E+11^{ns}	1.69E+10^{ns}	4.29E+10^{ns}	2.01E+10^{ns}	9.52E+08^{ns}	5.59E+09^{ns}	2.88E+10^{ns}	1.29E+10^{ns}	7.95E+09^{ns}	1.07E+09^{ns}	3.03E+11^{ns}	1.50E+10^{ns}
UNAMEN	1.45E+12^{ns}	2.91E+10^{ns}	2.99E+10^{ns}	2.95E+10^{ns}	2.39E+09^{ns}	1.38E+10^{ns}	3.69E+10^{ns}	1.84E+10^{ns}	3.16E+10^{ns}	2.79E+09^{ns}	7.42E+11^{ns}	2.06E+10^{ns}
TYPE	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
ORIGIN	ns	ns	*	ns	ns	*	ns	ns	*	ns	ns	ns
TYPE X ORIGIN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

WHEAT GRAIN	Integrase	Transposase	Aminoglycoside	β-lactam	FCA	MLSB	Sulfonamide	Tetracycline	Vancomycin	Multidrug	Total MGE (average)	Total ARG (average)
ORG_AG	2.33E+11^{ns}	8.81E+09^{ns}	9.42E+09^{ns}	1.50E+09^{ns}	3.76E+09^{ns}	8.12E+09^{ns}	1.91E+09^{ns}	3.15E+08^{ns}	-	1.45E+10^{ns}	1.21E+11^{ns}	5.65E+09^{ns}
ORG_FRES	1.66E+11^{ns}	8.87E+09^{ns}	5.56E+09^{ns}	9.55E+09^{ns}	1.58E+09^{ns}	7.52E+09^{ns}	1.43E+09^{ns}	2.80E+09^{ns}	-	1.33E+10^{ns}	8.74E+10^{ns}	5.95E+09^{ns}
ORG_SLU	2.25E+11^{ns}	2.68E+10^{ns}	2.77E+10^{ns}	8.24E+09^{ns}	9.85E+09^{ns}	2.63E+10^{ns}	2.58E+09^{ns}	4.32E+09^{ns}	-	2.37E+10^{ns}	1.26E+11^{ns}	1.47E+10^{ns}
CONV_AG	2.53E+11^{ns}	1.35E+10^{ns}	2.09E+10^{ns}	3.72E+09^{ns}	1.49E+09^{ns}	2.29E+09^{ns}	3.45E+09^{ns}	2.64E+09^{ns}	-	1.76E+10^{ns}	1.33E+11^{ns}	7.44E+09^{ns}

CONV_FRES	2.04E+11^{ns}	4.94E+09^{ns}	3.31E+09^{ns}	8.01E+09^{ns}	2.02E+08^{ns}	3.63E+09^{ns}	2.61E+08^{ns}	2.31E+08^{ns}	-	1.26E+10^{ns}	1.04E+11^{ns}	4.04E+09^{ns}
CONV_SLU	2.24E+11^{ns}	2.37E+10^{ns}	6.04E+09^{ns}	1.17E+09^{ns}	8.21E+08^{ns}	2.39E+09^{ns}	2.79E+09^{ns}	1.11E+09^{ns}	-	1.61E+10^{ns}	1.24E+11^{ns}	4.35E+09^{ns}
UNAMEN	4.41E+11^{ns}	2.44E+10^{ns}	4.89E+09^{ns}	1.51E+09^{ns}	3.15E+09^{ns}	3.38E+08^{ns}	2.04E+08^{ns}	3.53E+09^{ns}	-	2.68E+10^{ns}	2.33E+11^{ns}	5.77E+09^{ns}
TYPE	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns
ORIGIN	ns	ns	ns	ns	*	ns	ns	ns	-	ns	ns	ns
TYPE X ORIGIN	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns

Supplementary Table 7.2. Kendall's rank correlation coefficients between prokaryotic orders in lettuce and wheat soils and ARG and MGE-gene absolute abundances grouped by antibiotic family or MGE category, followed by Bonferroni's multiple comparisons test.

	Taxon/Antibiotic family or MGE category	Aminoglycoside	β-lactam	FCA	Integrase	MLSB	Multidrug	Sulfonamide	Tetracycline	Transposase	Vancomycin
Lettuce soil	**Acidimicrobiales**										0.45
	Acidobacteria 1		-0.47		-0.41						-0.40
	Ardenticatenales		-0.38			-0.46					
	B1-7BS				-0.39						
	BC-COM435				-0.45						
	Bdellovibrionales					-0.39					-0.43
	Blastocatellales					-0.38					
	Burkholderiales				-0.47	-0.44					-0.42
	Caldilineales							-0.38			
	Cellvibrionales					-0.42					
	Chitinophagales					-0.39					-0.38
	Chlorobiales					-0.50					
	Chthoniobacterales			-0.39	-0.48	-0.52					-0.49
	Cytophagales					-0.42					-0.39
	Desulfurellales										-0.41
	Fibrobacterales					-0.50					
	Flavobacteriales					-0.40			-0.40		-0.43
	Gaiellales										0.45
	Halanaerobiales				-0.38						

	HTA4		-0.39	-0.38			-0.51		-0.54
	Legionellales		-0.40	-0.43			-0.43		-0.50
	Leptospiriales				-0.39				
	Micrococcales				0.41				0.41
	Micromonosporales								0.42
	mle1-8							-0.42	
	NB1-j								-0.46
	Neisseriales						-0.39		
	Obscuribacterales		-0.42	-0.50	-0.39				-0.46
	Oceanospirillales			-0.44	-0.45				-0.42
	Oligoflexales				-0.45				
	Opitutales				-0.50				
	Planctomycetales	-0.41				-0.43			
	Propionibacteriales								0.39
	Pseudonocardiales				0.39		0.41		0.43
	Rhizobiales				0.45		0.41		
	Rickettsiales				-0.43				
	Rubrobacterales		0.39						0.38
	SAR324 clade		-0.48	-0.49	-0.38				-0.53
	SC-I-84					0.38			
	Selenomonadales					-0.41			
	Solirubrobacterales			0.38	0.46		0.39		0.59
	Spartobacteria 1								-0.40
	Vampirovibrionales		-0.47	-0.45					-0.38
Wheat soil	**Puniceicoccales**			-0.45					
	47209					-0.44			
	Kallotenuales			-0.41					
	Synechococcales					0.45			
	Unknown Deltaproteobacteria order 4				-0.44				
	Unknown Deltaproteobacteria order 5			-0.44					
	Limnochordales			0.39	0.41				

WN-HWB-116	0.46				0.42
S15A-MN16			0.39		
CPla-3 termite group			0.41		
Nostocales			0.38		
JG30-KF-CM45		-0.38			
Tepidisphaerales	0.38			0.39	0.41
Xanthomonadales		-0.47			

Supplementary Table 7.3. Relative abundances of bacterial orders in lettuce soils and wheat soils (only those orders that showed statistically significant differences are presented). Different letters indicate significant ($p<0.05$) differences according to one-way ANOVA and Duncan`s multiple range test. ORG_AG: aged manure from organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAMEN: unamended.

LETTUCE SOIL	ORG_AG	ORG_FRES	ORG_SLU	CONV_AG	CONV_FRES	CONV_SLU	UNAMEN
Acidobacteria group 22	4.71E-03^{cd}	6.20E-03^{b}	7.65E-03^{a}	4.27E-03^{cd}	5.01E-03^{bc}	3.46E-03^{d}	5.05E-03^{bc}
C0119	8.06E-04^{c}	1.03E-03^{bc}	7.08E-04^{c}	1.05E-03^{bc}	9.25E-04^{bc}	1.87E-03^{a}	1.27E-03^{b}
Chlorobiales	1.05E-03^{b}	1.32E-03^{ab}	1.07E-03^{b}	1.60E-03^{a}	9.38E-04^{b}	1.50E-03^{a}	5.20E-04^{c}
Cytophagales	1.33E-02^{d}	1.94E-02^{ab}	2.02E-02^{a}	1.79E-02^{ab}	1.49E-02^{cd}	1.67E-02^{bc}	1.37E-02^{cd}
KD4-96	1.65E-02^{d}	1.94E-02^{bcd}	1.51E-02^{d}	1.81E-02^{cd}	2.27E-02^{bc}	2.45E-02^{b}	3.17E-02^{a}
Nitrososphaerales	1.54E-02^{a}	1.66E-02^{a}	1.02E-02^{b}	1.27E-02^{b}	1.15E-02^{b}	1.58E-02^{a}	1.62E-02^{a}
Nitrospirales	2.96E-03^{b}	4.60E-03^{a}	3.44E-03^{b}	3.36E-03^{b}	2.90E-03^{b}	3.31E-03^{b}	3.04E-03^{b}
Obscuribacterales	4.09E-04^{ab}	3.87E-04^{ab}	2.74E-04^{bc}	3.16E-04^{bc}	2.32E-04^{cd}	4.57E-04^{a}	1.16E-04^{d}
Pseudonocardiales	5.99E-03^{b}	5.55E-03^{b}	5.74E-03^{b}	5.72E-03^{b}	6.08E-03^{b}	5.62E-03^{b}	8.76E-03^{a}
SAR324 clade	2.22E-04^{bc}	5.27E-04^{a}	2.53E-04^{bc}	2.12E-04^{bc}	2.66E-04^{bc}	3.28E-04^{b}	1.47E-04^{c}
SC-I-84	2.15E-02^{a}	9.75E-03^{b}	2.01E-02^{a}	1.92E-02^{a}	1.89E-02^{a}	1.32E-02^{b}	9.18E-03^{b}
Solibacterales	3.37E-03^{a}	3.57E-03^{a}	3.46E-03^{a}	3.43E-03^{a}	3.54E-03^{a}	2.79E-03^{a}	1.72E-03^{b}
Solirubrobacterales	2.85E-02^{bc}	2.54E-02^{bc}	2.29E-02^{c}	2.98E-02^{bc}	3.26E-02^{b}	2.56E-02^{bc}	4.09E-02^{a}
Vampirovibrionales	3.74E-04^{bc}	4.55E-04^{b}	2.76E-04^{cd}	3.74E-04^{bc}	2.23E-04^{cd}	6.34E-04^{a}	2.13E-04^{d}
Verrucomicrobiales	2.63E-03^{c}	4.88E-03^{a}	3.37E-03^{bc}	3.10E-03^{bc}	3.28E-03^{bc}	3.81E-03^{b}	2.71E-03^{c}
WHEAT SOIL	ORG_AG	ORG_FRES	ORG_SLU	CONV_AG	CONV_FRES	CONV_SLU	UNAMEN
Desulfurellales	1.97E-02^{ab}	1.85E-02^{bc}	1.43E-02^{d}	1.59E-02^{cd}	1.79E-02^{bc}	1.80E-02^{bc}	2.26E-02^{a}
Lineage IIb	1.19E-03^{a}	6.37E-04^{b}	6.65E-04^{b}	9.68E-04^{a}	1.09E-03^{a}	4.86E-04^{b}	6.94E-04^{b}
Rhodospirillales	1.86E-02^{c}	2.02E-02^{bc}	2.22E-02^{ab}	1.83E-02^{c}	1.90E-02^{c}	2.42E-02^{a}	1.97E-02^{bc}

Supplementary Table 7.4. Differences (based on Welch's *t*-test) in ARG and MGE-gene absolute abundances in lettuce soils and wheat soils & in lettuce plants and wheat grains, according to treatments. L: lettuce samples; W: wheat samples. ORG_AG: aged manure form organic farm; ORG_FRES: fresh manure from organic farm; ORG_SLU: slurry from organic farm; CONV_AG: aged manure from conventional farm; CONV_FRES: fresh manure from conventional farm; CONV_SLU: slurry from conventional farm; UNAMEN: unamended. ns: no significant; *: p<0.05; **: p<0.01; ***: p<0.001.

	Integrase		**Transposase**		**Aminoglycoside**		**β-lactam**		**FCA**		**MLSB**		**Sulfonamide**		**Tetracycline**		**Vancomycin**	**Multidrug**	
Treatment	Soil	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil	Soil	Plant
ORG_AG	ns	ns	ns	ns	W>L*	W>L*	W>L**	ns	ns	ns	ns	ns	ns	ns	W>L*	W>L*	W>L**	W>L**	W>L***
ORG_FRES	ns	ns	ns	ns	W>L*	ns	W>L**	ns	W>L*	W>L*	W>L*	ns	ns	ns	W>L*	ns	W>L*	ns	ns
ORG_SLU	ns	W>L*	ns	ns	W>L*	W>L*	W>L*	W>L*	ns	ns	ns	ns	ns	ns	W>L*	W>L*	ns	ns	W>L*
CONV_AG	ns	W>L*	ns	ns	ns	W>L*	W>L*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	W>L*
CONV_FRES	ns	W>L*	ns	ns	W>L*	ns	W>L**	ns	ns	ns	ns	ns	ns	ns	W>L*	ns	W>L*	ns	ns
CONV_SLU	ns	W>L*	ns	ns	W>L**	ns	W>L**	ns	ns	ns	ns	ns	ns	ns	ns	W>L*	ns	ns	W>L*
UNAMEN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

8. AGRICULTURAL SOILS AMENDED WITH THERMALLY-DRIED ANAEROBICALLY-DIGESTED SEWAGE SLUDGE SHOWED INCREASED RISK OF ANTIBIOTIC RESISTANCE DISSEMINATION

Abstract

The application of sewage sludge (SS) to agricultural soil can help meet crop nutrient requirements and enhance soil properties, while reusing an organic by-product. However, SS can be a source of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs), resulting in an increased risk of antibiotic resistance dissemination. We studied the effect of the application of thermally-dried anaerobically-digested SS on (i) soil physicochemical and microbial properties, and (ii) the relative abundance of 85 ARGs and 10 MGE-genes in soil. Soil samples were taken from a variety of SS-amended agricultural fields differing in three factors: dose of application, dosage of application, and elapsed time after the last application. The relative abundance of both ARGs and MGE-genes was higher in SS-amended soils, compared to non-amended soils, particularly in those with a more recent SS application. Some physicochemical parameters (*i.e.*, cation exchange capacity, copper concentration, phosphorus content) were positively correlated with the relative abundance of ARGs and MGE-genes. Sewage sludge application was the key factor to explain the distribution pattern of ARGs and MGE-genes. The 30 most abundant families within the soil prokaryotic community accounted for 66% of the total variation of ARG and MGE-gene relative abundances. Soil prokaryotic α-diversity was negatively correlated with the relative abundance of ARGs and MGE-genes. We concluded that agricultural soils amended with thermally-dried anaerobically-digested sewage sludge showed increased risk of antibiotic resistance dissemination.

8.1. Introduction

In the current scenario of increasing world population and environmental degradation, the transition to a Circular Economy model requires, among many other aspects, the reuse and sustainable management of wastes and by-products. The application of sewage sludge (SS) to soil is a common agricultural practice that can certainly lead to agronomic improvements such as, for instance: (i) increased soil organic matter (OM) and nutrient content (Lashermes et al., 2009); (ii) enhanced soil porosity and bulk density (Singh & Agrawal, 2007; Annabi et al., 2011); (iii) greater water holding capacity (Bulluck et

al., 2002); (iv) higher soil microbial activity (García et al., 1992); and (v) improved overall soil quality (Powlson et al., 2011). The application of organic amendments (*e.g.*, SS) into soil can also result in structural and functional changes in soil bacterial communities (Innerebner et al., 2006; Epelde et al., 2014).

However, the application of SS to agricultural soil as organic amendment can likewise lead to potential risks for human and environmental health, in particular, owing to the presence in SS of: (i) elevated concentrations of potentially toxic metals (Singh & Agrawal, 2007); (ii) organic contaminants (Alvarenga et al., 2015); (iii) nanoparticles (Fijalkowski et al., 2017); (iv) microplastics; and (v) pharmaceutical compounds (Martín et al., 2015), including antibiotics and their transformation products. These later chemical emerging contaminants (antibiotics and their transformation products) are often accompanied in SS by several biological contaminants of great concern, *i.e.,* antibiotic resistance genes (ARGs), antibiotic resistant bacteria (ARB) and mobile genetic elements (MGEs).

Most worryingly, the use, overuse and misuse of antibiotics for medical and veterinary use have promoted the emergence and spread of antibiotic resistance in the environment, including the soil ecosystem (Cytryn, 2013). Antibiotic resistant bacteria can transfer to other bacteria (including potential human pathogens) the ARGs they harbor through horizontal gene transfer (HGT) via a variety of MGEs such as plasmids, transposons, integrative conjugative elements, phages, integrons, genomic islands, etc. (Pruden et al., 2006; Zhu et al., 2013).

In the European Union, in 2014, the consumption of antimicrobials for medical and veterinary use, including animal production, reached a staggering value of 12,720 tonnes of active substance (ECDC, EFSA & EMA, 2017). It has been reported (Van Boeckel et al., 2017) that, worldwide, more than 73% of all antimicrobials are administered to animals for veterinary or food-producing purposes. Relevantly, a considerable amount (between 30 and 90%) of the antibiotics administered for human or veterinary purposes are excreted in the urine and feces, essentially unchanged or as active metabolites (Sarmah et al., 2006). Regrettably, wastewater treatment plants (WWTPs) are not designed to efficiently remove these emerging contaminants and, then, they are unsurprisingly regarded as hotspot for the emergence and dissemination of antibiotic resistance (Rizzo et al., 2013).

In SS, the concentration of widely use antibiotics ranges from $\mu g\ kg^{-1}$ to $mg\ kg^{-1}$ (Lillenberg et al., 2009; Nieto et al., 2010). Although there are certainly many differences between countries and

specific WWTPs, resulting in the impossibility to describe a common pattern, several authors (Zhang & Li, 2011; Rutgersson et al., 2020) detected lower levels of sulfonamides, macrolides and tetracyclines, compared to quinolones, in SS samples.

In the European Union, the most common method of SS disposal remains its application to agricultural soil, followed by thermal disposal and landfill (Christodoulou & Stamatelatou, 2016). The improper disposal of SS can lead to serious environmental risks such as, for instance, the contamination of aquifers and soils with a variety of potentially toxic inorganic and organic compounds (Sharma et al., 2017). The majority of legislations on SS management, such as Directive 86/278/ECC dealing with soil protection when SS is used in agriculture (European Commission, 1986), do not consider the abovementioned emerging contaminants. Actually, these legislations have traditionally been focused on total metal concentrations, both in the SS itself and in the amended agricultural soil, as well as on the presence of potential human pathogens.

The aim of this study was to assess the impact of the application of thermally-dried anaerobically-digested SS on: (i) soil physicochemical and microbial properties, including soil prokaryotic diversity and composition; and (ii) the presence and relative abundance of ARGs and MGE-genes in soil. In particular, regarding the application of SS, three factors were studied: dose of application, dosage of application, and elapsed time after the last application. The term "dose" refers here to a specific amount of SS applied at one time. Instead, the term "dosage" refers to the total amount of SS applied to those fields (*i.e.*, the sum of all the individual applications). The novelty of the study is supported by the following facts: (1) the study was carried out with samples taken from a high number of real agricultural farms, *i.e.* 20 farms; (2) the studied agricultural fields differed in the abovementioned three factors, thus allowing the assessment of their individual and combined influence on the main topic under study, *i.e.* antibiotic resistance; and (3) the SS had been thermally-dried and anaerobically-digested prior to their application which, *a priori*, could have considerably reduced the risk of antibiotic resistance spread. We hypothesized that the application of SS would enhance soil physicochemical and microbial properties, alter the composition of soil prokaryotic communities and, finally, increase the relative abundance of ARGs and MGE-genes in soil. Moreover, we expected these changes to be dependent upon the three factors mentioned above. This study was carried out with SS from a single WWTP. Nonetheless, SS composition and management (*e.g.*, storage, treatment, dose

and mode of application, etc.) can significantly differ between WWTPs. This fact must be taken into consideration when comparing our results with those from other studies.

8.2. Materials and methods

8.2.1. Experimental design

The study was carried out with soil samples collected from 20 real agricultural fields (ranging from ca. 1 to 14 ha) located in the Valley of Orba (Valdorba), province of Navarre (North of Spain). The Valley of Orba is composed of a series of valleys occupying a total area of 130 ha (46% of that area corresponds to agricultural land). Thermally-dried anaerobically-digested SS from a local WWTP had been applied to 13 out of those abovementioned 20 agricultural fields. The physicochemical properties of the SS were: pH = 8.1; dry matter content = 17.3%; C/N ratio = 5.6; total metal concentrations < 3, 73, 196, 40, 47 and 936 mg kg^{-1} dry weight-DW SS for Cd, Cr, Cu, Ni, Pb and Zn, respectively.

Regarding the application of SS, these 13 agricultural fields differed in the following three factors: (i) *dose of application* = 22, 33 and 44 t ha^{-1}; (ii) *dosage of application* = 22, 55, 77 and 99 t ha^{-1}; and (iii) *elapsed time after the last application* = 1, 2, 3 and 4 years ago. Seven fields had never been amended with SS and were then used as control unamended soils.

As described above, soil samples were collected from each agricultural field to study the impact of the application of SS on soil physicochemical and microbial properties, as well as on the presence and relative abundance of ARGs and MGE-genes in soil. In particular, three composite soil samples, each composed of 10 soil cores (depth: 0-10 cm) randomly taken in an area of 10 m^2, were collected per agricultural field, each of them corresponding to a different area within such field. The three areas were at least 50 meters apart from each other. At soil sampling times, the agricultural fields were planted with different crops (barley, beans, rapeseed, wheat). Soil samples were collected in polyethylene bags, protected from sunlight, and immediately transferred to the laboratory. The experimental design is summarized in Table 8.1.

Table 8.1. Experimental design: description of the 20 agricultural fields.

Field	SS applications (year)	Elapsed time after the last application (years)	Dose (t ha^{-1})	Dosage (t ha^{-1})	Current crop
1	2009 + 2013	4	33/22	55	Rapeseed
2	-		-	-	Barley
3	2007 + 2011 + 2015	2	33/22/22	77	Barley
4	-		-	-	Wheat
5	2006 + 2010 + 2014	3	44/33/22	99	Wheat
6	-		-	-	Wheat
7	2008 + 2012 + 2016	1	33/22/22	77	Wheat
8	-		-	-	Wheat
9	2006 + 2010 + 2014	3	44/33/22	99	Wheat
10	2013	4	22	22	Barley
11	-		-	-	Beans
12	2008 + 2012 + 2016	1	33/22/22	77	Barley
13	2007 + 2011 + 2015	2	33/22/22	77	Barley
14	2009 + 2013	4	33/22	55	Rapeseed
15	-		-	-	Barley
16	2006 + 2010 + 2014	3	44/33/22	99	Wheat
17	2008 + 2012 + 2016	1	33/22/22	77	Wheat
18	-		-	-	Wheat
19	2005 + 2009 + 2013	4	44/33/22	99	Wheat
20	2007 + 2011 + 2015	2	33/22/22	77	Rapeseed

8.2.2. Soil physicochemical properties

Prior to the determination of soil physicochemical parameters, soil samples were air-dried at room temperature until constant weight. The following parameters were determined in SS and soil samples according to standard methods (MAPA, 1994): OM content, pH, cation exchange capacity (CEC), electrical conductivity (EC), texture, water soluble organic carbon (WSOC), total nitrogen (N), Olsen phosphorus (P), and content of nitrate (NO_3^-), ammonium (NH_4^+) and potassium (K^+). Inductively coupled plasma-optical emission spectrometry (ICP-OES) was used for the determination of pseudo-total metal concentrations following aqua regia digestion (McGrath and Cunliffe, 1985). $CaCl_2$-extractable (0.01 M), $NaNO_3$ extractable (0.1 M) and low molecular weight organic acid (LMWOA) solution-extractable metal fractions in soil and SS were determined following Houba et al. (2000), Gupta & Aten (1993), and Feng et al. (2005), respectively.

8.2.3. Soil microbial properties

For the determination of soil microbial parameters, fresh soil samples were sieved to <2 mm and stored at 4 ºC for less than a month prior to their analysis. Microbial biomass carbon was determined according

to Vance et al. (1987). Soil respiration was measured following ISO 16072 (2002). For the molecular analyses, DNA was extracted from soil samples (0.25 g DW soil) using the Power Soil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Prior to DNA extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk et al., 1997). DNA concentration was determined using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). The extracted DNA was stored at -20 ºC until use.

Amplicon libraries preparation was carried out as described in Lanzén et al. (2016), using a dual indexed adapter with sequence-specific primers for prokaryotic communities, targeting the V4 region of the 16S rRNA genes. Primers were 519F (CAGCMGCCGCGGTAA) adapted from Ovreås et al. (1997) and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso et al. (2012). Sequencing was carried out with an Illumina MiSeq V2 platform and paired-end sequencing strategy (2 × 250 nt) at Tecnalia, Spain. Reads were merged, quality filtered and clustered into operational taxonomic units (OTUs) as described in Lanzén et al. (2016). The taxonomic classification was performed using CREST (Lanzén et al., 2012).

High-throughput real-time PCR (HT-qPCR) was employed to quantify the abundance of ARGs and MGE-genes using the nanofluidic qPCRBioMarkTM HD system with 48.48 and 96.96 Dynamic Array Integrated Fluidic Circuits (IFCs) (Fluidigm Corporation), following Urra et al. (2019b). A total of 96 validated primer sets (Hu et al., 2016) were used: 85 primer sets targeting ARGs conferring resistance against all major classes of antibiotics [10 aminoglycosides, 14 β-lactams, 5 FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol), 13 MLSB (macrolide, lincosamide, streptogramin B), 5 multidrugs, 4 sulfonamides, 24 tetracyclines and 10 vancomycines], 10 primer sets targeting MGE-genes (8 genes encoding transposases, 2 genes encoding integrases) and one primer set for the 16 rRNA gene. Measurements were conducted in the Gene Expression Unit of the Genomics Facility of SGIker – University of the Basque Country, Spain. Raw data were processed with the Fluidigm Real-Time PCR Analysis Software (v.3.1.3) with linear baseline correction and manual threshold settings. A threshold cycle (C_T value) of 31 was chosen, as the highest C_T value obtained in our study was 30.5. The detection of the ARGs and MGE-genes was considered positive when at least 3 of the 4 technical replicates for each sample were above the detection limit. The relative copy number was calculated as the proportion of the abundance of a given ARG or MGE-gene to the abundance of the 16S rRNA gene (Looft et al., 2012).

8.2.4. Statistical analysis

The calculation of α-diversity indices for the studied soil prokaryotic communities, as well as the treatment of 16S rRNA gene amplicon sequencing data, were performed with R package *vegan* (Oksanen et al., 2015). Rarefied richness was calculated to compensate for the observed variation in read numbers across samples (it was estimated by means of interpolating the expected richness at the lowest sample-specific sequencing depth). Differences in the abundance of prokaryotic taxa at family level among plots were determined, followed by Bonferroni's multiple comparisons test, using R software (v.3.5.1).

The statistical significance of the observed differences in the values of soil physicochemical properties, microbial properties and ARG and MGE-gene relative abundances between SS-amended and unamended soils, and ARG and MGE-gene relative abundances in our SS, were determined by Welch's t-test (for unequal variances and unequal sample sizes) using package *agricolae* of R software (v.3.5.1). All statistical tests were considered significant at $p<0.05$, except for Bonferroni correction.

Relationships between (i) SS experimental factors (presence/absence of SS, dose of application, dosage of application, elapsed time after the last application); (ii) soil physicochemical properties; (iii) abundance of ARGs and MGE-genes; (iv) the most abundant prokaryotic families; and (v) values of α-diversity indices were explored by redundancy analysis (RDA) using Canoco 5 (Ter Braak & Ŝmilauer, 2012). Response data were log transformed and centered, and the number of permutations was unrestricted. Redundancy analyses were performed after forward selection in which only those explanatory variables that contributed significantly to the analysis were taken into account. In order to study the influence of SS application on the relative abundance of ARGs and MGE-genes, the abovementioned SS experimental factors were used as explanatory variables. In the same way, RDAs were conducted to find out how much of the variability in the relative abundance of ARGs and MGE-genes could be attributed to the: (i) soil physicochemical properties; (ii) the α-diversity of soil prokaryotic communities; and/or (iii) the composition of soil prokaryotic communities (*i.e.*, the 30 most abundant families). The variables *field* and *current crop* were used as covariates. Kendall's rank correlation coefficients (followed by Bonferroni's multiple comparisons test) between prokaryotic taxa at family level and the relative abundance of ARGs and MGE-genes were obtained using R software (v.3.5.1). A network analysis was performed to explore the correlations between multi-resistant

prokaryotic families and ARGs and MGE-genes. Network visualization was conducted in Gephi platform.

A principal component analysis (PCA) was performed to reduce the dimensionality of the soil physicochemical properties (*i.e.*, dry matter, OM, WSOC, pH, CEC, EC, NO_3^-, NH_4^+, total N, Olsen P, K^+, clay and loam content). Two linear axes, which explained the maximum amount of variance, were selected for Structural Equation Modelling (SEM) analysis (Curiel Yuste et al., 2019). The first axis (PC1, 28.8% of the explained variance) was positively correlated with total N, K^+, Olsen P, OM, clay content and CEC. By contrast, PC1 was negatively correlated with WSOC, NH_4^+ and pH. The second axis (PC2, 17.7% of the explained variance) was positively correlated with NO_3^-, EC and loam content. The heavy metal (HM) pollution index was calculated as the mean of the ratios between the concentration of each specific HM and its corresponding regulatory limit according to "Law 4/2015, on prevention and correction of soil contamination in the Basque Country (BOE-A-2015-8272)". Structural equation models were used to assess the direct and indirect influence of biotic (prokaryotic diversity indices) and abiotic (dosage of application, elapsed time after the last application, soil physicochemical parameters, HM pollution index) factors on ARGs and MGE-genes. The variables *field* and *current crop* were considered as random factors in the SEM, which was performed using *piecewiseSEM* package in R (Lefcheck, 2016). The Shipley's direct separation test was used to assess the overall fit of the models. Models were accepted when Fisher's C statistic was above the significance level ($p<0.05$). The Akaike's Information Criterion (AIC) was used to perform accepted model comparisons. Standardized path coefficients, which describe the strength and sign of the relationship between two variables, were estimated by using the maximum likelihood algorithm (Shipley, 2018).

8.3. Results

8.3.1. Physicochemical and microbial properties of SS-amended and unamended soils

Soil physicochemical properties varied considerably among the 20 studied fields (Table 8.2). Soils had a loam or clay loam texture and showed the following physicochemical properties: OM content = 2.28%, WSOC = 99 mg kg^{-1}, pH = 8.4, CEC = 14.4 mEq 100 g^{-1}, EC = 0.153 mS cm^{-1}, NO_3^- content = 101.4 mg kg^{-1} and NH_4^+ content = 2.24 mg kg^{-1} (Table 8.2). Values of extractable metals were below the quantification limit. The application of SS significantly increased Olsen P and Zn content in SS-amended soils compared to unamended soils.

Table 8.2. Physicochemical and microbial properties of the 20 agricultural soils. *: p<0.05; **: p<0.01, based on Welch's t-test.

Property	Mean ± SD	Range	SS amended	Unamended
Dry matter (%)	86.5 ± 1.9	81.2 - 87.9	86.4 ± 2.2	86.6 ± 1.1
OM (%)	2.3 ± 0.4	1.8 - 3.0	2.3 ± 0.4	2.2 ± 0.4
WSOC (mg C kg^{-1} DW soil)	99.0 ± 35.5	54.2 - 154.4	96.4 ± 36.4	104 ± 34
pH	8.4 ± 0.1	8.3 - 8.5	8.4 ± 0.1	8.4 ± 0.1
CEC (mEq 100 g^{-1})	14.4 ± 3.1	10.4 - 19.4	14.9 ± 3.1	13.5 ± 2.8
EC (mS cm^{-1})	0.15 ± 0.02	0.13 - 0.18	0.15 ± 0.02	0.15 ± 0.02
NO_3^- (mg kg^{-1})	101 ± 69	41 - 226	103 ± 68	98.8 ± 72
NH_4^+ (mg kg^{-1})	2.2 ± 1.1	1.1 - 4.3	2.2 ± 0.9	2.4 ± 1.4
N (%)	0.16 ± 0.03	0.12 - 0.20	0.16 ± 0.03	0.15 ± 0.03
P (mg kg^{-1})*	25.0 ± 14.6	9.4 - 47.8	28.0 ± 14.6	19.4 ± 13.2
K (mg kg^{-1})	1372 ± 621	873 - 2854	1383 ± 578	1353 ± 708
Clay (%)	30.3 ± 4.8	25.8 - 36.2	30.3 ± 4.6	30.1 ± 5.2
Sand (%)	32.0 ± 5.2	25.6 - 39.7	32.0 ± 5.5	32.0 ± 5.0
Silt (%)	37.8 ± 3.6	34.5 - 41.5	37.7 ± 3.5	37.9 ± 3.8
Cd (mg kg^{-1} DW soil)	1.7 ± 0.50	1.1 - 2.7	1.7 ± 0.49	1.6 ± 0.53
Cr (mg kg^{-1} DW soil)	20.8 ± 5.9	13.3 - 29.8	21.4 ± 5.5	19.7 ± 6.5
Cu (mg kg^{-1} DW soil)	21.0 ± 5.9	13.9 - 30.6	22.0 ± 5.4	19.3 ± 6.4
Ni (mg kg^{-1} DW soil)	25.7 ± 5.1	18.8 - 33.2	26.5 ± 4.8	24.3 ± 5.3
Pb (mg kg^{-1} DW soil)	19.5 ± 5.4	12.8 - 26.4	20.2 ± 5.3	18.2 ± 5.3
Zn (mg kg^{-1} DW soil)**	59.3 ± 10.0	42.8 - 73.6	62.3 ± 8.5	53.9 ± 10.3
BR (mg C kg^{-1} DW soil h^{-1})	1.28 ± 0.21	0.98 - 1.56	1.28 ± 0.21	1.30 ± 0.19
MBC (mg C kg^{-1} DW soil)	636 ± 104	455 - 812	645 ± 103	618 ± 107
Richness (R)**	3536 ± 622	2670 - 4390	3390 ± 638	3802 ± 504
Shannon's index (H')*	7.0 ± 0.27	6.5 - 7.4	6.9 ± 0.28	7.1 ± 0.23
Simpson's index (D)*	0.997 ± 0.001	0.996 - 0.998	0.997 ± 0.001	0.998 ± 0.001
Pielou's evenness (J')	0.813 ± 0.020	0.782 - 0.839	0.811 ± 0.021	0.817 ± 0.020

Regarding soil microbial properties (Table 8.2), basal respiration (BR) values ranged from 0.98 (Field 1) to 1.56 (Field 17) mg C kg^{-1} DW soil h^{-1}. In turn, values of microbial biomass carbon (MBC) ranged from 455.1 (Field 11) to 812.4 (Field 14) mg C kg^{-1} DW soil. Similarly, values of prokaryotic α-diversity ranged between 2670 and 4390 for richness (R); 6.51 and 7.35 for the Shannon's index (H'); 0.996 and 0.998 for the Simpson's index (D); and 0.782 and 0.839 for Pielou's evenness (J'). Statistically significant (p<0.001) differences between agricultural fields were observed for all α-diversity parameters. Field 9 showed the lowest values for all the α-diversity indices determined here. By contrast, Field 7 showed the highest R, H' and D values (the highest J' value was observed in Field 6). In addition, significantly lower R, H' and D values were found in SS-amended *vs.* unamended soils.

Regarding soil prokaryotic community composition, 79.1, 67.3 and 34.5% of the 16S rRNA gene amplicon reads were taxonomically classified to order, family and genus rank, respectively. Out of the 2500 classified families, statistically significant differences in family abundance among

agricultural soils were found for only 13 families. Three of these 13 families were included among the 30 most abundant families detected in our study: *Cytophagaceae*, *Methylobacteriaceae* and *Xanthomonadales Incertae Sedis* (Supplementary Figure 8.1). In the SS itself, *Cytophagaceae* was the most abundant family, followed by *Nitrosomonadaceae*, *Xanthomonadales Incertae Sedis*, *Chitinophagaceae* and *Xanthomonadaceae*.

8.3.2. Antibiotic resistance in SS-amended *vs.* unamended soils

Out of the 85 studied ARGs, 77 were amplified by HT-qPCR in both SS-amended and unamended soil (74 ARGs were amplified in the SS itself). In addition, the 10 targeted MGE-genes were amplified by HT-qPCR in the SS itself and SS-amended/unamended soil samples.

The relative abundances of ARGs and MGE-genes in amended soils, unamended soils and SS, grouped by antibiotic family or type of MGE-genes, are shown in Figure 8.1. The relative abundances of ARGs and MGE-genes were higher in SS-amended soils, compared to unamended ones. In particular, the relative abundances of genes conferring resistance to β-lactams ($p<0.001$), aminoglycosides and transposases ($p<0.01$), integrase and FCA ($p<0.05$) were significantly higher in SS-amended *vs.* unamended soils. Gene relative abundances in the SS itself were several tens of times higher than in soil samples. In SS, transposase genes showed the highest abundance values, followed by aminoglycoside genes.

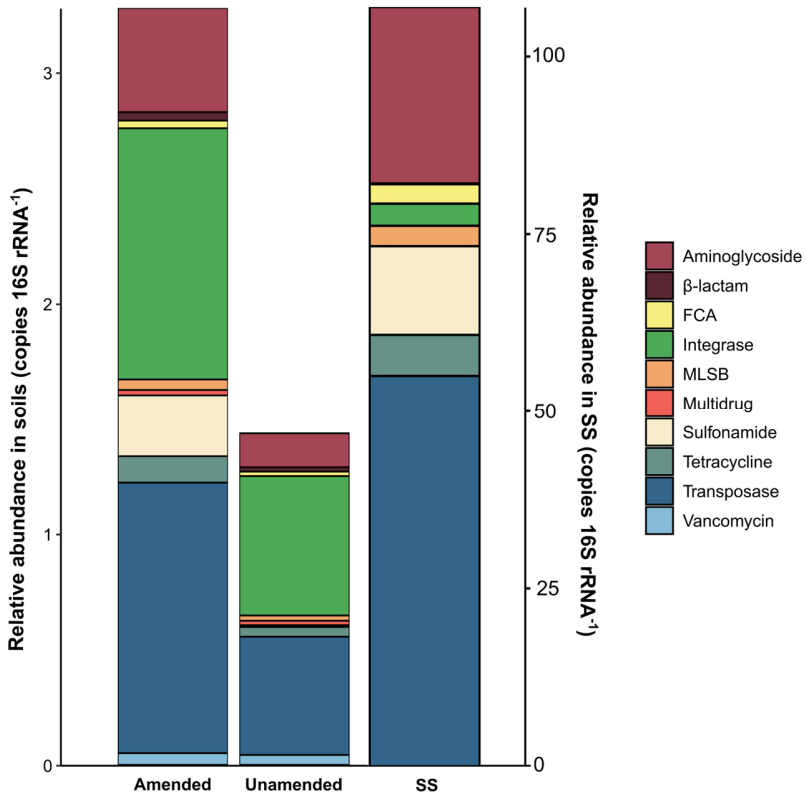


Figure 8.1. Relative abundances of ARGs and MGE-genes in amended soils, unamended soils and SS.

The effects of SS management (here, the term "SS management" includes the following three variables: dosage of application, elapsed time after the last application, and presence/absence of SS) on (i) soil physicochemical properties; (ii) the relative abundance of ARGs and MGE-genes; and (iii) the composition (at family rank) and α-diversity of soil prokaryotic communities, along with the interactions between these parameters, are summarized in Figure 8.2.

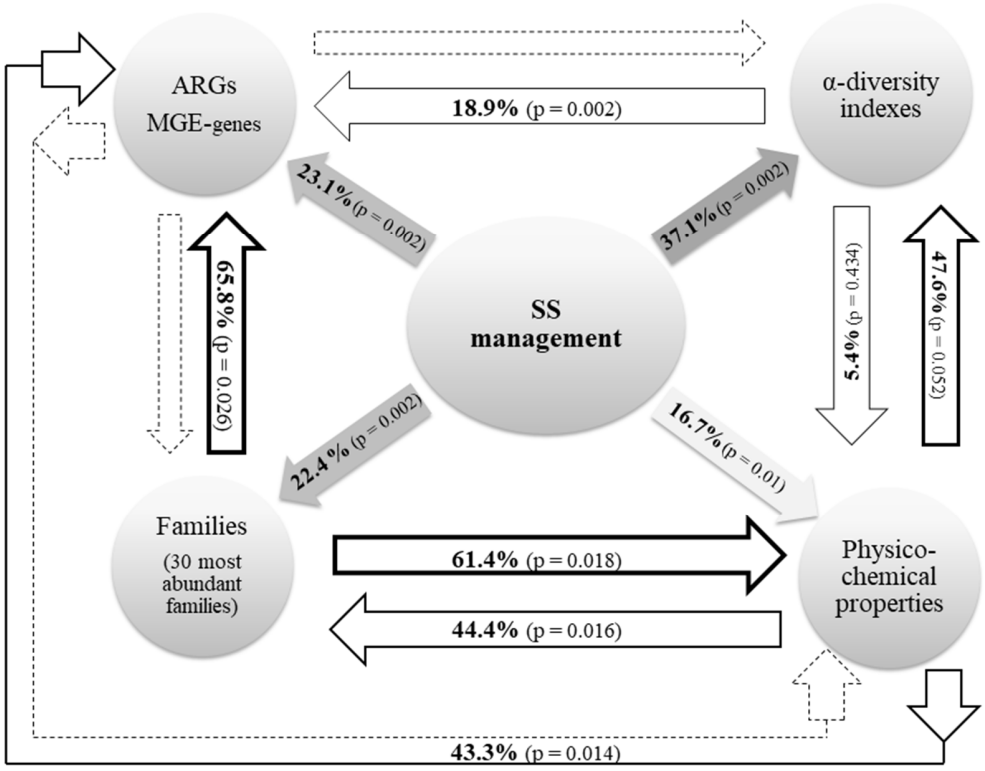


Figure 8.2. Radial diagram from RDA data. Solid lines represent a significant ($p<0.05$) effect of the corresponding variable. Dotted lines represent the lack of significant effect. The variable "SS management" includes the dosage of application, the elapsed time after the last application, and the presence/absence of SS. ARGs and MGE-genes: relative abundance (relative to the 16S rRNA gene) of ARGs and MGE-genes. α-diversity: richness, Shannon's diversity and Simpson's diversity. Physicochemical properties: OM, pH, CEC, EC, NO3-, NH4+, total N, Olsen P, K+, texture and pseudo-total metal concentrations (Cd, Cr, Cu, Ni, Pb and Zn).

Regarding the variation of ARG and MGE-gene relative abundances, the RDA represented in Figure 8.3 (23.1% variation explained, pseudo-F = 2.6, p = 0.002) shows that the presence of SS, an elapsed time of one year after the last application, and a dosage of application of 22 t SS ha^{-1} were the key factors explaining the distribution pattern of ARG and MGE-gene relative abundances in the studied soils. The factor *presence/absence of SS* was separated along RDA 1. The presence of SS was associated with increased ARG and MGE-gene relative abundances (Figure 8.2). Pertaining to soil physicochemical properties, 16.7% of the variation of the observed values was explained by SS management (pseudo-F = 1.7, p = 0.01). In addition, SS management significantly influenced the distribution of the 30 most abundant prokaryotic families (22.4% variation explained, pseudo-F = 2.5,

p = 0.002). Finally, 37.1% of the explained variation (pseudo-F = 4.9, p = 0.002) in α-diversity values (R, H' and D indices) was attributable to SS management.

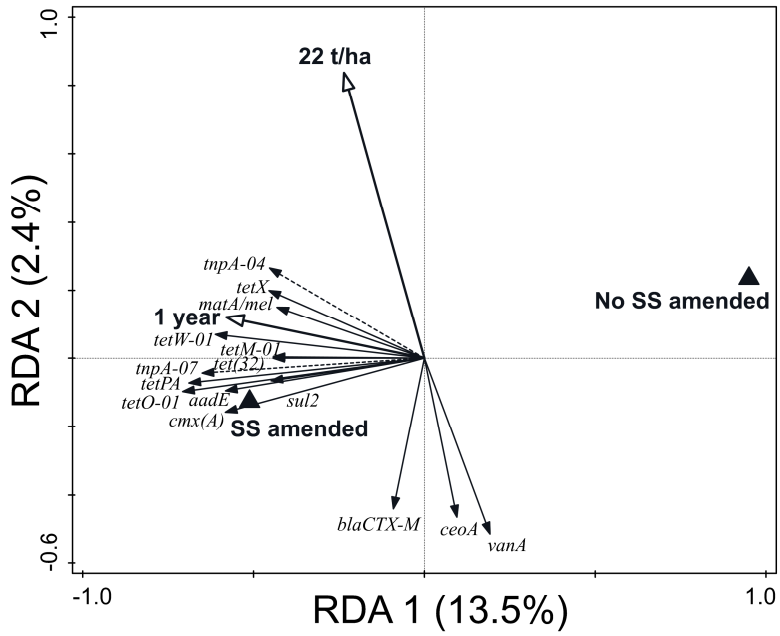


Figure 8.3. Biplot of the RDA performed with SS management (*i.e.*, dosage of application, elapsed time after the last application, and presence/absence of SS) as explanatory variables, ARGs and MGE-genes relative abundances as response variables, and field and current crop as covariates. Only statistically significant explanatory variables and response variables with the best fit are shown. The explanatory variables explained 23.1% of the variation in ARG and MGE-gene relative abundances.

The prokaryotic community composition, according to the 30 most abundant families, was significantly influenced by the physicochemical properties of the studied soils (44.4% variation explained, p = 0.016). On the other hand, the 30 most abundant families significantly (p = 0.018) explained 61.4% of the variation of soil physicochemical properties. Furthermore, 47.6% of the variation shown by the values of prokaryotic α-diversity (R, H' and D indices) was due to the physicochemical properties of the studied soils (nonetheless, this influence was not significant, p = 0.052). In turn, the distribution pattern of the values of soil physicochemical properties (5.4% variation explained, p = 0.434) was also influenced by soil prokaryotic α-diversity.

Similarly, 43.3% of the variation in ARG and MGE-gene relative abundances was explained by the values of soil physicochemical properties (pseudo-F = 1.5, p = 0.014) (Figure 8.4). Primarily, this variation was attributable to the pseudo-total Cu concentration (17.1%) but also to the values of

CEC and Olsen P. These physicochemical parameters were positively correlated with the relative abundance of most of the ARGs and MGE-genes studied here: pseudo-total Cu concentration and Olsen P were especially (positively) correlated with the abundance of tetracycline resistance genes. Values of CEC were mainly positively correlated with vancomycin resistance genes.

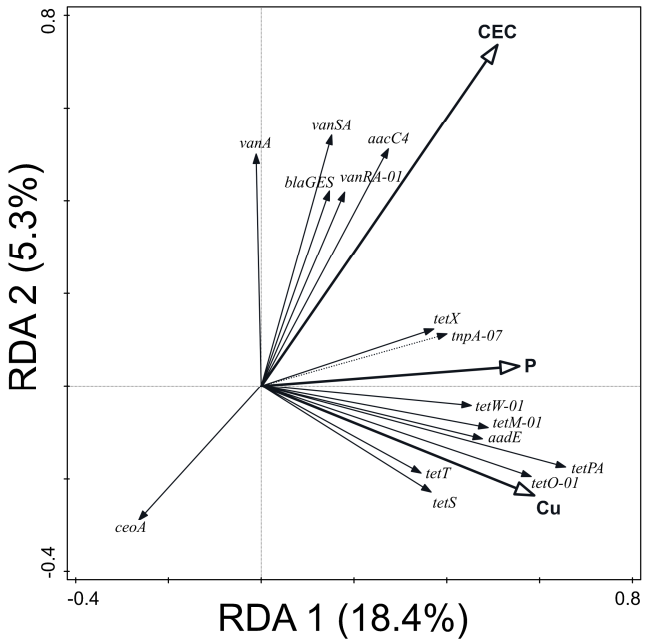


Figure 8.4. Biplot of the RDA performed with soil physicochemical properties (explaining 43.3% of the variation in ARG and MGE-gene relative abundances) as explanatory variables, the relative abundance of ARGs and MGE-genes as response variables, and field and current crop as covariates. Only statistically significant explanatory variables and response variables with the best fit are shown.

Furthermore, 65.8% of the variation in ARG and MGE-gene relative abundances was explained by the distribution of the 30 most abundant prokaryotic families (Figure 8.5). The prokaryotic families which contributed significantly were *Oxalobacteraceae*, *Sphingomonadaceae*, *Xanthomonadaceae*, *Flavobacteriaceae*, *Tepidisphaeraceae* and *Blastocatellaceae*. The abundance of *Blastocatellaceae*, *Oxalobacteraceae* and *Flavobacteriaceae* showed positive correlation with the relative abundance of *blaOXY* (gene conferring resistance to β-lactam antibiotics), *tetG-01* (gene conferring resistance to tetracyclines) and intl1 (class 1 integron integrase gene) genes. Particularly, the *Xanthomonadaceae* family positively explained the distribution of ARGs and MGE-genes.

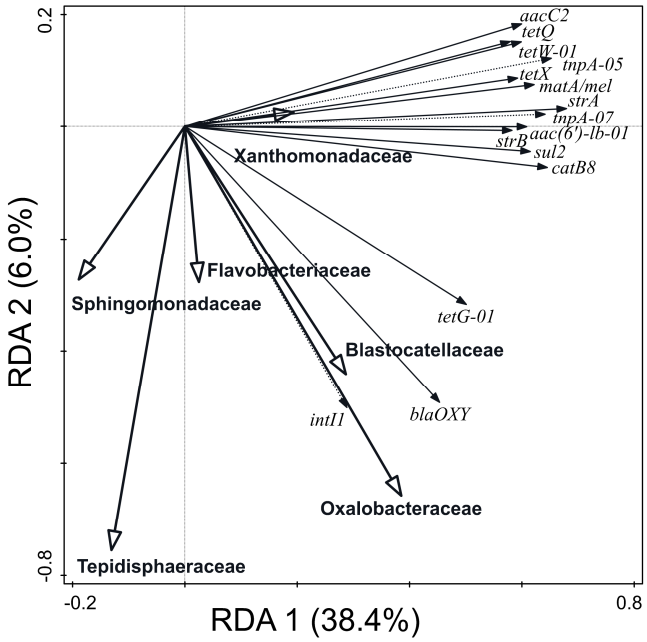


Figure 8.5. Biplot of the RDA performed with the 30 most abundant families (explaining 65.8% of the variation in ARG and MGE-gene abundances) as explanatory variables, the relative abundance of ARGs and MGE-genes as response variables, and field and current crop as covariates. Only statistically significant explanatory variables and response variables with the best fit are shown.

Moreover, 18.9% of the variation in the relative abundance of ARG and MGE-genes ($p = 0.002$) was attributable to prokaryotic α-diversity (Figure 8.6). The values of the Shannon´s and Simpson's index were negatively correlated with ARG and MGE-gene relative abundances. No correlation was found between richness and the distribution of ARGs or MGE-genes.

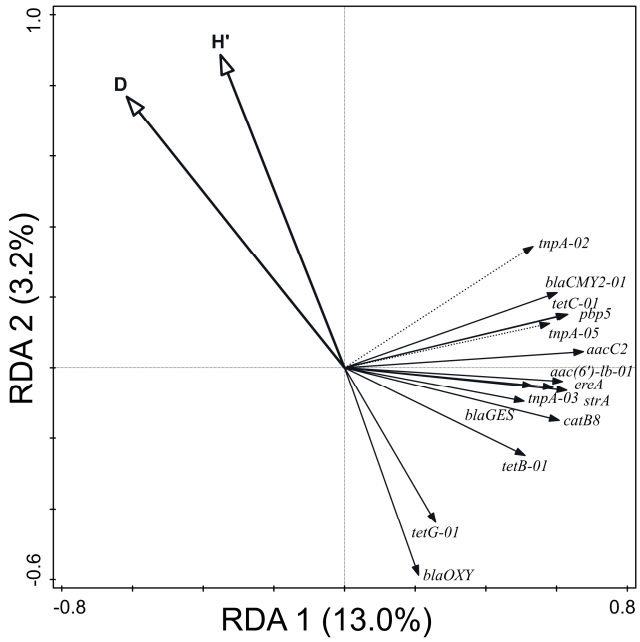


Figure 8.6. Biplot of the RDA performed with α-diversity indices (explaining 18.9% of the variation in the abundance of ARGs and MGE-genes) as explanatory variables, the relative abundance of ARGs and MGE-genes as response variables, and field and current crop as covariates. Only statistically significant explanatory variables and response variables with the best fit are shown.

Out of the 355 families that showed correlation with the relative abundance of at least one ARG or MGE-gene, 30 showed correlation with at least two genes that are known to confer resistance against several antibiotics. Among these 30 multi-resistant families, 12 showed significantly higher relative abundance values in SS-amended *vs.* unamended soils (Supplementary Table 8.1). The correlations between these 12 multi-resistant families and ARGs and MGE-genes were explored by network analysis (Figure 8.7). The modularity index was 0.465, suggesting that the network had a modular structure (Fortunato & Barthélemy, 2007). The network was divided into five modules (*i.e.*, clusters of nodes that interact more among themselves than with other nodes, compared with a random association). Regarding ARGs and MGE-genes, *intI1* and *blaOXY* had the greatest diversity in terms of possible multi-resistant hosts, with 10 and 8 families, respectively. Furthermore, *Chromatiaceae*, *Alcanivoracaceae* and *Opitutaceae* presented the highest diversity of ARGs and MGE-genes, with 22, 15 and 10 genes, respectively.

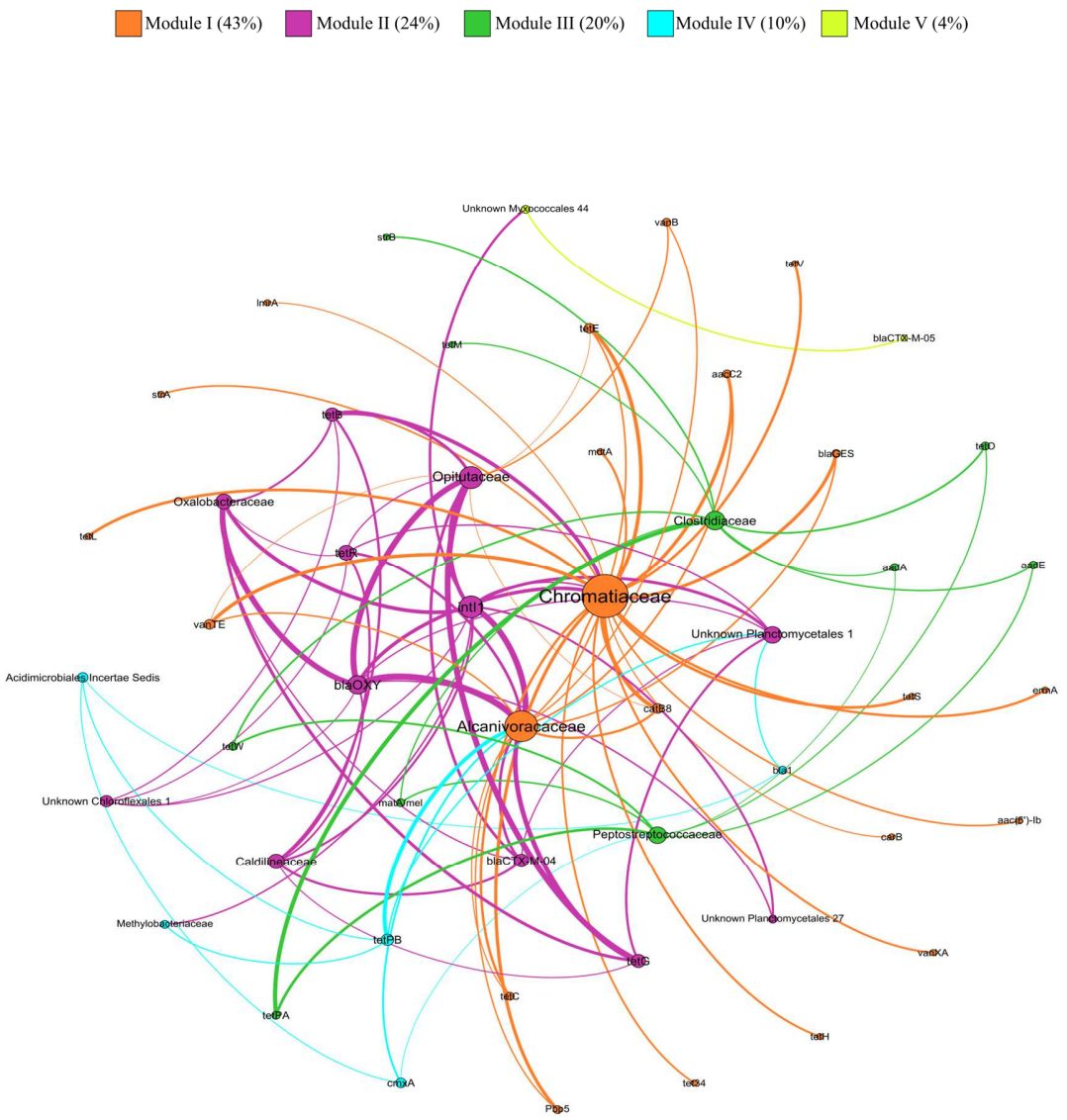


Figure 8.7. Network analysis of ARGs, MGE-genes (relative abundances) and 12 multi-resistant bacterial families based on Kendall's correlations. Node size is proportional to the number of connections (degree). An edge represents a significant correlation, where the edge thickness is proportional to Kendall's correlation coefficient (weight).

Since many studied variables showed correlation among themselves, a SEM analysis was performed to outline the direct or indirect influence of biotic and abiotic factors on the distribution of ARGs and MGE-genes (Figure 8.8). The HM pollution index was directly affected by many soil physicochemical properties, such as nutrient contents, OM, clay content, CEC and EC. The dosage of

SS application had a negative and positive influence on the Shannon´s and Simpson´s index, respectively. Specifically, the Simpson´s index (r^2 = 0.94) was directly affected negatively by ARGs and MGE-genes, unlike the HM pollution index and the Shannon´s index. Regarding the standardized regression weights, MGE-genes were primarily regulated by ARGs, followed by the elapsed time after the last application and the PC1 (r^2 = 0.86).

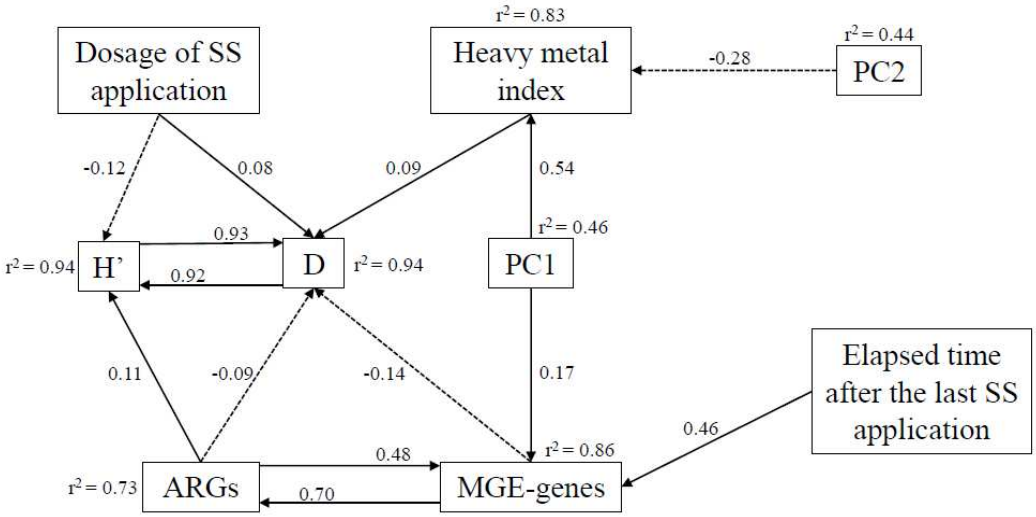


Figure 8.8. Structural equation models showing direct and indirect effects of prokaryotic diversity indices, dosage of SS application, elapsed time after the last application, HM pollution index, PC1 and PC2 on ARGs and MGE-genes. Arrows indicate positive and negative relationships by solid and dashed lines, respectively. Numbers next to arrows represent standardized estimated regression weights ($p < 0.05$). The r2 values indicate the proportion of variance explained for each variable. $\chi 2$ = 31.5, AIC = 133, P = 0.416, df = 34.

8.4. Discussion

The application of organic amendments to agricultural soil is a common practice because of its potential positive effects on soil quality (Epelde et al., 2018; Urra et al., 2019c). Furthermore, this practice reduces the need for energetically costly synthetic fertilizers, while reusing a material that would otherwise be treated as a waste. Relevantly, in the current scenario of climate change, the application of SS to agricultural soils can help increase their soil OM content (a pertinent objective for many agricultural soils with low OM content), thus enhancing their capacity to sequester carbon. Regrettably, the application of organic amendments to agricultural soil, such as SS, can likewise lead to potential risks for human and environmental health, in particular, due to the presence of traditional and emerging

contaminants (Singh & Agrawal, 2007; Alvarenga et al., 2015; Martín et al., 2015). In particular, nowadays, emerging contaminants (*e.g.*, pesticides, pharmaceuticals, nanomaterials, personal and house care products, etc.) are a matter of much concern (Epelde et al., 2018). The possible dissemination of ARGs is of special concern owing to the risk of transfer of ARGs to human pathogens (Marshall & Levy, 2011; Epelde et al., 2018).

In this study, SS-amended soils presented higher Olsen P contents than unamended soils, as previously described (Börjesson & Kätterer, 2018; Urra et al., 2019b). The rate and amount of SS application to agricultural soil is frequently based on the N requirement of crops. Then, other nutrients, such as P and K+, can be over-applied (Antoniadis et al., 2015). It is a well-known fact that SS from WWTPs can present elevated levels of P (Alvarenga et al., 2015), a fact that must be taken into consideration as this key nutrient is being depleted at an alarming rate due chiefly to its use in fertilizers. In accordance with other studies (Kirchmann et al., 2017; Bogusz & Oleszczuk, 2018; Urra et al., 2019b), in our study, the application of SS also resulted in significantly higher soil Zn concentrations. Sewage sludge application to agricultural soil can lead to accumulation of potentially toxic metals, with concomitant adverse effects for crop quality (Muchuweti et al., 2006). On the other hand, in accordance with previous studies (Lloret et al., 2016; Urra et al., 2019b), the application of SS resulted in reduced soil microbial diversity, with potential negative consequence for soil functioning and resilience (Delgado-Baquerizo et al., 2016). On the contrary, some authors found an increase in diversity in SS-amended soils (Chen et al., 2016; Bai et al., 2019).

The application of SS to soil can result in an increase of microbial biomass and activity, associated with the supply of easily available carbon sources for the soil microbial communities (Fernández et al., 2009; García-Gil et al., 2004; Sánchez-Monedero et al., 2004). The application of organic amendments has been frequently reported to enhance soil microbial activity and biomass (Das et al., 2017; Dinesh et al., 2010; Hernández et al., 2016; Reardon & Wuest, 2016; Zhen et al., 2014, Urra et al., 2019b). In our study, unexpectedly, no differences in microbial biomass and activity values were found between SS-amended and unamended soils. At soil sampling times, our agricultural fields were planted with different crops (barley, beans, rapeseed, wheat). Taking into account that our soil samples were collected from the topsoil (0-10 cm depth), it is possible that the expected stimulation of soil microbial biomass and activity, derived from the rhizodeposition of readily available carbon sources, might have veiled the positive effect of SS on soil microbial biomass and activity. It is also

feasible that, at sampling times, most of the easily available C substrates initially present in SS were long gone (elapsed times after the last SS application range from 1 to 4 years).

In our study, all the sampled soils and the SS itself met the EU Directive 86/278/EEC (European Commission, 1986) regarding total metal concentration values. However, the bioavailable fraction of soil metals is considered more relevant from an environmental risk point of view (Olaniran et al., 2013). Bioavailable metal concentrations in soil usually show an inverse relationship with soil pH (Rieuwerts et al., 1998). The agricultural soils sampled for this study have an alkaline pH (mean value: 8.4 ± 0.1), which could explain the fact that bioavailable metal levels were below the quantification limit, suggesting a lack of metal-induced ecotoxicity. Similarly, Urra et al. (2019b) found that the application of SS increased the total concentration of Cu and Zn in amended soils, but without affecting their bioavailability, possibly due to the high values of soil pH and OM content.

It is a well-known fact that metals might promote the spread of antibiotic resistance via co-selection mechanisms. In consequence, due to the current growing concern over the emergence and dissemination of antibiotic resistance, an increasing interest exists in the study of the generalities and particulars related to the role of metal contamination as selective agent in the proliferation of environmental antibiotic resistance. This co-selection depends on a variety of factors, including the nature and concentration of metal contaminants, as well as the type of antibiotic and its mechanism of action. In any case, the co-selection of antibiotic and metal resistance rests on two main mechanisms: (i) co-resistance, when different resistance determinants are present in the same genetic element; and (ii) cross-resistance, when the same genetic determinant is responsible for resistance to both antibiotics and metals (Baker-Austin et al., 2006). Due to the magnitude of the problem of metal contamination, as well as to the exceptionally long residence times of metals in the environment, metals represent a widespread and recalcitrant selection pressure contributing to the emergence, maintenance and spread of antibiotic resistance factors. In our study, total Cu concentration in soil was positively correlated with the relative abundance of both ARGs and MGE-genes. Other authors have reported that the selective pressure caused by the presence of metals in SS can result in increased abundance of ARGs (Gullberg et al., 2014). In a previous study on the long-term impact of SS on agricultural soil (Urra et al., 2019b), we found a positive correlation between soil Cu and Zn concentrations and the abundance of ARGs and MGE-genes in soils amended with thermally-dried anaerobically-digested SS. Similarly, we observed that all metals were positively correlated with, at least, one ARG (soil metal

concentrations also showed positive correlations with MGE-genes, particularly with the gene *tnpA-07*) (Urra et al., 2018).

The content of Olsen P explained a considerable percentage of the variation in the relative abundances of ARGs and MGE-genes in our soils, which could possibly be related to the abovementioned high P content of SS (Daneshgar et al., 2018). Soil CEC was also positively correlated with the relative abundance of ARGs, which could be, at least partly, due to the interaction (*e.g.*, sorption mechanisms) between antibiotics and soil surfaces in SS-amended soils (Tolls, 2001). The distribution and fate of antibiotics in soil hinge on their sorption coefficient value (K_d) which, in turn, depends on a variety of soil properties, such as soil pH, OM and CEC (Lützhøft et al., 2000; Chee-Sanford et al., 2009; Wegst-Uhrich et al., 2014). In any event, although environmental parameters such as soil pH and OM content are the most significant variables that affect antibiotic sorption in soil, it is important to take into consideration the concentrations used, the analytical method employed, and the transformations that can occur when determining K_d values (Wegst-Uhrich et al., 2014). Antibiotics with low K_d values are highly mobile (they are not strongly bound to soil particles) and, hence, more bioavailable and degradable. The principal route for antibiotic degradation in soil is aerobic biodegradation. Antibiotic degradation rates, usually expressed as half-lives, can range from days to years (Boxall et al., 2004). Macrolides and β-lactams have been reported to be less persistent in soil than tetracyclines and quinolones (Boxall et al., 2004).

As stated above, the application of organic wastes of anthropogenic origin, such as SS, can increase the risk of dissemination of ARGs into agricultural soil (Munir & Xagoraraki, 2011). Values of ARG relative abundance in our SS were higher compared to those observed by other authors: in anaerobically digested sludge from South Korea, Yoo et al. (2020) reported relative abundance values of 8.93×10^{-2}, 5.74×10^{-2} and 5.71×10^{-2} for MLSB, sulfonamide and tetracycline genes, respectively. Xu et al. (2020) found ARG relative abundance values of 3.3 to 5.1×10^{-1} in SS samples from two Chinese WWTPs operated via anaerobic/anoxic/oxic and anoxic zone nitrification techniques. Han & Yoo (2020) reported ARG relative abundance values from 5.80×10^{-5} to 1.20×10^{-1} and from 4.62×10^{-6} to 4.48×10^{-1} in activated and dewatered sludge, respectively. Differences in antibiotic consumption patterns, as well as in SS composition, treatment and management, are possibly responsible for the observed differences in ARG relative abundance values. In any case, it is strongly recommended that SS is properly treated prior to its application in order to minimize or prevent the

spread of ARB and ARGs from WWTPs to agricultural soils. This same recommendation was reported by Urra et al. (2019a) in their study on the impact of six different manure-derived amendments on agricultural soil quality. According to Sun et al. (2016), anaerobic digestion generally reduces integron abundance, eliminating the aerobic hosts of such integrons. The presence of the class 1 integron has been reported to be associated with the proliferation of ARGs in soil (Binh et al., 2008). In fact, the *intl1* gene was proposed as a proxy for anthropogenic contamination, due to its relationship with ARGs, disinfectants and metals (Gillings et al., 2015).

In any event, when interpreting data on antibiotic resistance in soil, it must not be forgotten that natural soil microbial communities are potentially a large environmental reservoir of antibiotic resistance (Allen et al., 2009). Although, in this study, thermally-dried and anaerobically-digested SS was used as amendment, out of the 85 ARGs measured here, 77 and 74 were detected in soil samples and SS itself, respectively. Furthermore, the 10 MGE-genes determined in our study were detected in both soil samples and SS.

In our study, the relative abundance of both ARGs and MGE-genes was higher in SS-amended soils, compared to non-amended soils, particularly in those with a more recent SS application, suggesting that the application of thermally-dried anaerobically-digested SS to agricultural soil can increase the risk of environmental antibiotic resistance, as previously described by Urra et al. (2019b). Rahube et al. (2014) reported the impact of fertilizing with anaerobically-digested *vs.* raw SS on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. Instead, Rutgersson et al. (2020) did not find evidence of antibiotic accumulation or enrichment of ARGs or ARB in soil amended with digested and stored SS at doses of up to 12 tons per hectare every four years.

The use (above all, the overuse and misuse) of antibiotics for animal, human and agricultural purposes has led to the emergence and dissemination, by HGT, of antibiotic resistance among bacteria. The spread of antibiotic resistance by HGT is an ancient phenomenon. Nonetheless, the use of antibiotics has raised the corresponding selective pressure, thus increasing HGT (von Wintersdorff et al., 2016). As abovementioned, our results showed a stimulatory effect of SS on the relative abundance of both ARGs and MGE-genes, especially when the elapsed time after the last application was only one year, in agreement with previous studies (Chen et al., 2016; Xie et al., 2016). *A priori*, the increased

relative abundances of ARGs and MGE-genes in SS-amended soils could be the consequence of: (i) an increase microbial growth induced by the input of organic carbon sources present in the SS itself; and/or (ii) the dissemination of ARGs from SS to soil microorganisms through HGT. Since the intensity of these processes will decrease over time after the application of SS, it is expected that the resistome risk will also decrease over time after the last application of SS. In a much shorter microcosm experiment, Chen et al. (2019b) found that risk scores for amended soils decreased to levels comparable to those of unamended soil after just 120 days. However, as stated before, the persistent accumulation of heavy metals in SS-amended soils provide a long-term selective pressure which can result in co-selection for antibiotic resistance (Song et al., 2017) and, thus, a persistent resistome risk.

Urra et al. (2019a) observed that genes encoding MGEs (*tnpA*, *intI1*) were positively correlated with ARGs, suggesting a risk of dissemination of antibiotic resistance via HGT in agricultural soils, as a result of the application of livestock manure-derived amendments. Garbisu et al. (2018) screened metal contaminated soil treated with organic wastes for the presence of MGEs, confirming the occurrence of conjugative IncP-1 and mobilizable IncQ plasmids, as well as of class 1 integrons, suggesting that bacteria from those soils had gene-mobilizing capacity with implications for the dissemination of resistance factors. Moreover, their data pointed out the role of spontaneous mutations in achieving low-level antibiotic resistance in a short time, which was associated with a trade-off in the capability to metabolize specific carbon sources (Garbisu et al., 2018).

Regarding prokaryotic taxa at phylum level, the two dominant phyla in SS were *Proteobacteria* and *Bacteroidetes*. Both phyla have previously been reported as dominant taxa in SS (Liao et al., 2018; Zhao et al., 2019) and can harbour ARGs (Sun et al., 2016). In accordance with previous studies (Zhang et al., 2019b), in our soils, *Xanthomonadaceae* was linked with the relative abundance of ARGs and MGE-genes. Interestingly, this family was the fifth most abundant family in the SS itself. In their study on the long-term impact of SS application of agricultural soil, Urra et al. (2018) found that some ARGs correlated positively with particular prokaryotic taxa, being Gemmatimonadetes the taxon with the greatest number of positive correlations at phylum level. However, no positive correlation was detected between prokaryotic taxa and genes encoding resistance to sulfonamides and FCA (Urra et al., 2018).

Interestingly, Shannon´s and Simpson's index values correlated negatively with ARG and MGE-gene relative abundances, as previously reported (Chen et al., 2019a), suggesting that a decrease

in prokaryotic diversity could facilitate the proliferation and spread of ARGs and MGE-genes in soil. Since microbial diversity is essential for ecosystem functioning and resilience (Delgado-Baquerizo et al., 2016), the impact of organic amendments on such diversity must be carefully studied.

According to data from our Kendall's correlation analysis, 30 families showed correlation with at least two genes that are known to confer resistance against several antibiotic families. Among these 30 multi-resistant families, 12 showed a higher abundance in SS-amended *vs.* unamended soils, pointing out to an increased risk of antibiotic resistance.

The network analysis is a powerful tool to identify key ARGs (with potential as indicators or proxies) and to delineate potential ARGs hosts (Chen et al., 2016). In our network analysis, the families *Chromatiaceae*, *Alcanivoracaceae* and *Opitutaceae* were identified as the most common potential hosts of ARGs and MGE-genes in SS-amended soils. Many species belonging to *Chromatiaceae* are photosynthetic sulfur bacteria which contribute to water purification in sewage lagoons (Holm & Vennes, 1970). The family *Alcanivoracaceae* contains bacterial species that harbor Type IV and Type II secretion system genes (Yakimov et al., 2019). The Type II pathway is associated with organisms that form biofilms (Sandkvist, 2001). Bacteria within biofilms can be 10-1000 times more resistant to antibiotics, compared to corresponding planktonic cells (Ceri et al., 1999). In accordance with Chen et al. (2019a), the family *Opitutaceae* was strongly correlated with a tetracycline resistance gene (*tetG*). The higher relative abundance shown by these three families in SS-amended soils, together with their positive correlation with the abundance of ARGs and MGE-genes, suggests their potentially important role in AR in SS-amended agricultural soils.

Structural equation models facilitate the study of complex interactions. In our study, soil physicochemical parameters (OM, CEC, EC, clay content, pH, soil nutrients) were major predictors of the HM pollution index. According to our SEM analysis, the composition and relative abundance of ARGs were mainly driven by MGE-genes, pointing out to the well-known role of HGT for the observed resistome (Smillie et al., 2011). The elapsed time after the last application contributed to the observed variation in MGE-genes diversity and abundance, suggesting that the acquisition of genetic material from non-parental lineages by HGT is dependent on such elapsed time. Our SEM confirmed the recognized crucial role of MGEs in shaping the patterns of ARGs and facilitating their dissemination. Indeed, in this study, MGE-genes (which were themselves affected by parameters such as soil OM and

nutrient contents, the elapsed time after the last application of SS, and the distribution of ARGs) have shown their critical role for AR dissemination in agricultural soils.

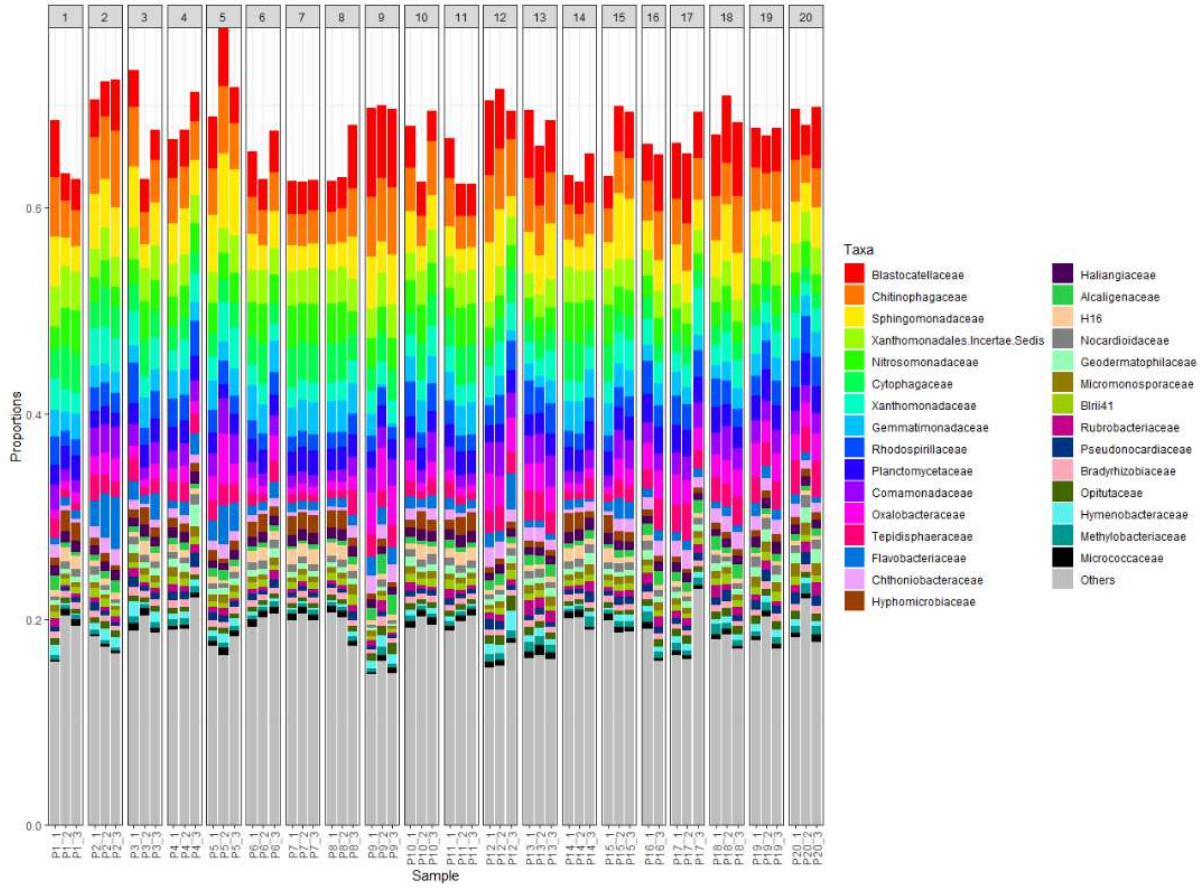
8.5. Conclusion

In our SS-amended soils, the composition of prokaryotic communities was mainly influenced by the soil´s physicochemical properties. The 30 most abundant families within the soil prokaryotic community accounted for a considerable percentage (66%) of the total variation of ARG and MGE-gene relative abundances. Soil prokaryotic α-diversity was negatively correlated with the relative abundance of ARGs and MGE-genes, suggesting that a decrease in soil prokaryotic diversity could facilitate the proliferation and spread of ARGs and MGE-genes in soil. The families *Chromatiaceae*, *Alcanivoracaceae* and *Opitutaceae* were identified as the most common potential hosts of multi-resistant genes in SS-amended soils. According to the SEM performed here, the diversity and abundance of MGE-genes are critical for AR dissemination in agricultural soils. We concluded that agricultural soils amended with our thermally-dried anaerobically-digested SS showed increased risk of antibiotic resistance dissemination. Much research and awareness is needed for (i) a safe reduction of antibiotic consumption and (ii) the development of innovative treatments for SS (prior to its application) in order to reduce the risk of emergence and spread of ARGs and MGE-genes in SS-amended agricultural soils and then crops. Until then, SS should be applied conservatively in agricultural soils.

8.6. Supplementary information

Supplementary Table 8.1. Relative abundance of multi-resistant bacterial families in SS-amended and unamended soils. *: p<0.05; **: p<0.01; ***; p<0.001.

Family	Amended	Unamended
Acidimicrobiales Incertae Sedis **	1.43E-04	9.75E-05
Alcanivoracaceae **	1.65E-04	3.05E-05
Caldilineaceae **	1.71E-03	1.29E-03
Chromatiaceae	1.39E-05	1.21E-05
Chthoniobacteraceae	1.05E-02	9.29E-03
Clostridiaceae **	5.15E-04	3.18E-04
Methylobacteriaceae *	5.94E-03	4.86E-03
Oceanospirillaceae	1.82E-03	1.63E-03
Opitutaceae *	6.52E-03	5.42E-03
Oxalobacteraceae **	2.17E-02	1.53E-02
Peptostreptococcaceae **	5.00E-04	2.60E-04
Rhizobiaceae	2.09E-03	2.01E-03
Tepidisphaeraceae	1.94E-02	1.62E-02
Unknown Bdellovibrionales family 1	3.67E-05	2.72E-05
Unknown Bdellovibrionales family 29	4.22E-06	5.42E-06
Unknown Chlamydiales family 2	1.38E-05	1.93E-05
Unknown Chloroflexales family 1 *	1.44E-04	9.62E-05
Unknown Myxococcales family 44 ***	5.74E-06	1.07E-06
Unknown Oligoflexales family 1	3.50E-05	2.34E-05
Unknown Phycisphaerales family 6	2.59E-05	1.73E-05
Unknown Planctomycetales family 1 **	7.04E-05	3.70E-05
Unknown Planctomycetales family 15	2.24E-05	1.74E-05
Unknown Planctomycetales family 2	7.95E-05	6.15E-05
Unknown Planctomycetales family 27 *	1.49E-05	6.96E-06
Unknown Planctomycetales family 69	6.14E-06	6.11E-06
Unknown Tepidisphaerales family 10	3.75E-05	2.46E-05
Unknown Tepidisphaerales family 4	9.16E-05	8.98E-05
Verrucomicrobiaceae	3.21E-03	2.88E-03
Vicinamibacter family incertae sedis	6.22E-04	4.83E-04
Xiphinematobacteraceae	1.17E-03	9.94E-04



Supplementary Figure 8.1. Barplots of the 30 most abundant bacterial taxa at family rank grouped by agricultural field.

9. ORGANIC AMENDMENT TREATMENTS FOR RESISTOME RISK REDUCTION IN SOIL-CROP SYSTEMS

Abstract

Agricultural fertilization with organic amendments of animal origin often leads to antibiotic resistance dissemination. In this study, we evaluated the effect of different treatments (anaerobic digestion, biochar application, ozonation, zerovalent iron nanoparticle application, and spent mushroom substrate addition) on the resistome in dairy cow manure-derived amendments (slurry, manure, and compost). Anaerobic digestion and biochar application resulted in the highest reduction in antibiotic resistance gene (ARG) and mobile genetic element (MGE) gene abundance. These two treatments were applied to cow manure compost, which was then used to fertilize the soil for lettuce growth. After crop harvest, ARG and MGE gene abundance in the soil and lettuce samples was determined by droplet digital PCR and high-throughput qPCR. Prokaryotic diversity in cow manure-amended soils was determined using 16S rRNA metabarcoding. Compared to untreated compost, anaerobic digestion led to a 38% and 83% reduction in *sul2* and *intl1* absolute abundances in the soil, respectively, while biochar led to a 60% reduction in *intl1* absolute abundance. No differences in lettuce gene abundances were observed among treatments. The potential bacterial hosts for soil ARGs and MGE genes were explored using network analysis. We conclude that amendment treatments can minimize the risk of antibiotic resistance in agroecosystems.

9.1. Introduction

Antibiotic resistance is an ancient phenomenon (D'Costa et al., 2011); however, anthropogenic activities have increased the prevalence of antimicrobial-resistant microorganisms (Cytryn, 2013; Holmes et al., 2016), causing serious human health problems. The transfer of animal and environmental resistomes to humans is a matter of great concern for policymakers. Consequently, in 2006, the European Union banned the use of antibiotics for animal growth promotion [Regulation (EC) No 1831/2003]. Moreover, the EU Farm to Fork Strategy aims to reduce the overall EU sales of antimicrobials for farmed animals and aquaculture by 50% by 2030 (European Commission, 2020).

Although these regulations control the excessive use of antibiotics in livestock farming, none are related to the management and/or treatment of organic amendments of animal origin. Organic

amendments of animal origin, which are commonly used for agricultural fertilization, are a source of antibiotic contamination because a considerable percentage (30–90%) of antibiotics administered to livestock are discharged in the urine and feces (Kumar et al., 2005). The application of these organic amendments to soil as organic fertilizers can lead to the emergence and dissemination of antibiotic-resistant bacteria (ARB) harboring antibiotic resistance genes (ARGs) in soils and crops (Chee-Sanford et al., 2009). The principal mechanism underlying the spread of antibiotic resistance is horizontal gene transfer (HGT) via various mobile genetic elements (MGEs), such as conjugative plasmids, integrative conjugative elements, integrons, and transposons (Heuer et al., 2011).

The many benefits of organic amendments to agricultural soils and crops are undeniable (Epelde et al., 2018). Therefore, there is an urgent need to develop and implement management practices and treatments to reduce the abundance of ARGs and MGEs in animal-based organic amendments. Possible strategies for achieving this include sanitizing the amendments under certain environmental conditions (*e.g.*, temperature, oxygenation) or with other substances, as well as seeking to immobilize ARB and ARGs by adding agents with a very high surface-to-volume ratio. Anaerobic digestion is the process of microbial decomposition of organic matter in the absence of oxygen (Youngquist et al., 2016). Traditionally, it has been used to reduce the volume of solids from wastewater treatment, producing biogas and other organic compounds (Ma et al., 2015). Nevertheless, its use to treat livestock manure is increasing; this process can reduce antibiotics, ARB, and ARGs in manure (Youngquist et al., 2016; Wang et al., 2021b). In addition, ozone disinfection destroys bacterial cell membranes (Thanomsub et al., 2002) and is widely used in wastewater treatment plants and the food industry. This disinfection process can also remove ARGs (Sousa et al., 2017).

On the other hand, biochar is a carbon rich material with a large surface area-to-volume ratio generated from the pyrolysis of biomass in the absence of oxygen (Park et al., 2011). It is used as a soil amendment to increase soil carbon sequestration, enhance soil fertility and productivity, reduce the bioavailability of organic compounds (Jeffery et al., 2015), and remove antibiotics (Krasucka et al., 2021). Moreover, nanoscale zerovalent iron particles (nZVI) show high reactivity towards a broad range of pollutants and have a large surface-area-to-volume ratio (Ken & Sinha, 2020). For example, they are used for the removal of polycyclic aromatic hydrocarbons and metal(oid)s (Zhao et al., 2016), as well as bacteria and viruses (Diao & Yao, 2009; Shi et al., 2012). They are mainly applied in groundwater remediation and, to a lesser extent, soil remediation (Anza et al., 2019). Finally, five

kilograms of spent mushroom substrate (SMS) are generated in the production of one kilogram of fresh mushrooms [for example, Spain produces 166,010 tons of mushrooms per year (FAOSTAT, 2020a)]. Spent mushroom substrate is therefore generated in large quantities, and for many years, it has accumulated in landfills and has become an environmental problem. SMS has been used in the production of compost (Polat et al., 2009) and animal feed (Fazaeli & Masoodi, 2006), as well as for enzyme extraction (Phan & Sabaratnam, 2012), bioremediation (Lau et al., 2003), and removal of antibiotics by combining their adsorption and biodegradation mechanisms (Mayans et al., 2021).

In the present study, we evaluated the potential of 11 organic amendment management processes to reduce the abundance of ARGs and MGE genes; the amendments used were cow slurry, manure, and compost. Subsequently, the most promising management strategies were applied to a compost that was used to fertilize soil and grow lettuce plants in a microcosm experiment. Thus, we quantified ARGs and MGE genes in (i) amendments, (ii) amended soils, and (iii) lettuce plants. In addition, we studied the relationship between the structural diversity of soil bacterial communities and the abundance of ARGs and MGE genes. We hypothesized that the abundance of ARGs and MGE genes would be lower in the treated amendments than in the untreated controls, and consequently be lower in the amended soils and lettuce plants grown in these soils.

9.2. Materials and methods

9.2.1. Preliminary experiment

The amendments used in this study were provided by a dairy cow farm located in Basque Country (Spain). Three types of amendment (slurry, manure, and compost) were used. The slurry was collected from the outlet of a slurry pond, manure was collected from cow beddings (made from feces, urine, and wheat straw), and compost was sampled from manure piles that had been stored for six months. Both the manure and compost samples were collected and placed in polyethylene bags, whereas the slurry sample was placed in a plastic barrel. The samples were immediately transferred to the laboratory and stored at 4 °C until further use. The physicochemical properties of the amendments are presented in Supplementary Table 9.1.

The following 11 management processes were tested in the preliminary experiment: anaerobic digestion at 55 ºC (AD 55 ºC), 75 ºC (AD 75 ºC), and 90 ºC (AD 90 ºC); biochar addition at the rate of

0.5% (biochar 0.5%), 2% (biochar 2%), and 5% dry weight (DW; biochar 5%); zerovalent iron nanoparticle addition (nZVI) at 1% (nZVI 1%) and 2% DW (nZVI 2%); ozonation at 2 ppm (ozonation), and spent mushroom substrate addition (SMS) at 1:0.5 (SMS 1:0.5) and 1:1 w:w (SMS 1:1).

For anaerobic digestion, 16, 23, and 80 g FW of compost, manure, and slurry, respectively, were placed in syringes, and distilled water was added to make a final volume of 80 mL. These samples were incubated at the corresponding temperatures in an incubator for 21 d. The rest of the management was carried out at room temperature in darkness. First, 100 g FW of each amendment were placed in plastic pots. Biochar was manually added to the amendments and thoroughly mixed. It had the following physicochemical properties: DW = 53%, pH = 7.5, organic matter (OM) = 69%, electrical conductivity (EC) = 4.1 mS cm^{-1}, total N = 0.7 %, K = 13 g kg^{-1}, P = 2.2 g kg^{-1}, and metal concentrations = 0.56, 18, 23, 14, and 103 mg kg^{-1} for Cd, Cr, Cu, Ni, and Zn, respectively. Zerovalent iron nanoparticles (Nanofer 25S, aqueous dispersion of Fe(0) nanoparticles, NANO IRON s.r.o., Czech Republici) were added to the amendments in the form of slurry and thoroughly mixed; nZVI were applied twice, one week apart. For ozonation, the amendments were placed on a tray and subjected to ozone exposure in an ozonation chamber (2 ppm) for three weeks. Finally, the SMS obtained from the Mushroom Research Technological Center of La Rioja (Spain) was mixed with the amendments. The SMS had the following physicochemical properties: pH = 4.65, OM = 71%, total N = 0.49%, K = 4.3 g kg^{-1}, and P = 0.62 g kg^{-1}. All the management processes had their respective untreated controls, which were under the same experimental conditions. For every treatment, two DNA extractions were performed at the beginning (day 0) and at the end of the process (day 21).

9.2.2. Microcosm experiment

Before the beginning of the microcosm experiment, the following physicochemical properties were again determined in the compost, according to standard methods (MAPA, 1994): DW = 24%, pH = 9.1, C/N ratio = 14.6, EC = 0.54 mS cm^{-1}, and metal concentrations = 0.49, 29, 229, 19, 9.4, 868 mg kg^{-1} DW for Cd, Cr, Cu, Ni, Pb, and Zn, respectively. The experimental soil was collected from the upper 30 cm layer of a semi-natural grassland field, which, to our knowledge, has never been amended. After collection, the soil was sieved to < 4 mm particles and was filled in 2 kg pots. The soil had the

following physicochemical properties: clay loamy texture, OM = 5.4%, pH = 6.0, EC = 0.04 mS cm^{-1}, total N = 0.22%, Olsen P = 2.3 mg kg^{-1} DW soil, and a K^+ content of 97 mg kg^{-1} DW soil.

The following treatments were tested in the microcosm experiment: (i) untreated compost, (ii) compost subjected to anaerobic digestion at 75 ºC for 21 d (anaerobic digestion), (iii) biochar 5% w/w to compost (biochar compost), (iv) biochar 5% w/w to soil (biochar soil), (v) mineral fertilization [NPK, N as NH_4NO_3 (33.5%), P as P_2O_5 (18%), and K as K_2O (60%)], and (vi) unamended treatment. The compost dose was adjusted to provide an equivalent of 150 kg N ha^{-1} for lettuce plants. The compost was manually incorporated into the soil, thoroughly mixed (homogenized), and left to stabilize for two weeks. Lettuce seedlings were planted and bottom-watered every 2–3 days during the experimental period. The microcosm experiment was carried out in a growth chamber under the following controlled conditions: 14/10 h light/dark cycle, 20/16 ºC day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 µmol photon m^{-2} s^{-1}. After one month of growth, the plants were harvested, and soil samples were collected.

9.2.3. Quantification of antibiotic resistance genes and mobile genetic elements

DNA was extracted from the amendments and soil samples (0.25 g DW) using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions, and from the plant samples using the innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). The concentrations of amendments, soil, and plant DNA were quantified using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA). The extracted DNA was stored at -20 ºC until further analysis.

Absolute quantification of ARGs and MGE genes in the preliminary and microcosm experiments was conducted using a droplet digital PCR (ddPCR, Bio-Rad Laboratories Inc., Hercules, CA, USA). Two sulfonamide (*sul1* and *sul2*) and two tetracycline (*tetA* and *tetX*) resistance genes, as well as two MGE genes (*intl1* and *tnpA-04*) and the 16S rRNA gene were analyzed (see primers and PCR conditions in Supplementary Table 9.2). Prior to generating droplets for ARGs and MGE genes, the DNA was digested with the XbaI restriction enzyme (Takara Bio, CA, USA) according to the manufacturer's instructions. The reaction mixture (total volume of 25 µL) consisted of 12.5 µL of QX200 ddPCR EvaGreen Supermix (Bio-Rad), 0.50 µL each of forward and reverse primers (final concentration 10 nM each), 1.0 µL of the digested DNA extract (for 16S rRNA quantification, the

DNA was diluted to 0.1 ng μL^{-1}), and 10.5 Milli-Q water. The reaction mixture was added to a 96-well plate, sealed with foil, homogenized by vortexing, and centrifuged at 1000 g for 1 min. Aliquots of 21 µL ddPCR reaction mixture were dispensed into the sample well of the DG8 Droplet Generation Cartridge (Bio-Rad), and 70 µL of the QX200 Droplet Generation Oil for EvaGreen were added to the oil wells. Droplet generation was performed using a QX200 Droplet Generator (Bio-Rad). The generated droplets were transferred to a 96-well plate and incubated at 180 °C using a PX1 PCR plate sealer. The 96-well plate was then transferred to a C1000 Touch Thermal Cycler for PCR amplification. After thermal cycling, the plate was transferred to a QX200 droplet reader for data acquisition. Data analysis was performed using the QuantaSoft software (v.1.4., Bio-Rad). Target gene copies were quantified in duplicate, and positive and no-template controls were included in each ddPCR assay.

Furthermore, the abundance of ARGs and MGE genes in each sample of the microcosm experiment was analyzed using customized primer sets in a SmartChip qPCR system (TakaraBio) by Resistomap Oy (Helsinki, Finland). A total of 96 and 24 validated primer sets were used for soils and plants, respectively (see the list of primers in Supplementary Table 9.3); they were selected because they tested positive in a previous pre-screening analysis carried out with 384 genes. The quantified genes included (i) 76 and 16 primer sets (for soils and plants, respectively) targeting the ARGs conferring resistance against all major classes of antibiotics [aminoglycoside, β-lactam, FCA (phenicol, quinolone), MLSB (macrolide, lincosamide, streptogramin B), multidrug (*i.e.*, those conferring resistance to more than one antibiotic), other (peptide, triclosan, mercury, hydrocarbon), sulfonamide, tetracycline, trimethoprim, and vancomycin]; (ii) 17 and 5 primer sets (for soils and plants, respectively) targeting MGE genes; and (iii) additional taxonomic reference genes (for Bacteroidetes, Firmicutes, and 16S rRNA).

PCR cycling conditions and initial data processing were the same as described previously (Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017). A threshold cycle (C_T) of 27 was used as the detection limit (Zhu et al., 2013). Detection of each ARG or MGE gene was considered positive when two out of three technical replicates for each sample were detected. The $2^{-\Delta C_T}$ method was used to calculate the ARG and MGE gene relative abundances, normalized to the abundance of the 16S rRNA reference gene [where $\Delta C_T = C_{T(target\ gene)} - C_{T(16S\ rRNA\ gene)}$ (Livak & Schmittgen, 2001)].

9.2.4. Amplicon sequencing of soil bacterial communities

The prokaryotic 16S rRNA hypervariable region V4 was targeted using 519F [adapted from Ovreås et al. (1997)] and 806R (Caporaso et al., 2012) adapter-linked primer pairs as described by Lanzén et al. (2016). Pair-ended sequencing was performed using Illumina MiSeq at the Genomics Facility of SGIker (University of the Basque Country, Spain). Quality control of the reads was performed using FASTQC software (Andrews, 2010). PCR primers were removed from the sequences using Cutadapt (Martin, 2011). The resulting FASTQ files were further analyzed via *QIIME2* (Bolyen et al., 2019) as follows: sequences were imported into QIIME2 as *PairedEndSequencesWithQuality*, after which both reads were joined by the *qiime vsearch join-pairs* plugin. Then, low-quality reads were filtered using the *QIIME quality-filter Q-score-joined* command with the default options. In the next step, deblur (Amir et al., 2017) was used for denoising (qiime deblur denoise-16S), after which the resulting reads were classified using the *qiime feature-classifier classify-sklearn* and *silva-132-99-nb-classifier.qza* command as the reference model for assigning a taxonomical classification to the ASVs. The singletons were removed by the *QIIME feature-table filter-features* command, and contaminant and unclassified ASVs were also removed by the *QIIME taxa filter-table*. Finally, a table summarizing the ASVs was created using a *qiime feature-table summarize* command.

9.2.5. Statistical analysis

In the preliminary experiment, the removal rates were calculated following Yang et al. (2014) by first subtracting the abundances obtained on day 21 from those obtained on day 0 and then from those of the control treatment. Heatmaps were visualized with ggplot2 (Wickham, 2016).

In the microcosm experiment, statistically significant differences among the treatments in the abundance of ARGs and MGE genes ($p < 0.05$) for soil and lettuce were assessed with ANOVA and Tukey's post-hoc tests using the agricolae R package (de Mendiburu, 2020). Ordinary least squares (OLS) regression models were used to assess the relationships between ARG and MGE gene abundances obtained by HT-qPCR analysis using R, and the results were visualized using the ggplot2 package (Wickham, 2016). The analysis of similarities (ANOSIM) test was used to compare the mean of ranked dissimilarities between treatments to the mean of ranked dissimilarities within treatments. A network analysis was performed to explore the Pearson's correlations ($P < 0.05$) between soil bacterial classes and ARGs and MGE genes obtained from HT-qPCR analysis using the Hmisc (Harrell et al.,

2021) and reshape2 (Wickham, 2007) R packages, and network visualization was conducted in Gephi (v 0.9.2).

9.3. Results

9.3.1. Preliminary study with the three amendments

The ARG and MGE gene removal rates in each treatment for every amendment were evaluated (Fig. 9.1). Positive values indicated that the management process was successful in gene elimination; conversely, negative values indicated that compared to that in the control treatment, gene abundance increased from day 0 to day 21 in the studied treatment. To determine the success of the management processes in terms of the removal rate of the studied genes, we investigated both the number of genes reduced and the magnitude of their removal. In slurry, the AD 55 °C treatment showed the greatest success in removing all tested genes; the following most successful treatments were the biochar treatments for all genes except *tetA*. On the other hand, the ozonation treatment presented the opposite trend, showing a significant increase in all genes except *tetA*. The treatments with the highest removal rates for each specific gene were as follows: biochar 0.5% for *sul1*; biochar 2% for *sul2*, *tetX*, *intl1*, and *tnpA-04*; and ozonation for *tetA*. Regarding the manure, the nZVI 2% treatment had the greatest success in removing all tested genes, followed by the AD 55 ºC, AD 75 ºC, biochar 2%, biochar 5%, and SMS 1:0.5 treatments, which failed only with the *tetA* gene. In contrast, the ozonation and SMS 1:1 treatments increased the removal rates of five out of the six genes. The following treatments had the highest deletion rates for each specific gene: AD 75 ºC for *sul1* and *sul2*; biochar 5% for *tetX*, *intl1*, and *tnpA-04*; and nZVI 2% for *tetA*. In addition, the AD 55 ºC, AD 75 ºC, and biochar 5% treatments showed the greatest success in removing ARGs and MGE genes in the compost. On the other hand, the nZVI 1%, ozonation, SMS 1:0.5, and SMS 1:1 treatments showed the opposite trend, increasing the removal rate of every gene. The treatments with the highest removal rates for each specific gene were AD 55 ºC, AD 75 ºC, and AD 90 ºC for *tetA*, *intl1*, and *tnpA-04*, respectively, and biochar 5% for *sul1*, *sul2*, and *tetX*.

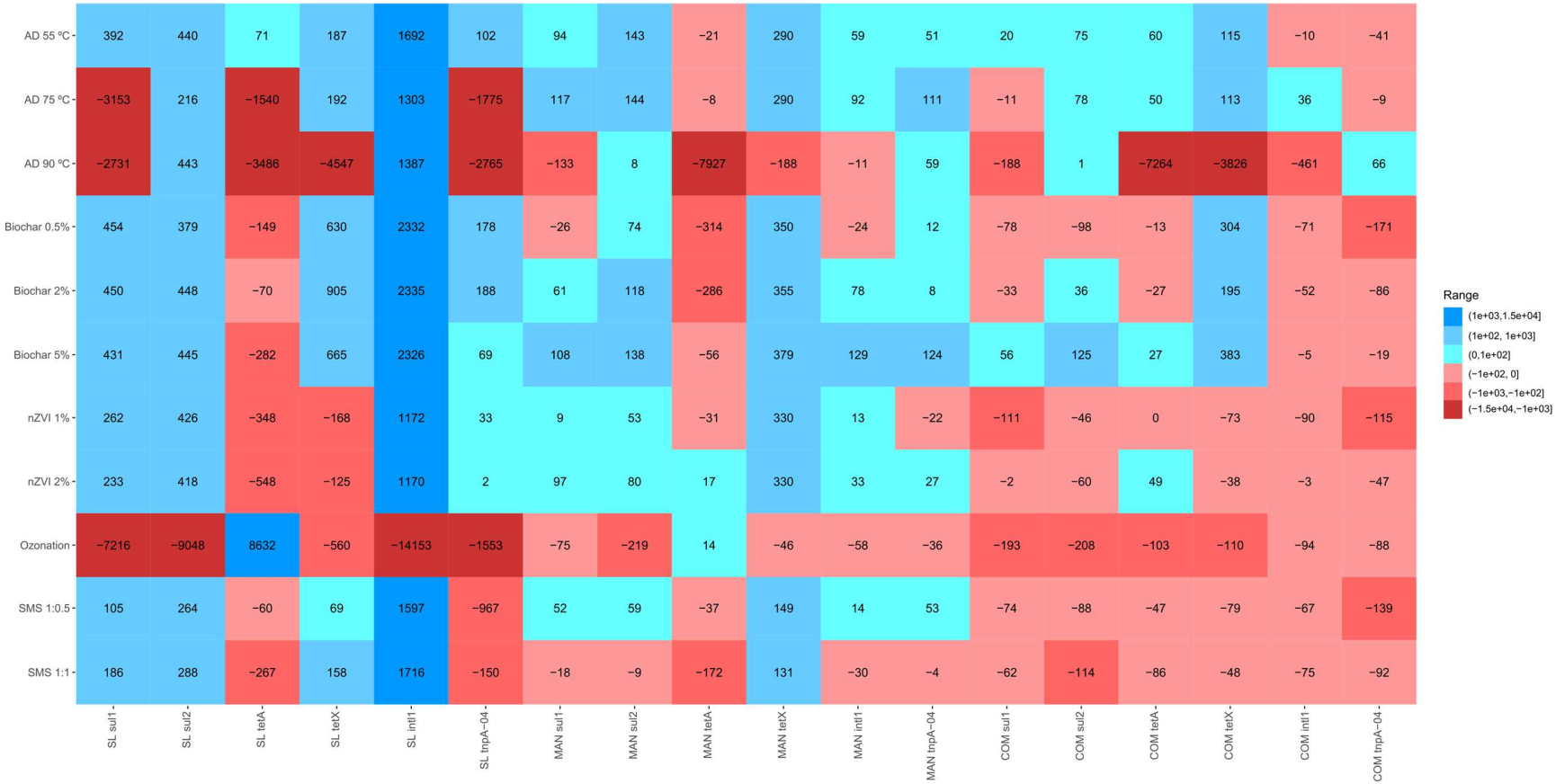


Figure 9.1. Removal rates of antibiotic resistance genes (ARGs) and mobile genetic element (MGE) genes under different treatments in three organic amendments. Treatments: AD: anaerobic digestion; nZVI: zerovalent iron nanoparticles; SMS: spent mushroom substrate. Amendments: SL: slurry; MAN: manure; COM: compost.

9.3.2. Soil and lettuce resistome and mobilome

Of the three investigated amendments, we selected compost for the microcosm experiment because it is commonly used to fertilize lettuce. The compost management treatments AD 75 ºC and biochar 5% were chosen because they were the most promising treatments in the preliminary study. Additionally, a 5% biochar addition to the soil was tested.

The absolute abundances of the genes were obtained from ddPCR for each treatment (Fig. 9.2). The abundance of *sul1* was significantly lower in the NPK and unamended treatments than that in the biochar soil treatment. Similarly, the abundance of *sul2* was lower in the anaerobic digestion, NPK, and unamended treatments than in the untreated compost and biochar soil treatments. Moreover, untreated compost, NPK, and unamended treatments showed lower *tetA* abundance than those in anaerobic digestion, biochar compost, and biochar soil treatments (the difference was not statistically significant between untreated compost and biochar compost). Regarding the MGE genes, anaerobic digestion, biochar compost, NPK, and unamended treatments presented a lower *intl1* gene abundance than that in the untreated compost. Finally, the abundance of *tnpA-04* was lower in the unamended treatment than in the untreated compost treatment.

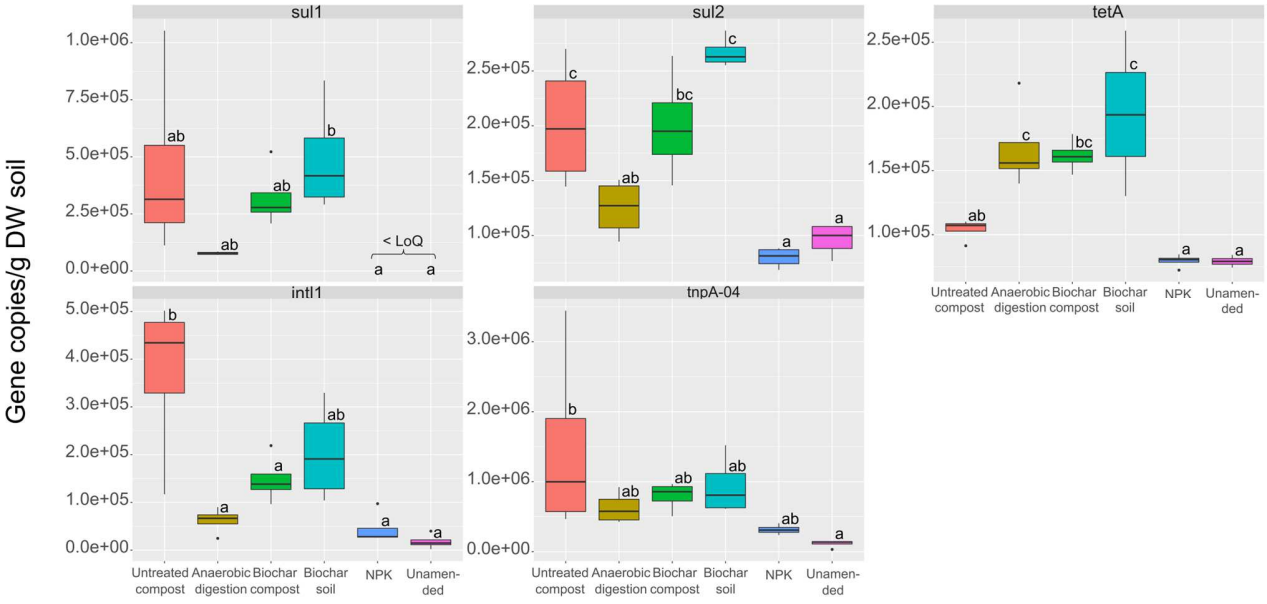


Figure 9.2. Absolute abundances of antibiotic resistance genes (ARGs) and mobile genetic element (MGE) genes in the soil samples according to the ddPCR analysis, expressed as gene copies per g of DW soil. Different letters indicate statistically significant differences among treatments ($p < 0.05$) according to Tukey's post hoc test. LoQ: limit of quantification.

The absolute abundance of the ARGs in the soil samples varied in the following increasing order, expressed as average gene copies × 10^5 g^{-1} DW soil: NPK (1.8), unamended (1.9), anaerobic digestion (3.8), biochar compost (7.0), untreated compost (7.7), and biochar soil (9.6). Similarly, the abundance of MGE genes increased in the following order: unamended (1.3), NPK (3.6), anaerobic digestion (6.9), biochar compost (9.5), biochar soil (11), and untreated compost (19). The most abundant gene in the studied soils was *tnpA-04* in all treatments, followed by *sul1* in the biochar compost, biochar soil, and untreated compost treatments; *sul2* in the NPK and unamended treatments; and *tetA* in the anaerobic digestion treatment. In contrast, the least abundant gene in almost all treatments was *tetX*, with values were under the limit of quantification, with the NPK treatment being an exception in which the least abundant gene was *sul1*.

With respect to lettuce plants, no statistically significant differences were found among the treatments in terms of ARG and MGE gene abundances (Fig. 9.3). The most abundant gene in all treatments (expressed as gene copies 10^5 g^{-1} DW lettuce) was *tnpA-04* (2.3 to 6.0, in untreated compost and biochar soil treatments, respectively), except in the biochar soil treatment, in which *sul2* was most abundant (6.7). In contrast, the abundances of *sul1*, *tetA*, and *tetX* were below the quantification limit.

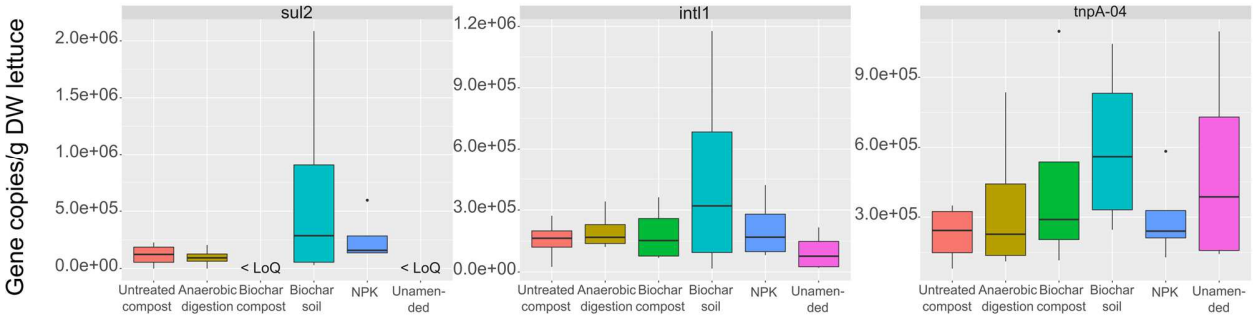


Figure 9.3. Absolute abundances of antibiotic resistance genes (ARGs) and mobile genetic element (MGE) genes in the lettuce samples according to the ddPCR analysis, expressed as gene copies per g of DW lettuce. No statistically significant differences were found among the treatments. LoQ: limit of quantification.

The results of HT-qPCR showed that out of the 76 ARGs and 17 MGE genes targeted in the soils, 69 and 17 genes were amplified, respectively. Out of the 16 ARGs and five MGE genes targeted in lettuce plants, eight and three genes were amplified, respectively. No statistically significant differences in individual genes or gene families were detected between the treatments in any soil (Supplementary Figure 9.1) or lettuce plant (Supplementary Figure 9.2). Moreover, we compared the relative abundance of individual genes in both compartments. We found significant differences in at

least 16 of the 21 common genes studied between soils and lettuce (Supplementary Table 9.4). In all cases, the relative abundance of the genes was higher in soils than in lettuce.

Furthermore, the ordinary least squares (OLS) regression model showed that the abundance of MGE genes was linearly and positively correlated with the abundance of ARGs in the studied soils (R^2 = 95.3, $p < 0.001$) (Fig. 9.4A) and lettuce plants (R^2 = 37.2, $p < 0.01$) (Fig. 9.4B).

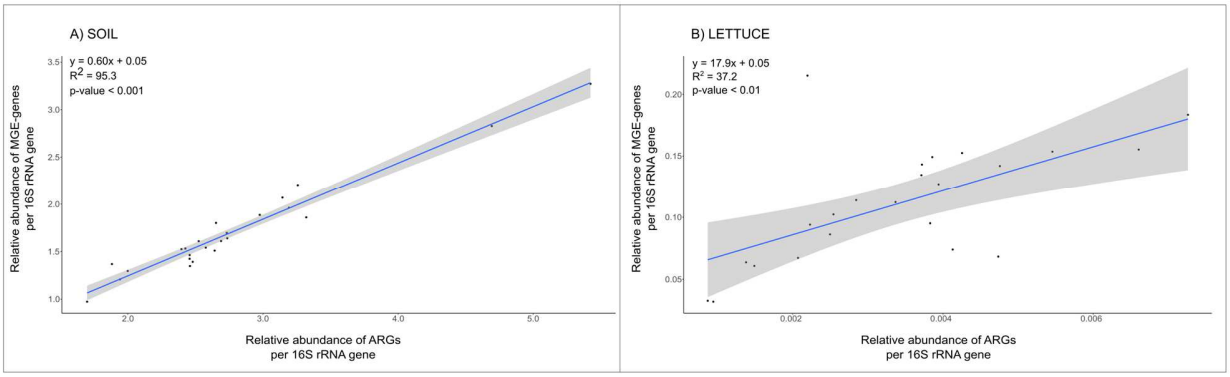


Figure 9.4. Results of the ordinary least squares (OLS) regression analysis of the relationship between the relative abundance of antibiotic resistance genes (ARGs) and mobile genetic element (MGE) genes in (A) soils and (B) lettuce plants. The line represents the best-fit curve. The shaded area represents the 95% confidence interval for the fitted OLS regression.

9.3.3. Soil bacterial community composition

Regarding the soil bacterial community composition, 99%, 93%, 84%, 47% and 6% of the reads were taxonomically classified at the class, order, family, genus, and species levels, respectively. The two most abundant classes were Alphaproteobacteria and Verrucomicrobiae, accounting for more than 60% of the relative abundance of bacteria; the exception was the anaerobic digestion treatment, in which the second most abundant class was Bacilli (Supplementary Figure 9.3). The values of the Shannon's diversity index (H') ranged from 3.87 (in both the unamended and biochar compost treatments) to 4.12 (in the NPK treatment). No statistically significant differences were found among the treatments in terms of (i) taxa at the class level, (ii) H' index, or (iii) composition according to the ANOSIM test.

The network analysis was constructed between the relative abundances of the ARGs and MGE genes obtained in the HT-qPCR analysis and the relative abundances of bacterial classes to identify the potential hosts harboring ARGs and MGE genes (Fig. 9.5). We included the genes and classes that presented at least one significant correlation (*i.e.*, 64 ARGs, 16 MGE genes, and 18 taxa). Regarding

the ARGs and MGE genes, *mefA_2* and *cmlV* presented the highest diversity in terms of possible hosts (*i.e.*, five and four taxa, respectively). Moreover, MB-A2-108, Thermoleophilia, and Bacteroidetes correlated positively with the highest numbers of ARGs and MGE genes (*i.e.*, 74, 12, and 10 genes, respectively). In contrast, the following ARGs presented the highest number of negative correlations with potential hosts: *aac(6')-Iy*, *aph3-III*, *qnrB*, and *ttgB* (3, 2, 2, and 2, respectively). Remarkably, all seven correlations between Gammaproteobacteria and the ARGs were negative.

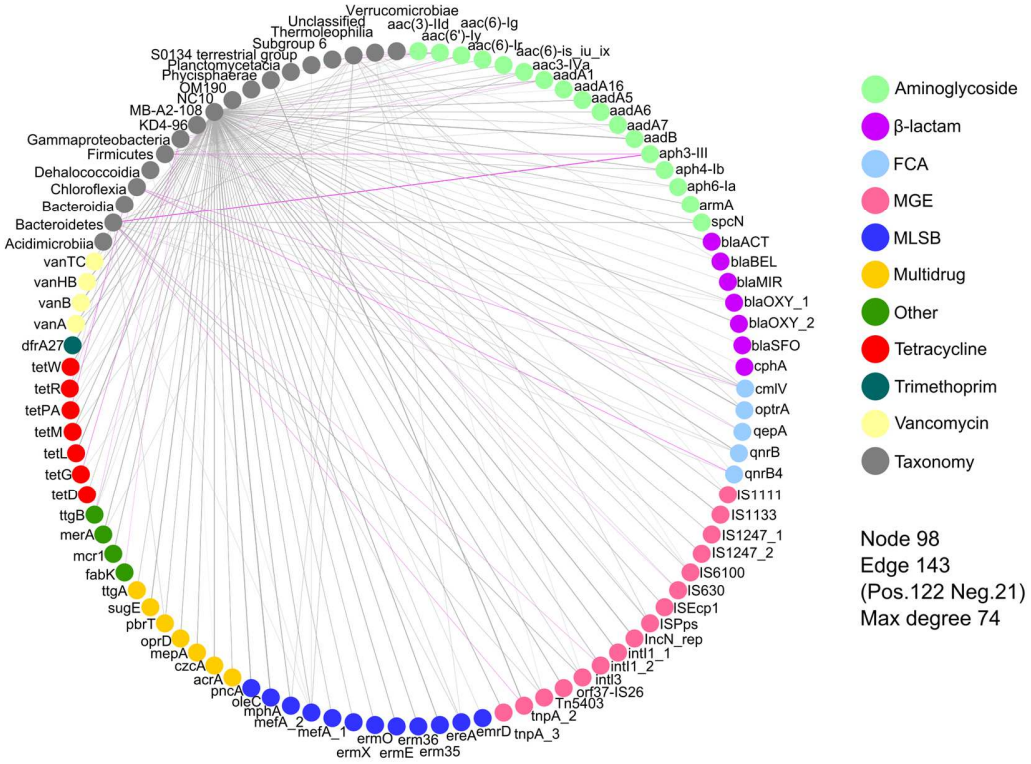


Figure 9.5. Networks revealing the correlation patterns (Pearson correlations; $p < 0.05$) of bacterial taxa (at the class and phylum levels) with antibiotic resistance genes (ARGs) and mobile genetic element (MGE) genes in all treatments. The nodes with different colors represent different classes of ARGs, MGE genes, and bacterial taxa. Grey and pink lines represent positive and negative correlations, respectively.

9.4. Discussion

Organic amendments of animal origin are important sources of antibiotics and ARGs (Congilosi & Aga, 2021). Their dissemination in the environment and the associated risks to human and animal health are complex problems that require urgent action. Even though composting offers an environmentally friendly approach to remove antibiotics or ARGs to some extent (Oliver et al., 2020),

some antibiotics and ARGs remain in the organic amendments. Therefore, it is essential to develop effective treatment methods for organic amendments of animal origin to reduce the risk.

The effectiveness of different management processes may differ depending on the type of organic amendment and whether it is in a solid, liquid, or semi-liquid state. In the present study, the best management processes were specific to each organic amendment. In the slurry, anaerobic digestion at 55 ºC resulted in the reduction of the abundance of the six studied genes, followed by biochar treatments. In the manure, the nZVI 2% treatment had the highest efficacy. On the other hand, anaerobic digestion at 55 and 75 ºC and biochar at 5% were the most effective treatments for the compost. In general, the success was lower in compost than in the other two amendments, probably because it was more difficult for the treatment effect to spread well in this solid matrix.

Previous research suggested that anaerobic digestion may be able to remove pathogens and ARGs, with thermophilic digestion being particularly effective (Beneragama et al., 2013; Sun et al., 2016). In a previous study, the thermophilic digestion of dairy manure reduced the relative abundance of *intl1*, *sul1*, *sul2*, and *tetX* (Sun et al., 2016). Similarly, compared to conventional composting, hyperthermophilic composting at 90 °C significantly reduced the abundance of ARGs of tetracyclines, sulfonamides, and MGE genes (Liao et al., 2018). In contrast, an increase in *sul1*, *sul2* (Sun et al., 2019), and *tetA* (Zou et al., 2020) was reported as a result of thermophilic digestion of cattle and pig manure, respectively. These inconsistent results may be attributable to the different characteristics of the raw materials, digestion parameters, and bacterial community composition, among others. Remarkably, the impact of anaerobic processes on ARG reduction could be gene specific (He et al., 2020). In the present study, anaerobic digestion was among the best treatments for the three amendments. Surprisingly, the success rate was lower at higher digestion temperatures.

Similarly, the application of biochar to the three amendments (slurry, manure, and compost) was a promising treatment. The effectiveness was higher with higher rates of biochar addition, especially in the case of compost. A previous study (Cui et al., 2016) evaluated the effects of applying rice straw biochar and mushroom biochar during chicken manure composting on ARG removal (including *sul1*, *sul2*, *tetA*, and *tetX* genes). Mushroom biochar addition to chicken manure composting resulted in a higher removal rate than that in the control, whereas rice straw biochar addition yielded the opposite results, which may be related to the higher abundance of pathogenic bacteria carrying ARGs (Cui et al., 2016). Biochar application may potentially alter the resistome profile as a

consequence of the changes in bacterial community composition, which is the primary determinant of ARG content (Forsberg et al., 2014). Biochar can also adsorb heavy metals and reduce their bioavailability, thus reducing the selective pressure on ARB (Li et al., 2017c).

Nanoparticles disrupt intracellular metabolism, damage DNA, and inhibit cell proliferation (Bondarenko et al., 2012). They can also enhance HGT via oxidative stress and increase the expression of mating pair formation (Wang et al., 2018b). In the present study, the application of nZVI 2% had the greatest success in removing all tested genes, but the removal rate was negative for almost every gene in the case of the compost. Another study also reported an increase in the relative abundance of *intl1*, *sul2*, and *tetC* in composted chicken manure 21 d after the application of 100 and 600 mg kg^{-1} of nZVI (Qiu et al., 2022). However, 42 d after the nZVI application, the relative abundance of the abovementioned genes decreased (Qiu et al., 2022).

In the present study, regarding ozonation, the abundances of ARGs and MGE genes increased in all three organic amendments (except for *tetA* in slurry and manure) compared to those in the control treatments. These results can be attributed to the consumption of ozone by organic constituents present in the amendments, such as humic substances, carbohydrates, and fatty acids (Zeng et al., 2014). Moreover, ozone damages the cell surface before releasing DNA, and intact ARGs may be transferred through HGT (Pak et al., 2016). In another study, the inactivation of ARGs increased in municipal wastewater when the ozone concentration increased from 27 to 61 mg L^{-1} (Zhuang et al., 2015).

Finally, the enrichment of ARGs and MGE genes observed in both manure and compost after SMS application (observed only with the 1:1 dose in manure) might be attributable to (i) the nutrients provided by the SMS, which led to the proliferation of bacterial hosts carrying ARGs and MGEs in the amendment or (ii) SMS-derived ARG and MGE survival and transfer to both manure and compost.

The results of the microcosm experiment with lettuce plants differed depending on the specific genes and the method used to quantify them. The resistome and mobilome risk tendency discrepancy detected between ddPCR and HT-qPCR may be attributable to the reaction volume (21 µL vs. 100 nL in ddPCR vs. HT-qPCR, respectively), primer sequences employed, annealing temperature (specific vs. general), and abundance units (absolute vs. relative). Droplet digital PCR generates up to 20,000 droplets and a PCR reaction occurs in each droplet. In terms of sensitivity, the lower limit of detection of ddPCR was 10 times more efficient than that of qPCR (Park et al., 2021). Therefore, we believe that HT-qPCR is useful for quantifying a wide range of genes simultaneously, but the results obtained

should be confirmed with another method, such as ddPCR. In our case, we chose to study tetracyclines and sulfonamides in detail because they accounted for 31% and 8% of the total antimicrobial sales for use in food-producing animals in 31 European countries in 2018, respectively (EMA, 2020). Although sulfonamides are used less frequently than tetracyclines, the main sulfonamide resistance genes (*sul1* and *sul2*) are often located on transposable elements of self-transferable or mobilizable broad-host-range plasmids and occur in a wide range of bacterial species (Heuer et al., 2004). Furthermore, both antibiotics were categorized as highly important antimicrobials for human medicine by the World Health Organization (WHO, 2019a). Tetracycline and sulfonamide resistance genes have frequently been reported in manure and soil (Zhu et al., 2013; Guo et al., 2018).

In terms of the results obtained by ddPCR in the studied soils, anaerobic digestion led to a 38% and 83% reduction in *sul2* and *intl1* absolute abundance, respectively, compared to those in the untreated compost. In another study, dairy manure application resulted in higher abundances of *intl1* and *sul1* in soils fertilized with slurry digestate and untreated slurry than those in mineral fertilization and unamended soils (Nõlvak et al., 2016). Furthermore, Nõlvak et al. (2016) reported a higher abundance of *tetA* in mineral-fertilized soils than in slurry digestate, untreated slurry, or unamended soils. The application of biochar to compost also led to a 60% reduction in *intl1* gene abundance compared with that in the untreated compost. In another study, the application of 0.5% biochar to soil decreased the ARG abundance in unplanted soil, but failed to remove ARGs from soil planted with *Brassica chinensis* L. (Chen et al., 2018). As expected, in general, we found that the application of compost increased the soil resistome and mobilome, as both mineral fertilization and unamended soil treatments presented the lowest ARG and MGE abundances.

Fortunately, we detected lower abundances of ARGs and MGE genes in lettuce plants than in soil using HT-qPCR. Moreover, *sul1*, *tetA*, and *tetX* gene abundances were below the quantification limit in all treatments of lettuce plants according to ddPCR. These results were consistent with the findings of previous studies indicating that the resistome and mobilome are more diverse and abundant in rhizosphere soil than in plants after the application of organic amendments (Chen et al., 2018; Zhu et al., 2017b; Jauregi et al., 2021c). In addition, we did not find statistically significant differences between the treatments in terms of ARG and MGE gene abundance. In contrast, Zhu et al. (2017) found 8-fold higher ARG abundance in lettuce amended with compost from animal manure or manure mixed with crop residues than in conventionally produced lettuce. Furthermore, ordinary least squares regression models pointed to the key role of MGEs in shaping the pattern of ARGs.

No differences were found between treatments in terms of soil bacterial diversity. Therefore, we can argue that the changes in the resistome and mobilome were not a consequence of the modification of the bacterial communities. Despite being very rare in our samples, the MB-A2-108 class harbored the highest resistome and mobilome risks, demonstrating a positive and significant correlation with 74 of the 93 studied genes. This class is characteristic of deep subsoil environments (Seuradge et al., 2017) and was found to be positively correlated with soil pH and negatively correlated with soil C and N contents (Curd et al., 2018). The class Thermoleophilia was abundant and showed a positive correlation with several ARGs and MGE genes. According to Wu et al. (2021), Thermoleophilia showed a positive correlation with the *intl1*, *sul1*, and *sul2* genes in sediment samples. Moreover, Wu et al. (2021) detected a higher abundance of Thermoleophilia in tetracycline-spiked soils than in the control soils.

In addition, the multidrug gene *mefA* was detected in the anaerobic digestion, biochar soil, and untreated compost treatments. The network analysis revealed that *mefA* was positively correlated with five bacterial classes belonging to Actinobacteria, Chloroflexi, and Gemmatimonadetes, indicating that inter-phylum HGT may have occurred. The acquisition of *mefA* via HGT has previously been demonstrated by Gabashvili et al. (2020) and has been detected in at least 66% of both unmedicated and tetracycline-medicated swine fecal samples (Looft et al., 2012). Finally, we found negative correlations between Gammaproteobacteria and seven ARGs conferring resistance to aminoglycosides, FCA, multidrugs, tetracycline, and triclosan, suggesting that members of this class did not harbor the studied genes. Instead, the abundances of nine ARG genes (out of 38), *intl1*, and Gammaproteobacteria were positively correlated in soils amended with mineral fertilization, cow manure, and pig manure (Peng et al., 2017).

In summary, anaerobic digestion and biochar addition could be beneficial for reducing the risk of resistome and mobilome in organic amendments of animal origin before they are applied to soils. However, further confirmatory studies are necessary before applying these methods on a large scale. Ideally, the treatment or a combination of treatments should be inexpensive and feasible for application on livestock farms.

9.5. Supplementary information

Supplementary Table 9.1. Physicochemical properties of the organic amendments studied in the preliminary experiment.

Property	Compost	Manure	Slurry
Dry matter (%)	32.61	22.42	6.56
Electrical conductivity (mS cm^{-1})	1.03	1.08	1.00
pH	8.69	8.91	8.67
Organic matter (% DW)	66.23	86.74	78.27
Organic C (%)	12.56	11.31	2.99
N (%)	1.00	0.58	0.32
C/N	12.56	20.56	29.85
K_2O (% DW)	2.10	3.10	4.44
P_2O_5 (% DW)	0.59	0.70	1.16
Cd (mg kg^{-1} DW)	0.15	0.06	0.14
Co (mg kg^{-1} DW)	1.68	1.40	3.25
Cr (mg kg^{-1} DW)	7.06	6.74	7.08
Cu (mg kg^{-1} DW)	55.31	50.71	116.94
Ni (mg kg^{-1} DW)	10.63	4.61	10.78
Pb (mg kg^{-1} DW)	2.72	1.44	2.76
Zn (mg kg^{-1}DW)	203.07	193.97	441.4

Supplementary Table 9.2. Primers and PCR conditions for droplet digital PCR (ddPCR) analysis.

Gene	Primers	Amplicon size (bp)	Cycling conditions	Reference
16S rRNA	F: CCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	194	95 ºC for 5 min 40 cycles of 95 ºC for 30 s and 60 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	Muyzer et al., 1993
tetA	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210	95 ºC for 5 min 40 cycles of 95 ºC for 30 s and 62.5 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	Ng et al., 2001
tetX	F: AGCCTTACCAATGGGTGTAAA R: TTCTTACCTTGGACATCCCG	278	95 ºC for 5 min 40 cycles of 95 ºC for 30 s and 57.5 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	Diehl & Lapara, 2010
sul1	F: CCGTTGGCCTTCCTGTAAAG R: TTGCCGATCGCGTGAAGT	67	95 ºC for 5 min	Heuer & Smalla, 2007

			40 cycles of 95 ºC for 30 s and 60 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	
sul2	F: CGGCTGCGCTTCGATT R: CGCGCGCAGAAAGGATT	60	95 ºC for 5 min 40 cycles of 95 ºC for 30 s and 60 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	Heuer et al., 2008
intl1	F: GCCTTGATGTTACCCGAGAG R: GATCGGTCGAATGCGTGT	196	95 ºC for 5 min 40 cycles of 95 ºC for 30 s and 61 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	Barraud et al., 2010
tnpA-04	F: CCGATCACGGAAAGCTCAAG R: GGCTCGCATGACTTCGAATC	101	95 ºC for 5 min 40 cycles of 95 ºC for 30 s and 61 ºC for 1 min, with a ramping rate of 2.5 ºC s^{-1} 4 ºC for 5 min 90 ºC for 5 min	Zhu et al., 2013

Supplementary Table 9.3. Primer sets used in high-throughput qPCR (HT-qPCR) analysis.

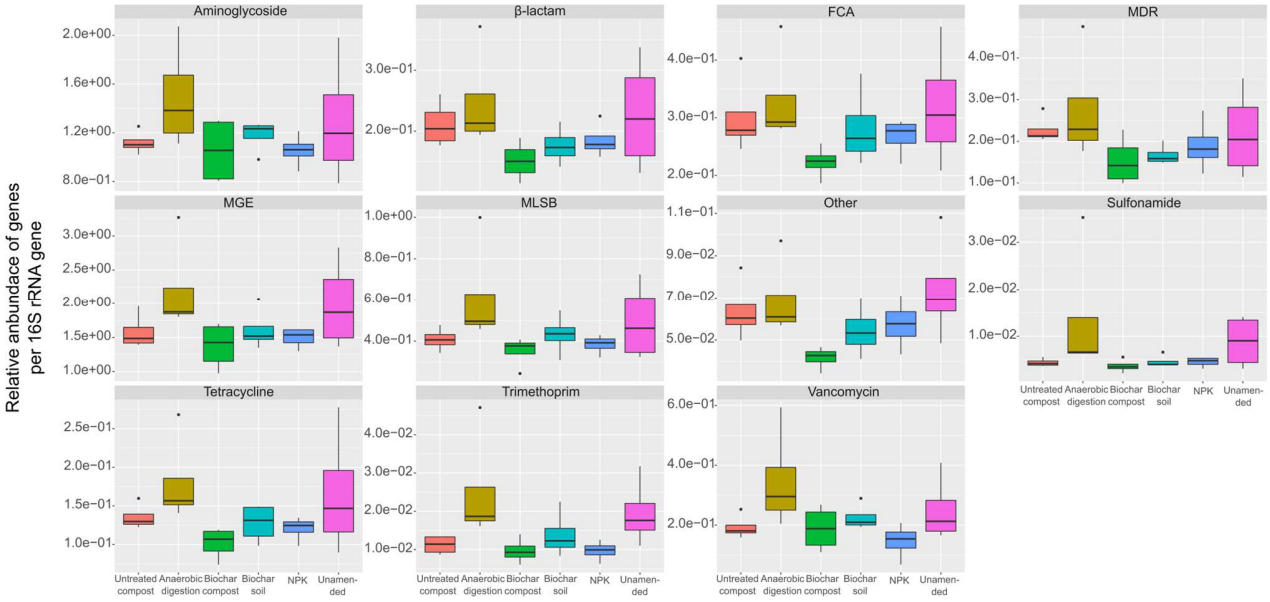
Gene	Forward Primer	Reverse Primer	Target antibiotics (major)
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	16S rRNA
Bacteroidetes	GGARCATGTGGTTTAATTCGATGAT	AGCTGACGACAACCATGCAG	Taxanomic
Firmicutes	GGAGYATGTGGTTTAATTCGAAGCA	AGCTGACGACAACCATGCAC	Taxanomic
aac(3)-iid_iia	CGATGGTCGCGGTTGGTC	TCGGCGTAGTGCAATGCG	Aminoglycoside
aac(6)-ig	GCGATGTTAGAAGCCTCAATTCG	CACACTTCGGCCTGTCGAA	Aminoglycoside
aac(6)-ir	GCTATAACGATCAGCAGCAAGC	CGCGATGCATGGCATGAC	Aminoglycoside
aac(6)-is_iu_ix	AAGCTTACTCTGGCCTGATCATG	TGCCTGAACGTCGATATTCAGG	Aminoglycoside
aac(6')-Iy	GCCTCAATCCGCCACGATTA	ACGCGCTCTGTTTCCTCAAA	Aminoglycoside
aac3-IVa	CCAACACGACGCTGCATC	GCTGTCGCCACAATGTCG	Aminoglycoside
aac6-aph2	CCAAGAGCAATAAGGGCATACCAA	GCCACACTATCATAACCACTACCG	Aminoglycoside
aadA1	TGTACGGCTCCGCAGTG	CACGGAATGATGTCGTCGTG	Aminoglycoside
aadA16	ACGGTGGCCTGAAGCC	GAATTGCAGTTCCCGTCTGG	Aminoglycoside
aadA5	ATCACGATCTTGCGATTTTGCT	CTGCGGATGGGCCTAGAAG	Aminoglycoside
aadA6	CCATCGAGCGTCATCTGGAA	CCCGTCTGGCCGGATAAC	Aminoglycoside
aadA7	CACTCCGCGCCTTGGA	TGTGGCGGGCTCGAAG	Aminoglycoside
aadB	CCTGCTTGGTGGGCAGAC	CGGCACGCAAGACCTCAA	Aminoglycoside
aph3-III	CAGAAGGCAATGTCATACCACTTG	GACAGCCGCTTAGCCGAA	Aminoglycoside
aph4-Ib	GGGAACACCGTGCTCACC	GTTGGTCCCGTGCAGGTC	Aminoglycoside
aph6-Ia	CGCTGGGAGCTGAAGAGG	AGCATCGTGCTGCTCTCC	Aminoglycoside
apmA	GGCGCACATGCATTCATCA	CTATACTCCAGTCCCACCATTTGA	Aminoglycoside
armA	TCTTCGACGAATGAAAGAGTCG	GCTAATGGATTGAAGCCACAACC	Aminoglycoside
spcN	GCTATGTGCTGGTGGACTGG	GGAACCACTCGACGAACTCG	Aminoglycoside

strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside
bla1	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA	β-lactam
blaACT	AAGCCGCTCAAGCTGGA	GCCATATCCTGCACGTTGG	β-lactam
blaBEL-nonmobile	ATGTCCATGGCACAGACTGTG	CCTGTCTTGTCACCCGTTACC	β-lactam
blaMIR	CGGTCTGCCGTTACAGGTG	AAAGACCCGCGTCGTCATG	β-lactam
blaOXY_1	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT	β-lactam
blaOXY_2	AAAGGTGACCGCATTCGC	CCAGCGTCAGCTTGCG	β-lactam
blaSFO	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT	β-lactam
cphA	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC	β-lactam
intI1_1	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	Integrons
intI1_2	CGAAGTCGAGGCATTTCTGTC	GCCTTCCAGAAAACCGAGGA	Integrons
intl3	CAGGTGCTGGGCATGGA	CCTGGGCAGCATCACCA	Integrons
acrA	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC	MDR
cefa_qacelta	TAGTTGGCGAAGTAATCGCAAC	TGCGATGCCATAACCGATTATG	MDR
czcA	GCCTTGTTCATCGGCGAAC	GGCAATGTCGCCTTCGTTC	MDR
emrD	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC	MDR
mepA	ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT	MDR
oprD	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA	MDR
pbrT	GATGCGCACTGGGCTTG	TCGGAATATGCGGAAATGCG	MDR
sugE	CTTAGTTATTGCTGGTCTGCTGGA	GCATCGGGTTAGCGGACTC	MDR
ttgA	ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA	MDR
IncN_rep	AGTTCACCACCTACTCGCTCCG	CAAGTTCTTCTGTTGGGATTCCG	MGE
IS1111	GTCTTAAGGTGGGCTGCGTG	CCCCGAATCTCATTGATCAGC	MGE
IS1133	GCAGCGTCGGGTTGGA	ACGCGTTCGAACAACTGTAATG	MGE
IS1247_1	CGGCCGTCACTGACCAA	TCGGCAGGTTGGTGACG	MGE
IS1247_2	TGGATCGACCGGTTCCAT	GCTGACCGAGCTGTCCATGT	MGE
IS6100	CGCACCGGCTTGATCAGTA	CTGCCACGCTCAATACCGA	MGE
IS630	CCGCCACCAGTGTGATGG	TTGGCGCTGACTGGATGC	MGE
ISEcp1	CATGCTCTGCGGTCACTTC	GACGCACCTTCTTGATGACC	MGE
ISPps	CACACTGCAAAAACGCATCCT	TGTCTTTGGCGTCACAGTTCTC	MGE
orf37-IS26	GCCGGGTTGTGCAAATAGAC	TGGCAATCTGTCGCTGCTG	MGE
Tn5403	AAGCGAATGGCGCGAAC	CGCGCAGGGTAAACTGC	MGE
tnpA_2	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC	MGE
tnpA_3	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	MGE
trbC	CGGYATWCCGSCSACRCTGCG	GCCACCTGYSBGCAGTCMCC	MGE
ereA	GATAATTCTGCTGGCGCACA	GCAGGCGTGGTCACAAC	MLSB
erm35	CCTTCAGTCAGAACCGGCAA	GCTGATTTGACAGTTGGTGGTG	MLSB
erm36	GGCGGACCGACTTGCAT	TCTGCGTTGACGACGGTTAC	MLSB
ermE	GTCACGCAGCTGGAGTTCG	CGGTGAAGCACAGCTCGAC	MLSB
ermO	GAGTACGCCCGCAAACG	GCGTTCGATCCGGAGGA	MLSB
ermX	TGATGACGGCTCAGTGG	GTGCACCAGCGCCTGA	MLSB
lnuB	GGATCGTTTACCAAAGGAGAAGG	AGCATAGCCTTCGTATCAGGAA	MLSB

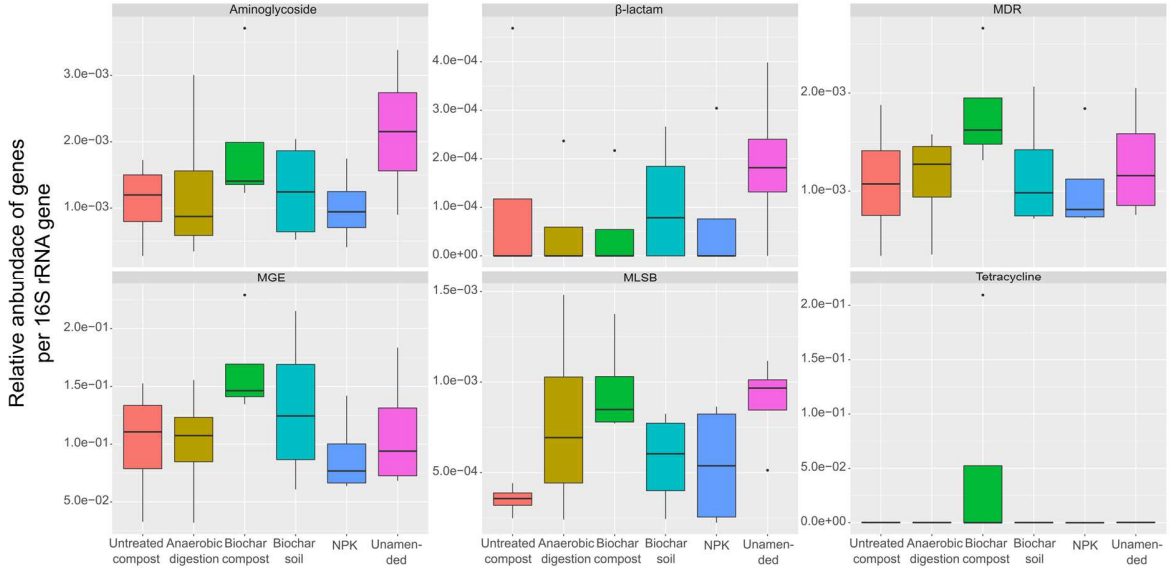
lnuC	GGGTGTAGATGCTCTTCTTGGA	CTTTACCCGAAAGAGTTTCTACCG	MLSB
mefA_1	TAATTATCGCAGCAGCTGGTTC	GTTCCCAAACGGAGTATAAGAGTG	MLSB
mefA_2	CCGTAGCATTGGAACAGCTTTT	AAACGGAGTATAAGAGTGCTGCAA	MLSB
mphA	TCAGCGGGATGATCGACTG	GAGGGCGTAGAGGGCGTA	MLSB
oleC	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG	MLSB
pncA	GCAATCGAGGCGGTGTTC	TTGCCGCAGCCAATTCA	MLSB
vat(A)	ATGAACGGAGCGAATCATCGG	CCATACCGATCCAAACGTCATTTC	MLSB
bacA	ATCCGCGGCACCCTGA	CCTGCTTGATGGACTTGATGAAGA	Other
fabK	CAGGAGCAGGAAATCCAAGC	CCAGCTTCCATTCCTTCTGC	Other
mcr1	CACATCGACGGCGTATTCTG	CAACGAGCATACCGACATCG	Other
merA	GTGCCGTCCAAGATCATG	GGTGGAAGTCCAGTAGGGTGA	Other
ttgB	TCGCCCTGGATGTACACCTT	ACCATTGCCGACATCAACAAC	Other
catQ	AGGTGCACTTACAGTATGACTGC	AACGTGGGAAGTTCTCGTCATAC	Phenicol
cmlV	GCCCTCATCACCGTCTTCG	GGACGTTGGCGATGGAGAG	Phenicol
optrA	GGTGGATGAAGTCCGTACGG	AGGTTAGACCTCCAAGAGCCA	Phenicol
qepA	GGGCATCGCGCTGTTC	GCGCATCGGTGAAGCC	Quinolone
qnrB	GCGACGTTCAGTGGTTCAGA	GCTGCTCGCCAGTCGAA	Quinolone
qnrB4	TCACCACCCGCACCTG	GGATATCTAAATCGCCCAGTTCC	Quinolone
sul3	TCCGTTCAGCGAATTGGTGCAG	TTCGTTCACGCCTTACACCAGC	Sulfonamide
tetD	AATTGCACTGCCTGCATTGC	GACAGATTGCCAGCAGCAGA	Tetracycline
tetG	TCGCGTTCCTGCTTGCC	CCGCGAGCGACAAACCA	Tetracycline
tetL	ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT	Tetracycline
tetM	GGAGCGATTACAGAATTAGGAAGC	TCCATATGTCCTGGCGTGTC	Tetracycline
tetPA	GGAAACCTTAGTTCAGTGACTTGG	CCCATTTAACCACGCACTGAA	Tetracycline
tetPB	TGGGCGACAGTAGGCTTAGAA	TGACCCTACTGAAACATTAGAAATATACCT	Tetracycline
tetR	CCGTCAATGCGCTGATGAC	GCCAATCCATCGACAATCACC	Tetracycline
tetW	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC	Tetracycline
dfrA27	GCCGCTCAGGATCGGTA	GTCGAGATATGTAGCGTGTCG	Trimethoprim
vanA	GGGCTGTGAGGTCGGTTG	TTCAGTACAATGCGGCCGTTA	Vancomycin
vanB	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT	Vancomycin
vanHB	GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT	Vancomycin
vanTC	ACAGTTGCCGCTGGTGAAG	CGTGGCTGGTCGATCAAAA	Vancomycin

Supplementary Table 9.4. Comparison of the relative abundances of antibiotic resistance genes and mobile genetic element genes in soil (S) and lettuce (L) samples for each treatment, based on Tukey's *post hoc* test. ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

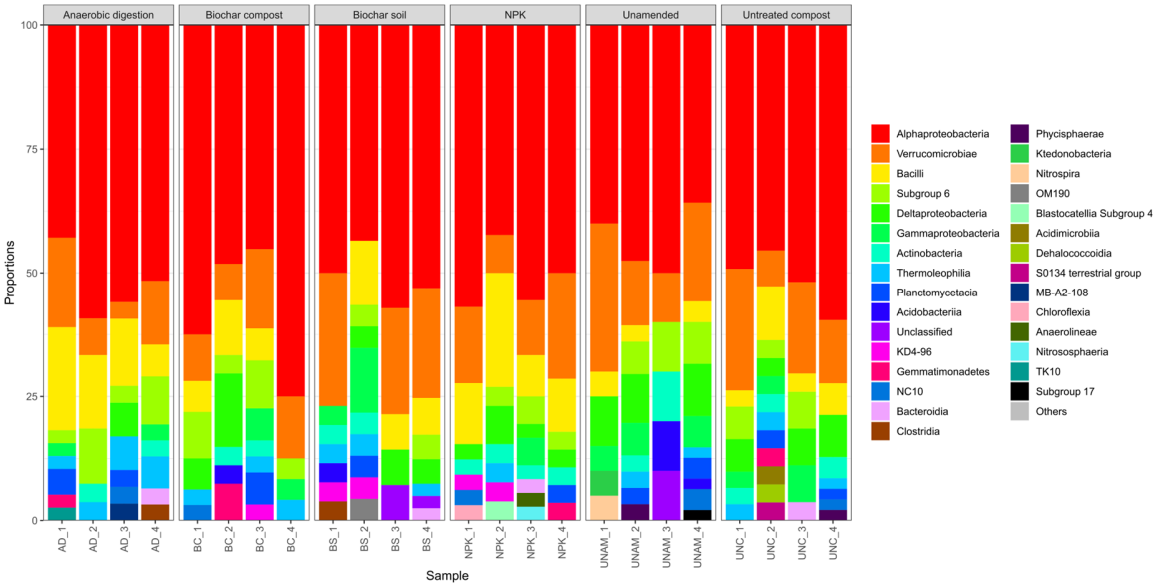
Gene	Untreated compost	Anaerobic digestion	Biochar compost	Biochar soil	NPK	Unamended
aac(6)-Ig	S > L ***	S > L **	S > L **	S > L ***	S > L ***	S > L *
aac3-IVa	S > L ***	S > L **	S > L *	S > L ***	S > L **	S > L **
aadA5	S > L *	ns	ns	ns	ns	S > L *
blaBEL-nonmobile	ns	ns	ns	ns	ns	ns
cmlV	S > L ***	S > L **	S > L **	S > L ***	S > L *	S > L ***
czcA	S > L ***	S > L **	S > L **	S > L ***	S > L ***	S > L **
erm35	S > L **	S > L *	S > L *	S > L **	S > L **	S > L **
ermX	S > L ***	S > L **	S > L ***	S > L ***	S > L ***	S > L **
fabK	S > L ***	S > L ***	S > L ***	S > L ***	S > L **	S > L *
intI1_1	S > L ***	S > L **	S > L ***	S > L ***	S > L ***	S > L **
intI1_2	ns	ns	ns	S > L *	ns	ns
IS6100	S > L ***	S > L **	S > L **	S > L ***	S > L ***	S > L *
ISEcp1	S > L ***	S > L *	S > L **	S > L **	S > L **	S > L **
mefA	S > L ***	S > L *	S > L **	S > L ***	S > L **	S > L *
mepA	S > L ***	S > L **	S > L ***	S > L **	S > L ***	S > L **
optrA	S > L ***	S > L *	S > L ***	S > L ***	S > L ***	S > L ***
orf37-IS26	S > L ***	S > L **	S > L ***	ns	S > L ***	S > L **
spcN	S > L ***	S > L ***	S > L **	S > L ***	S > L ***	S > L ***
tetPA	ns	ns	ns	ns	ns	ns
ttgA	S > L ***	S > L *	S > L ***	S > L ***	S > L ***	S > L **



Supplementary Figure 9.1. Relative abundance (via HT-qPCR analysis) of antibiotic resistance genes and mobile genetic element genes in the studied soils.



Supplementary Figure 9.2. Relative abundance (via HT-qPCR analysis) of antibiotic resistance genes and mobile genetic element genes in lettuce samples.



Supplementary Figure 9.3. The 30 most abundant bacterial classes in the studied soils.

DISCUSIÓN GENERAL

Se estima que la población mundial aumente aproximadamente hasta 10.000 millones de personas para el año 2050 (UN, 2019). Asimismo, se prevé que el consumo total de alimentos se incremente entre un 35% y un 56% en el periodo comprendido entre 2010 y 2050 (van Dijk et al., 2021). Estas tendencias globales presentan unos fuertes desafíos para la agricultura y la alimentación, ambas actividades frecuentemente ligadas al uso intensivo de recursos y, a su vez, responsables a menudo de la degradación del suelo y la pérdida de biodiversidad, entre otros impactos ambientales adversos. Por ello, se necesitan agroecosistemas que protejan y potencien los recursos naturales, al tiempo que aumenten la productividad agrícola. En este escenario, es imperativa una transición hacia sistemas de producción más sostenibles. El cambio en el paradigma de la economía lineal actual hacia una Economía Circular se sustenta en el principio de la circularidad de los materiales y la energía, de cara a conservar el capital natural mediante la optimización en el uso de recursos (Velenturf & Phunell, 2021). Por otra parte, un modelo económico circular impulsaría varios de los Objetivos de Desarrollo Sostenible en el marco de la iniciativa impulsada por las Naciones Unidas: el fin de la pobreza, el hambre cero, la salud y bienestar, una energía asequible y no contaminante, el trabajo decente y el crecimiento económico, una producción y consumo responsables, la vida de los ecosistemas terrestres, etc. Análogamente, la Bioeconomía es un modelo económico basado en el empleo de materias primas renovables de origen biológico que pretende, entre otros objetivos, ahondar en la interconexión entre economía, medioambiente y sociedad, y lograr un mejor equilibrio entre la conservación de los recursos naturales y las actividades económicas (IACGB, 2020).

En relación con la actividad agrícola, y en el marco de la deseada transición hacia una Europa neutra en emisiones, el objetivo de la Comisión Europea de lograr que un 25% de las tierras agrícolas se enmarquen dentro del ámbito de la agricultura ecológica para el año 2030, como parte de la estrategia "De la Granja a la Mesa" (COM/2020/381 final), impacta directamente en el uso de enmiendas orgánicas para fertilizar los suelos agrícolas. La producción ecológica está regulada por una normativa europea (Reglamento 2018/848) en la que se excluye el uso de fertilizantes químicos de síntesis, favoreciendo así el uso de enmiendas orgánicas de origen vegetal y/o animal. Estas últimas tienen que proceder de ganaderías ecológicas y/o de ganadería extensiva, y con una aplicación máxima de 170 kg de N ha^{-1} para evitar la contaminación ambiental por exceso de nutrientes.

Los suelos desempeñan un papel fundamental en la salud de los ecosistemas terrestres, proporcionando servicios ecosistémicos de *aprovisionamiento* (alimentos, agua, madera, fibras, recursos medicinales, etc.), de *regulación* (control biológico de plagas, regulación de la calidad del aire y la fertilidad de los suelos, prevención de la erosión, secuestro y almacenamiento de carbono, reciclaje de residuos, polinización, etc.), de *soporte* (espacios vitales para las plantas y animales, conservación de la biodiversidad), y *culturales* (actividades de recreo, cultura, arte, turismo, experiencias espirituales, etc.) (MEA, 2005). La creciente sobreexplotación antrópica del ecosistema edáfico, sumado a las prácticas de gestión insostenibles de este recurso natural limitado y los efectos adversos del cambio climático, están poniendo en riesgo el suministro de los citados servicios ecosistémicos con consecuencias potencialmente devastadoras para la supervivencia y/o el bienestar de la humanidad.

El empleo de enmiendas orgánicas como fertilizantes agrícolas no es una práctica novedosa promovida recientemente por la transición hacia una economía circular o el impulso a la agricultura ecológica. De hecho, en los sistemas agroganaderos, la generación de subproductos orgánicos y su posterior empleo para fertilizar los suelos agrícolas ha sido tradicionalmente, y es actualmente, una práctica muy habitual que contribuye a incrementar la productividad agrícola a la vez que puede, si se lleva a cabo adecuadamente, mejorar la salud del suelo. Esta práctica conlleva beneficios potenciales asociados, como pueden ser un aumento de la biomasa y actividad microbiana edáfica, en comparación con la fertilización mineral (Francioli et al., 2016). La cantidad de nitrógeno total añadida a los suelos a través de la aplicación de enmiendas orgánicas de origen vacuno alcanza cifras de relevancia por su magnitud y potencial impacto (expresado en 10^6 toneladas de N): 15, 3.6 y 0.27 a nivel mundial, europeo y nacional, respectivamente (FAOSTAT, 2020b). Sin embargo, y como columna vertebral de este trabajo, la aplicación de enmiendas orgánicas a los suelos agrícolas conlleva riesgos potenciales con relación a la emergencia y diseminación de resistencias antimicrobianas (AMR) en el medioambiente.

Con el fin de evaluar la idoneidad, en términos de beneficios y riesgos asociados, de las enmiendas orgánicas para uso agrícola, llevamos a cabo el estudio presentado en el Capítulo 5 de esta tesis doctoral. Además de cuantificar la presencia de contaminantes químicos (metales, aromáticos, hidrocarburos halogenados, hidrocarburos orgánicos volátiles, hidrocarburos totales de petróleo, pesticidas, ftalatos y otros compuestos) y biológicos (*Escherichia coli*, *Salmonella* spp.) , en dicho capítulo propusimos un Índice de Calidad de Enmiendas (AQI: Amendment Quality Index) basado en parámetros fisicoquímicos (contenido en materia orgánica y nutrientes) y microbiológicos (actividad,

biomasa y diversidad microbiana). Entre las enmiendas analizadas, los composts de vacuno procedentes de ganadería intensiva y ganadería ecológica no mostraron riesgo alguno en relación con la presencia de contaminantes químicos o biológicos potencialmente peligrosos y obtuvieron los valores más elevados del AQI. Aun así, consideramos preciso mejorar la caracterización de las enmiendas orgánicas, con una mayor exhaustividad en términos de parámetros cuantificados, así como la evaluación de los riesgos y beneficios potenciales de la aplicación de estas enmiendas en suelos agrícolas para, entre otros aspectos, incidir en la mitigación de la problemática de la AMR.

A lo largo de la historia, las enfermedades infecciosas han sido la causa principal de mortalidad humana (Aminov, 2010). El descubrimiento y la producción de antibióticos fue uno de los logros médicos más importantes del siglo XX y de toda la historia de la medicina en general (Aminov, 2010). Los antibióticos son herramientas indispensables para tratar infecciones bacterianas tanto en salud humana como en veterinaria. Sin embargo, al poco tiempo de introducir los antibióticos en la rutina clínica, tradicionalmente se ha detectado la presencia e incremento progresivo de bacterias resistentes a los citados antibióticos como consecuencia de la aparición de mutaciones con valor selectivo-adaptativo o la adquisición de genes de resistencia a antibióticos (ARGs). Actualmente, los residuos de antibióticos y sus productos de transformación se consideran contaminantes emergentes de interés debido a su amplia distribución y a sus efectos potencialmente negativos sobre los ecosistemas terrestres y acuáticos. Los productos de transformación de los antibióticos pueden mantener sus propiedades bioactivas. Además, concentraciones subinhibitorias de estos compuestos químicos ejercen a menudo una presión selectiva suficiente para inducir y mantener los ARGs (Sandergren, 2014). Por otra parte, el retorno de la susceptibilidad de las bacterias en ausencia de la presión selectiva ejercida por los antibióticos es incierto y objeto de mucha investigación por parte de la comunidad clínica y científica (Knöppel et al., 2017; Lopatkin et al., 2017; Andersson et al., 2019; Rajer & Sandegren, 2022). De acuerdo con la "hipótesis del reservorio", es necesario un umbral de concentración de antibióticos para generar una presión selectiva sobre los microorganismos expuestos y, consecuentemente, inducir y mantener las resistencias en el medioambiente (Heinemann et al., 2000).

El perfil del resistoma de las deyecciones animales se suele asociar a la exposición a los antibióticos más comunes en veterinaria, mientras que el resistoma del suelo se asocia a mecanismos de resistencia naturales (Sengupta et al., 2013). En el Capítulo 4 se realizaron entrevistas a ganaderos de cinco explotaciones de vacuno de leche de la CAPV. En estas entrevistas, nos informaron sobre el

uso de antibióticos (nombre comercial del antibiótico, motivo del uso del antibiótico, dosis, fecha de administración, etc.) realizado en las fechas previas a nuestros muestreos (ver Capítulo 4). En general, los antibióticos específicos administrados en esas fechas al ganado, de acuerdo con la información proporcionada por los ganaderos, no tuvo el reflejo esperado, en términos de las resistencias concretas detectadas en nuestro estudio, en el perfil del resistoma observado en ninguno de los compartimentos ambientales evaluados. Por ejemplo, los principales ARGs asociados a elementos genéticos móviles (MGEs) en muestras de deyecciones animales estaban relacionados con la resistencia a aminoglucósidos, mientras que el uso de aminoglucósidos no fue común en ninguna de las explotaciones evaluadas según la información reportada.

Asimismo, en el Capítulo 7 no observamos ninguna relación entre los antibióticos detectados en las enmiendas orgánicas y su perfil del resistoma, lo cual puede ser debido, al menos parcialmente, a la persistencia de los ARGs en dichas enmiendas en ausencia de los antibióticos específicos vinculados a su emergencia y diseminación (léase, con posterioridad a la degradación de los antibióticos en dichas matrices). En este sentido, de los 57 antibióticos analizados en un primer análisis, solo se detectaron colistina y marbofloxacina. Ulteriormente, a los dos meses del primer análisis, únicamente se detectó colistina a una concentración de 117 $\mu g\ kg^{-1}$ en el estiércol fresco procedente de la granja convencional. Estos resultados ponen de manifiesto la rápida degradación de algunos antibióticos en las enmiendas orgánicas. La vida media de un antibiótico se define como "el tiempo que tarda la concentración de un antibiótico en disminuir a la mitad de su valor inicial" (Paterson et al., 2016). Esta métrica puede explicar la variación en la persistencia de los antibióticos en el medioambiente en función del tipo y del compartimento ambiental. Por ejemplo, los aminoglucósidos, β-lactámicos, macrólidos y sulfamidas muestran una vida media en el estiércol < 30 días, mientras que las fluoroquinolonas y tetraciclinas presentan una vida media de 100 días (Boxall et al., 2006).

En todo caso, las comunidades bacterianas pueden cooperar para sobrevivir a la exposición a los antibióticos mediante interacciones de resistencia colectiva, tolerancia colectiva y de protección a la exposición (Bottery et al., 2021). En la *resistencia colectiva*, las comunidades bacterianas son capaces de sobrevivir a una exposición de antibiótico que sería letal a nivel de células individuales, aumentando la concentración mínima inhibitoria (MIC) de la comunidad e incrementando la capacidad de sus miembros para resistir la acción del antibiótico. La formación de biofilms y la secreción de moléculas de señalización desencadenan el aumento del nivel de resistencia al aumentar la expresión de determinados genes de resistencia (Bottery et al., 2021). La *tolerancia colectiva* viene dada, entre

otros factores, por la ralentización del metabolismo celular, la muerte celular y la sucesiva liberación de determinantes de resistencia beneficiosas para las bacterias supervivientes mediada por antibióticos y la producción de moléculas de señalización (Meredith et al., 2015). En este caso, la MIC de la comunidad no aumenta. Por último, la *protección a la exposición* consiste en reducir la concentración del antibiótico y proporcionar así protección a las especies sensibles cercanas (Vega & Gore, 2014). Las tres interacciones específicas de supervivencia a la exposición de antibióticos requieren de una población con una densidad suficientemente alta que pueda sobrevivir a una dosis de exposición de antibiótico que sería letal para una población de baja densidad. Estas interacciones pueden darse mediante la formación de biofilms, proporcionando protección frente a la exposición de antibióticos, limitando su difusión e inactivándolos (Singh et al., 2017). Además, los biofilms pueden incrementar los niveles de resistencia al aumentar la expresión de los mecanismos de resistencia individuales que ofrezcan protección a toda la comunidad (Høiby et al., 2010). Por último, en el centro de los biofilms se reduce la actividad metabólica debido a la limitación de nutrientes y oxígeno, lo que puede inducir estados celulares tolerantes.

En relación con este tema, algunos estudios basados en métodos cultivables como la MIC han considerado que la AMR se genera cuando las bacterias cultivables del suelo crecen a una concentración de antibiótico de 20 mg L^{-1} en el medio de cultivo (D'Costa et al., 2006; Bhullar et al., 2012). En nuestro caso, en el Capítulo 6 se observó un aumento significativo de la tolerancia de la comunidad bacteriana cultivable, a partir de 20 mg kg^{-1} suelo de exposición a la oxitetraciclina (OTC), en la fase de selección del método PICT de inducción de la tolerancia a nivel de comunidad. El umbral de exposición del antibiótico en el medio de cultivo es diferente al umbral de exposición necesario en el suelo para seleccionar las bacterias resistentes, debido en parte a los procesos de degradación y adsorción que condicionan la biodisponibilidad y la persistencia del antibiótico (Pan & Chu, 2016). En este estudio, la presión selectiva generada por la aplicación repetida de OTC (tres eventos de contaminación artificial, uno cada 10 días) fomentó la adaptación de las comunidades bacterianas cultivables. En consecuencia, se podría recomendar limitar la presencia de OTC por debajo de 20 mg kg^{-1} en las enmiendas antes de aplicarlas al suelo para evitar un aumento en la tolerancia de las comunidades microbianas edáficas. La presión de selección ejercida por los antibióticos puede beneficiar a las bacterias resistentes a antibióticos, ya que desplazan a las bacterias sensibles. En ocasiones, la concentración subletal de antibiótico es suficiente para mantener los ARGs y promover la HGT (Gullberg et al., 2014). En esta línea, la seguridad medioambiental de los nuevos productos

farmacéuticos veterinarios se determina mediante la evaluación del impacto medioambiental de las directrices GL6 y GL38 (VICH, 2000; VICH, 2004; EMA, 2016). En dicha evaluación, si la concentración prevista en el suelo supera los 100 µg kg^{-1}, se precisa una evaluación más detallada de los efectos ambientales que consisten en dos estudios sobre la toxicidad microbiana (VICH, 2000; VICH, 2004; EMA, 2016). Además, muchos productos farmacéuticos existentes no han sido sometidos a una reevaluación desde la entrada en vigor de la directriz vigente (Kools et al., 2008). En cuanto a la técnica PICT, aunque la evaluación de la tolerancia a una sustancia química permite ciertamente inferir posibles relaciones causales entre la exposición y los efectos biológicos, sin embargo, no proporciona información acerca de los mecanismos de tolerancia, los genes de resistencia y diseminación implicados, ni sobre la estructura de las comunidades bacterianas. En este sentido, su combinación con métodos moleculares ofrecería un análisis más completo.

En este trabajo se ha empleado la técnica de PCR cuantitativa de alto rendimiento (HT-qPCR) para cuantificar el resistoma y el moviloma en el sistema enmienda-suelo-planta (Capítulo 7), en el sistema enmienda-suelo (Capítulo 8) y en el sistema suelo-planta (Capítulo 9). En el Capítulo 7, los suelos de lechuga enmendados con estiércol envejecido procedente de ganadería ecológica o purín procedente de ganadería convencional mostraron mayor potencial de movilidad del resistoma (*i.e.*, mayor riesgo de diseminación de resistencias) que el resto de los tratamientos aplicados, con valores superiores de abundancias relativas de MGEs *versus* abundancias relativas de ARGs. Las lechugas fertilizadas con estiércol envejecido procedente de ganadería ecológica mostraron la misma tendencia. Por su parte, tanto los suelos cultivados con trigo como los granos de este cereal mostraron valores superiores de abundancias relativas de ARGs *versus* abundancias relativas de MGEs. En el Capítulo 8 se cuantificó el mismo conjunto de genes en el lodo de depuradora anaeróbicamente digerido y en los suelos enmendados o no con este lodo. En ambos compartimentos ambientales (*i.e.*, lodo y suelos), las abundancias relativas de los MGEs fueron superiores a las abundancias relativas de ARGs. Por último, en el Capítulo 9 se empleó un conjunto de genes y un equipo-técnica de cuantificación diferente para la evaluación del efecto de los tratamientos aplicados sobre el resistoma y moviloma. Aun así, se observó la misma tendencia en los suelos y en las plantas de lechuga en términos de magnitud estimada del resistoma y moviloma: en promedio, la abundancia relativa de MGEs fue mayor que la de los ARGs. Además, en los Capítulos 7 y 8 se empleó un valor de umbral de corte del ciclo (C_T) superior al empleado en el Capítulo 9 (31 *vs.* 27). En relación con esto, Stedtfeld et al. (2018) observaron que el valor de C_T de 31 sobrestimaba la abundancia relativa de los ARGs hasta 10 veces. En este trabajo,

calculando las abundancias con un C_T de 27 y 31, respectivamente, las abundancias relativas de los ARGs fueron superiores en suelos enmendados con compost (Capítulo 9) que en suelos enmendados y no enmendados con lodos de depuradora (Capítulo 8). En cambio, el promedio de las abundancias relativas de los MGEs obtenido mediante HT-qPCR fue superior en los suelos enmendados con lodos de depuradora *versus* compost con un C_T de 31 y 27, respectivamente.

En ambos ensayos realizados a escala microcosmos con el fin de evaluar el efecto de la aplicación de enmiendas orgánicas de origen vacuno sobre el resistoma en el sistema suelo-planta (Capítulos 7 y 9), se cuantificaron las abundancias absolutas de los ARGs y MGEs mediante diferentes métodos. En el Capítulo 7, las abundancias relativas obtenidas mediante HT-qPCR fueron convertidas en valores de abundancias absolutas basándonos en la cuantificación absoluta del gen 16S rRNA. En cambio, en el Capítulo 9 las abundancias se obtuvieron vía PCR digital en gotas (ddPCR). Por ello, no es sorprendente que, como consecuencia del empleo de diferentes métodos de cuantificación en los Capítulos 7 y 9, los resultados alcanzados fueron asimismo diferentes. En este sentido, las medias de los valores de abundancias absolutas de ARGs y MGEs de los suelos enmendados fueron varios órdenes de magnitud superiores (3-5 y 5-6 para los ARGs y MGEs, respectivamente) en el Capítulo *versus* el Capítulo 9. Igualmente, en las plantas de lechuga se mantuvo la diferencia observada en los suelos enmendados, siendo las medias de las abundancias absolutas de ARGs y MGEs entre 3 y 5 órdenes de magnitud superiores en el Capítulo 7 que en el Capítulo 9. Por un lado, los cálculos llevados a cabo en el Capítulo 7 para obtener las abundancias absolutas de los ARGs y MGEs pudieron introducir sesgos en la cuantificación (Crossette et al., 2021). Especialmente para aquellas muestras que presentan una baja concentración de genes, la ddPCR es una técnica más eficaz que la qPCR, lo que permite evaluar con mayor fiabilidad la diseminación de ARGs y MGEs en suelos tratados con enmiendas orgánicas (Cavé et al., 2016). Por el contrario, la técnica HT-qPCR proporciona la posibilidad de analizar simultáneamente un número elevado de genes (hasta 384 genes en el caso de nuestro sistema HT-qPCR). Por ello, según la pregunta concreta objeto de la investigación, será necesario optar por una de estas dos técnicas o, en su caso, combinarlas (y con otras técnicas disponibles en el mercado) para una cuantificación lo más precisa y exhaustiva posible de la magnitud del resistoma y moviloma.

Las estaciones depuradoras de aguas residuales (EDAR) se consideran importantes reservorios de ARGs, MGEs y bacterias resistentes a antibióticos (ARB), ya que los residuos de antibióticos que entran en las EDAR ejercen una fuerte presión selectiva sobre las comunidades bacterianas presentes

en dichas instalaciones. Además, se ha observado que los ARGs y ARB son mucho más abundantes en los lodos de depuradora que en las aguas residuales que conforman el efluente de las EDAR (Calero-Cáceres et al., 2014). En este trabajo, en el Capítulo 8, observamos un incremento de la magnitud del resistoma y moviloma en los suelos enmendados con lodos de depuradora. Cuando se aplican lodos de depuradora a los suelos agrícolas, el aporte de ARGs y ARB presentes en los propios lodos, junto con la estimulación del crecimiento y actividad de las poblaciones bacterianas edáficas derivada de la entrada de materia orgánica y nutrientes fácilmente asimilables, contribuyen a la potenciación del resistoma intrínseco del suelo, hecho que se observó con más claridad en aquellos suelos en los que la aplicación de lodos era más reciente. En este sentido, el tiempo transcurrido desde la última aplicación de los lodos (se muestrearon suelos en los que el tiempo transcurrido desde la última aplicación de los lodos era de 1, 2, 3 o 4 años) fue un parámetro más relevante que la dosis (22, 33 o 44 t ha^{-1}) o la dosificación (*i.e.*, cantidad total de lodos aplicada: 22, 55, 77 o 99 t ha^{-1}) de aplicación.

Como se ha mencionado anteriormente, la exposición de bacterias ambientales a concentraciones subinhibitorias de antibióticos favorece la selección de resistencias y su diseminación mediante eventos de transferencia horizontal de genes (HGT). La HGT promueve intercambios genéticos entre cepas y especies bacterianas, aumentando así el potencial del riesgo del resistoma en nuevos huéspedes y, de forma concomitante, la diseminación de este problema. A este respecto, los MGEs son módulos genéticos que intervienen en la evolución y adaptación de las bacterias, desempeñando un papel crucial en la emergencia y diseminación de la resistencia a los antibióticos, al promover la movilidad del DNA en los entornos intra y extracelular. Por ello, el seguimiento de los genes asociados a los MGEs y HGT es clave para la monitorización de la AMR. En los últimos años se ha propuesto la cuantificación de la abundancia relativa del gen de la integrasa *intl1* como un biomarcador de nivel de contaminación antrópico (Gillings et al., 2015). En el análisis de modelos de ecuaciones estructurales (SEM) del Capítulo 8 observamos que el predictor principal de la composición y abundancia de los ARGs en los suelos fueron los MGEs. No es sorprendente que los suelos enmendados con lodos de depuradora, enmiendas de origen urbano que habitualmente contienen numerosos contaminantes emergentes, tengan una mayor abundancia de MGEs que los suelos prístinos, ya que estos elementos genéticos contribuyen a la supervivencia y adaptabilidad de las bacterias. El aporte a los suelos de los MGEs presentes en los propios lodos de depuradora, junto con el aumento de los MGEs presentes intrínsecamente en las bacterias nativas del suelo derivado de la aplicación de dichos lodos (a través de su efecto estimulador del crecimiento bacteriano), se refleja habitualmente en

un incremento en la magnitud del resistoma de los suelos. De forma similar, los modelos de regresión del Capítulo 9 mostraron que la abundancia relativa de MGEs estaba correlacionada de forma lineal y positiva con la abundancia relativa de ARGs, siendo la correlación más fuerte en las muestras de suelo enmendadas con compost de vaca que en las de plantas cultivadas en esos mismos suelos. En este sentido, es esencial considerar el importante papel que desempeñan los MGEs en la diseminación de ARGs, ya que el control de estos "impulsores genéticos de la transmisión de genes" es clave para mitigar la creciente expansión del resistoma ambiental. En el Capítulo 4, los genes del moviloma asociados a ARGs disminuyeron hasta desaparecer a lo largo de la vía de exposición "deyecciones-enmiendas-suelos-plantas" en los sistemas agroganaderos estudiados. En consecuencia, el riesgo del resistoma disminuyó a lo largo de esta vía de exposición y las plantas mostraron los menores valores de riesgo entre todos los compartimentos ambientales muestreados.

En otro orden de cosas, los ecosistemas que presentan una elevada biodiversidad son a *priori* más estables y, por consiguiente, menos vulnerables a perturbaciones que los ecosistemas con menor biodiversidad (Delgado-Baquerizo et al., 2016). La combinación de la composición, distribución y disponibilidad de los nutrientes conforma, en parte, el hábitat funcional de una determinada población. En consecuencia, la competencia por los nutrientes es un factor modulador de las invasiones biológicas. Las especies invasoras pueden ser más competitivas que las autóctonas si son más eficientes extrayendo recursos. De forma similar, el perfil del resistoma en el origen de la vía de exposición (deyecciones animales y humanas) puede emplearse, con la requerida precaución, como un indicador del riesgo potencial de invasión de ARGs en el entorno agroganadero a través de los diversos compartimentos ambientales de la citada vía de exposición. Por otro lado, el suelo es el compartimento ambiental con mayor biodiversidad del planeta (Wall et al., 2012). Por lo tanto, este compartimento se convierte en un punto crítico donde los antibióticos, las bacterias resistentes a antibióticos (ARBs) y el resistoma proveniente de las enmiendas orgánicas interaccionan con la enorme diversidad bacteriana autóctona. En este sentido, la diversidad bacteriana edáfica representa un factor clave que regula el éxito en que los microorganismos invasores puedan establecerse (van Elsas et al., 2012) Para que la invasión tenga éxito se tienen que establecer poblaciones de especies no autóctonas suficientes, y además las características de los suelos (contenido de materia orgánica, gradientes de nutrientes, estructura espacial de las comunidades bacterianas, etc.) determinan la presencia potencial de nichos ecológicos disponibles (Mallon et al., 2018; Castledine et al., 2020; Billet et al., 2022). A pesar de que el microbioma de las deyecciones animales es menos competitivo en el entorno edáfico que en el intestino

de los animales, las bacterias presentes en las enmiendas orgánicas pueden colonizar los suelos al aplicarlas a los mismos (Chee-Sandford et al., 2009; Billet et al, 2022). Por lo tanto, es crucial mantener y aumentar la diversidad microbiana de los ecosistemas. Asimismo, la degradación y disminución de la biodiversidad del suelo pueden hacer que los suelos se conviertan en ecosistemas más vulnerables ante alteraciones ambientales, perdiendo su potencial de resistencia y resiliencia. Este escenario podría generar una mayor emergencia y diseminación de resistencias a antibióticos.

Por lo expuesto anteriormente, en todos los capítulos de este trabajo se ha evaluado simultáneamente tanto el resistoma como la composición procariota de los suelos objeto de investigación. En el Capítulo 7, en presencia de las plantas de lechuga, los valores menores de diversidad de Shannon y riqueza bacteriana del suelo no enmendado que los suelos enmendados (excepto los suelos enmendados con estiércol envejecido de la ganadería ecológica) se relacionaron con una mayor abundancia absoluta de resistencias a la vancomicina. De manera similar, los suelos estudiados en el Capítulo 4 mostraron mayor diversidad bacteriana y menor diversidad en el perfil del resistoma que las deyecciones y las enmiendas orgánicas. Por otro lado, en el Capítulo 8, el 66% de la variación de las abundancias relativas de los ARGs y MGEs en los suelos enmendados y no enmendados con lodos de depuradora se explicó por la composición de las 30 familias de bacterias más abundantes. Cabe destacar que 30 familias mostraron correlación con al menos dos genes que confieren resistencia a diferentes clases de antibióticos. Además, 12 de estas familias multirresistentes fueron más abundantes en los suelos enmendados con lodos de depuradora que en los no enmendados. Es decir, la aplicación de lodos de depuradora puede incrementar el número de taxones que alberguen múltiples ARGs. Estos resultados sugieren que la diversidad y composición del microbioma condicionan en gran medida el perfil del resistoma, de acuerdo con numerosos estudios (Forsberg et al., 2014; Chen et al., 2018; Bottery et al., 2022).

Para el estudio de la diversidad y composición bacteriana, el método de la secuenciación de amplicones del gen 16S rRNA está muy extendido. El 16S rRNA es un gen universal altamente conservado y su secuencia se ha caracterizado completamente en numerosos organismos (Janda & Abbott, 2007). El análisis bioinformático empleado en los Capítulos 7 y 8 se basó en agrupar las secuencias en unidades taxonómicas operativas (OTUs). Esta agrupación, basada en una similitud del 97% entre las secuencias, reduce el tamaño del conjunto de datos a la hora de analizarlos. En el Capítulo 7, con la metodología basada en la clasificación taxonómica por OTUs, el 73, 53 y 22% de las lecturas de suelos cultivados con lechuga, y el 68, 51 y 20% de las lecturas de suelos cultivados con trigo se

clasificaron en los niveles de orden, familia y género, respectivamente. En el Capítulo 8 los porcentajes de OTUs clasificados fueron superiores: 79, 67 y 35% en los niveles de orden, familia y género, respectivamente. En cambio, en el Capítulo 9, la agrupación se llevó a cabo en variantes de secuencias de amplicones (ASVs). Las ASVs hacen referencia a secuencias de DNA únicas y distinguen cambios de un único nucleótido (Callahan et al., 2017). De esta forma, las ASVs pueden proporcionar una clasificación más precisa y comparable entre distintos estudios. En el Capítulo 9, se consiguieron clasificar el 93, 84 y 47% de las lecturas en los niveles de orden, familia y género, respectivamente. A pesar de que la clasificación taxonómica fue más detallada al emplear ASVs frente a OTUs, el rango de valores del índice de diversidad de Shannon fue menor en los suelos estudiados en el Capítulo 9 (3.9–4.1) que en los Capítulos 7 (6.8–7.1) y 8 (6.5–7.4). Cabe especular que las herramientas bioinformáticas empleadas agruparan en ASVs secuencias muy relacionadas pero pertenecientes a taxones distintos, disminuyendo artificialmente la diversidad Shannon de las muestras (Prodan et al., 2020).

Análogamente, un aspecto relevante en la temática que nos ocupa es la posible contribución de otros factores ambientales al aumento de la abundancia de ARGs mediante mecanismos de co-selección. La selección simultánea de resistencias a antibióticos y metales pesados en ecosistemas contaminados por dichos metales se ha observado durante décadas (Timoney et al., 1978; Seiler & Berendonk, 2012; Dickinson et al., 2019; Wang et al., 2021a). Esto ocurre a través de procesos de co-resistencia (distintos determinantes de resistencia a metales y antibióticos presentes en el mismo elemento genético), resistencia cruzada (el mismo determinante confiere resistencia a metales y antibióticos) y resistencia co-regulatoria (la exposición a un contaminante puede inducir la resistencia a otro contaminante por la respuesta reguladora indirecta) (Baker-Austin et al., 2006). En este sentido, el principal mecanismo subyacente a la presencia de resistencias bacterianas es la actividad de bombas de expulsión activa que pueden regular la presencia intracelular tanto de metales pesados como de antibióticos. Así, aunque sólo esté presente un agente co-seleccionador (el antibiótico o el metal pesado), la resistencia puede ser simultáneamente conferida frente a ambos agentes. Dado que, entre otras actividades humanas, la industria y la agricultura han contribuido y contribuyen a la contaminación de los ecosistemas con metales pesados, sumado al hecho de que estos elementos metálicos no puede ser degradados y persisten, por ello, en los ecosistemas durante periodos de tiempo extremadamente largos, la co-selección entre las resistencias a estos contaminantes metálicos y a los antibióticos representa un riesgo sustancial que debe ser urgentemente abordado bajo la perspectiva

One Health. En este trabajo, los niveles de metales biodisponibles en los suelos del Capítulo 8 estaban por debajo del límite de cuantificación. A este respecto, la concentración biodisponible de los metales se considera más relevante que la concentración total en términos de riesgo medioambiental (Olaniran et al., 2013). Sin embargo, la aplicación de lodos aumentó significativamente el contenido de zinc en los suelos. Además, la concentración de cobre fue el parámetro fisicoquímico que explicaba en mayor medida la variación en la abundancia relativa de ARGs y MGEs. El cobre se correlacionó sobre todo con los genes de resistencia a las tetraciclinas.

Además de los metales pesados, se ha observado que la contaminación con determinados herbicidas, hidrocarburos aromáticos policíclicos y microplásticos también puede generar y alterar la AMR (Chen et al., 2017b; Comont et al., 2020; Liao et al., 2021). Algunos herbicidas, como el glifosato, pueden alterar la resistencia bacteriana, al seleccionar mutaciones asociadas con la desintoxicación de xenobióticos y mecanismos de resistencia a los antibióticos (Cummins et al., 2013; Liao et al., 2021). A pesar de que este tipo de compuestos son específicos para las plantas, pueden asimismo provocar efectos adversos en otros organismos (*i.e.*, en organismos no-diana) (Liao et al., 2021). Este fenómeno puede deberse a un aumento de la actividad de las bombas de expulsión activa, cambios en los genes diana de los antibióticos o su degradación enzimática (Suzuki et al., 2014; Hall & Mah, 2017; Zampieri et al., 2017). Además, los herbicidas pueden aumentar la permeabilidad de las membranas celulares y promover la HGT mediante plásmidos (Liao et al., 2021). Por otro lado, la contaminación por microplásticos (residuos plásticos de tamaño inferior a 5 mm) es actualmente motivo de gran preocupación debido a los riesgos que conlleva para la salud de los ecosistemas y la salud humana (Campanale et al., 2020). Entre otras fuentes, los microplásticos pueden llegar al suelo agrícola por degradación de los plásticos que se emplean para el acolchado, el uso de fertilizantes orgánicos de calidad mejorable, la deposición atmosférica y el riego con aguas residuales (Weithmann et al., 2018; Evangeliou et al., 2020; Jiang et al., 2020; Li et al., 2020). Por ejemplo, se detectaron entre 510 y 495 mil partículas de microplástico por kg de peso seco de lodos de depuradora (Hatinoğlu & Sanin, 2021), y se estimó que la aplicación de lodos de depuradora incorpora anualmente 26,042 toneladas de microplásticos al suelo en Europa (Mohajerani & Karabatak, 2020). En esta línea, en cultivos como el brócoli, zanahoria y lechuga se cuantificaron 126,150, 50,550 y 101,950 partículas de microplástico por gramo de vegetal (Oliveri Conti et al., 2020). Los microplásticos (y muchos otros componentes de la plastisfera) representan nuevos nichos ecológicos para la formación de biofilms, estructuras biológicas muy vinculadas a las resistencias a los antibióticos y a la transferencia horizontal

de genes (Zettler et al., 2013). De esta forma, los microplásticos actúan como vectores de la diseminación de ARGs, dado que los biofilms que se forman en su superficie aportan condiciones favorables para la conjugación vía plásmidos conjugativos (Eckert et al., 2018). Las interacciones de los contaminantes mencionados (antibióticos, metales pesados, herbicidas, microplásticos) pueden modificar la emergencia y la diseminación de la resistencia a antibióticos en el medioambiente, pero hasta ahora apenas se ha investigado su impacto (excepto en el caso de los metales).

Sea como fuere, no hemos querido limitar este trabajo a un diagnóstico de la emergencia y diseminación ambiental de AMR como consecuencia de la aplicación de enmiendas orgánicas de origen animal o humano. También hemos considerado importante tratar de aportar soluciones de cara a reducir este riesgo. Una de las estrategias podría ser el fomentar la ganadería ecológica frente a la convencional. En el Capítulo 7 se estudió el posible impacto del uso de antibióticos en ganadería convencional frente a su limitado uso en ganadería ecológica sobre el resistoma ambiental a lo largo de la vía de exposición enmienda orgánica-suelo-planta. La producción ecológica está regulada a nivel europeo y se define como "…un sistema general de gestión agrícola y producción de alimentos que combina las mejores prácticas en materia de medio ambiente y clima, un elevado nivel de biodiversidad, la conservación de los recursos naturales y la aplicación de normas exigentes sobre bienestar animal y sobre producción…" (Reglamento 2018/848). Respecto a la ganadería ecológica, se especifica la profilaxis como la principal gestión zoosanitaria, complementada con determinadas medidas de limpieza y desinfección. En ganadería ecológica se pueden utilizar antibióticos en condiciones estrictas y para evitar el sufrimiento de los animales. En cualquier caso, cuando un animal reciba más de tres tandas de tratamiento con antibióticos en un plazo de 12 meses, ni los animales tratados ni los productos derivados de ellos podrán venderse como productos ecológicos. En Europa, tanto para la ganadería convencional como para la ecológica, se prohibió el uso de antibióticos con fines de engorde en 2006 (Reglamento 1831/2003). En este trabajo, no se obtuvo un patrón diferenciador del resistoma en las enmiendas de origen convencional *versus* las de origen ecológico (Capítulo 7). De hecho, entre las enmiendas objeto de estudio, fue el tipo de enmienda, no su origen, la variable que marcó diferencias significativas, siendo el purín (tanto de origen convencional como ecológico) la enmienda con una mayor abundancia de ARGs y MGEs (resistencia a aminoglucósidos, MLSB, multidrogas y tetraciclinas; transposasas), en comparación con el estiércol fresco o envejecido. Otros factores de producción como la densidad de la población de animales en la explotación, la higiene

y el manejo de los animales también pueden influir en la relación entre el uso de antibióticos y el resistoma (Noyes et al., 2016).

En este mismo orden de ideas, para mitigar el citado riesgo, es fundamental minimizar la presencia de residuos de antibióticos, ARGs, MGEs y bacterias potencialmente patógenas en las enmiendas orgánicas, ya que son un importante punto de entrada del resistoma al medio ambiente agrario. El compostaje es un proceso controlado de descomposición de la materia orgánica en condiciones aeróbicas que da lugar a un producto agrícola estable denominado compost. Los principales objetivos del compostaje son disminuir el volumen de las enmiendas orgánicas, minimizar la cantidad de semillas en ellas presente, higienizarlas de cara a eliminar potenciales patógenos y, finalmente, estabilizar los sustratos orgánicos. Sin embargo, el compostaje es un proceso que puede ser empleado para reducir el riesgo del resistoma, como consecuencia principalmente de su capacidad para la higienización. Un proceso de compostaje habitual se desarrolla en tres fases según la temperatura (Storey et al., 2015; Fu et al., 2021): la primera *fase mesófila* (< 45 ºC) se inicia a temperatura ambiente y va aumentando debido a la actividad de los microorganismos. En esta fase se produce calor, se libera CO_2 y disminuye el pH (Sundberg et al., 2004). En la *fase termófila* (> 45 ºC), la segunda fase del compostaje, la temperatura sube hasta alcanzar valores de 70 ºC por la acción de la fermentación. En esta fase se destruyen los patógenos, se inhibe la germinación de semillas, se libera amoníaco y el pH asciende. La última fase, denominada *fase de maduración*, se alarga durante meses a temperatura ambiente. Aunque en muchas explotaciones agroganaderas es habitual apilar las deyecciones animales durante muchos meses e incluso años antes de su uso como enmiendas orgánicas, este proceso de "almacenamiento-apilamiento previo a su empleo" no es un verdadero compostaje (no obstante, es frecuente referirse a estas deyecciones apiladas, de forma errónea, como composts). El auténtico compost de estiércol es un producto higienizado y estabilizado, obtenido mediante descomposición biológica aeróbica (incluyendo la fase termófila) bajo condiciones controladas (Real Decreto 999/2017). Además, el compost de estiércol debe cumplir las siguientes características: contenido mínimo de materia orgánica total del 35%, humedad máxima del 40%, relación C/N < 20 y no contener impurezas ni inertes de ningún tipo tales como piedras, gravas, metales, vidrios o plásticos. En muchas explotaciones no se alcanzan las citadas condiciones del proceso de compostaje, ni las características propias del producto.

A pesar de que el compostaje representa uno de los procesos de gestión de deyecciones animales más comunes en los sistemas agroganaderos, algunos estudios sugieren que el compostaje

convencional no reduce eficazmente las abundancias de los ARGs ni los MGEs (Zhu et al., 2013; Su et al., 2015; Wang et al., 2015a). La reducción de los ARGs se suele observar a corto plazo, pero suelen repuntar tras finalizar el proceso de compostaje, debido a los cambios en la composición de la comunidad bacteriana y los eventos de HGT en la fase de maduración (Zhang et al., 2016; Guo et al., 2019). Una alternativa prometedora es el compostaje aeróbico hipertermofílico, donde en la fase de fermentación se alcanzan temperaturas superiores a 90 ºC (Oshima & Moriya, 2008). Liao et al. (2018) observaron que la reducción de ARGs y MGEs durante el compostaje hipertermofílico de los lodos de depuradora fue mayor (89%) que durante el compostaje convencional (49%). Además, constataron que la reducción de MGEs desempeñó un papel crucial en la eliminación de ARGs durante el compostaje hipertermofílico. No obstante, existen limitaciones prácticas y logísticas para poder realizar estos procesos de compostaje hipertermofílico en las propias explotaciones ganaderas, lo que implica el traslado de las deyecciones animales a instalaciones preparadas a este efecto con el consiguiente incremento de costes y huella ambiental. Algunos tratamientos de compostaje a temperaturas termófilas emplean aditivos (*e.g.*, biochar, arcilla, zeolita, etc.) para tratar de aumentar la tasa de eliminación de los ARGs, a través de la reducción de la biodisponibilidad de los metales pesados (con la consiguiente disminución de la presión selectiva vía co-selección) o alterando la composición de las comunidades microbianas (Cui et al., 2016; Awasthi et al., 2019).

En este trabajo, en el Capítulo 8, los lodos de depuradora se sometieron a un tratamiento de digestión anaerobia y a una posterior deshidratación antes de ser aplicados al suelo. Tras estos tratamientos, los lodos digeridos y deshidratados cumplían con los requisitos legales respecto a los niveles de metales pesados y patógenos humanos (European Commision, 1986; Orden AAA/1072/2013). Aun así, pudimos comprobar que no fue un proceso efectivo en relación con la eliminación de ARGs y MGEs. Estos resultados ponen de manifiesto la necesidad de desarrollar e implementar tratamientos más efectivos en las EDAR con el fin de garantizar una calidad óptima de los lodos de depuradora como enmiendas orgánicas, minimizando el riesgo asociado al resistoma y moviloma. Por otro lado, en el Capítulo 9 se propusieron una serie de procesos de manejo aplicables a enmiendas orgánicas de sistemas agroganaderos (*i.e.*, purín, estiércol y compost). En general, la digestión anaerobia y la adición de biochar resultaron eficaces para reducir la abundancia de ARGs y MGEs en los tres tipos de enmiendas testadas. Posteriormente, en un ensayo en microcosmos, el compost fue sometido a varios tratamientos y, posteriormente, aplicado al suelo. La digestión anaerobia redujo la abundancia de los genes *sul2* e *intl1* en un 38 y 83%, respectivamente, mientras que la adición

de biochar redujo en un 60% la abundancia de *intl1*, en comparación con el suelo enmendado con el compost no tratado. Es necesario realizar más estudios antes de aplicar estos tratamientos a gran escala. Lo ideal sería que el tratamiento o la combinación de tratamientos fueran baratos y factibles de aplicar en las propias explotaciones ganaderas.

Otra posible alternativa para la mitigación del resistoma en el suelo es la utilización de lombrices (Huang et al., 2018; Zhu et al., 2021). El alimento que ingieren las lombrices (estos animales ingieren partículas de suelo y digieren los restos orgánicos) se somete a una variedad de reacciones físico-bioquímicas en el intestino, provocando cambios relevantes en los perfiles de composición microbiana del sustrato digerido (Domínguez et al., 2017). De esta forma, a través de cambios en la composición de las especies bacterianas, las lombrices tienen el potencial de reducir los ARGs móviles y clínicamente relevantes del suelo (Zhu et al., 2021). Análogamente, antes de añadir las enmiendas orgánicas al suelo, si se les somete a un proceso de vermicompost, las lombrices pueden reducir el resistoma y el moviloma del residuo orgánico, reduciendo las bacterias hospedadoras que albergan los ARGs (Huang et al., 2018). En este contexto, cabe destacar que aproximadamente el 90% del horizonte superficial de los suelos puede ser consumido por las lombrices en un periodo de 40 años (Drake & Horn, 2007; Pass et al., 2014). Por otra parte, dada la amplia distribución de estos animales en los suelos de nuestro planeta, la reducción del resistoma mediante la utilización de lombrices podría suponer una aportación significativa a nivel global.

En definitiva, desde el punto de vista de la exposición humana, los resultados obtenidos en este trabajo son positivos, ya que se observó una disminución del riesgo a lo largo de la vía de exposición estudiada, *i.e.* deyecciones animales-suelo-cultivo agrícola-humanos. Sin embargo, bajo la perspectiva de *One Health* [Una sola salud (Alianza Tripartita & PNUMA, 2021)], no debemos olvidar que algunos de los compartimentos ambientales estudiados (*i.e.*, las deyecciones animales-enmiendas) mostraron un riesgo elevado. En relación con esto, el concepto EcoHealth (EcoSalud) ofrece un enfoque sistémico al igual que el de *One Health*, pero está menos orientado a la relación estricta entre salud humana y salud animal (Zinsstag, 2012). El enfoque EcoHealth pretende lograr una visión más amplia de la salud y el bienestar a través de disciplinas de las ciencias naturales, sociales y de la salud (Charron, 2012). Así, el EcoHealth podría favorecer el desarrollo de enfoques ecosistémicos en el contexto *One Health*, ya que reclama que los propios ecosistemas deben estar sanos y ser diversos para la supervivencia humana.

Actualmente es extremadamente complejo identificar y, sobre todo, dimensionar el riesgo del resistoma derivado de las abundancias y la diversidad-composición de los ARGs para la salud humana. Para ello, se deberían establecer los niveles de base de concentración de antibióticos, y de la magnitud y naturaleza del resistoma y del moviloma, en cada compartimento ambiental, con el fin de establecer niveles de referencia. Un metaanálisis sobre la distribución de los ARGs en el medioambiente reveló que la abundancia típica de la mayoría de ARGs se situaba en un intervalo de entre 10^{-5} y 10^{-3} copias de ARGs por cada copia de 16S rRNA, que corresponde aproximadamente a una copia de ARG en mil bacterias (Abramova et al., 2022). A pesar de que estos valores no se deben considerar como un límite máximo aceptable, indican el rango de referencia de abundancias de ARGs en el medioambiente. En este trabajo, los suelos de los Capítulos 4 y 7 que no recibieron ningún tipo de fertilización no mostraron unos niveles de resistoma y moviloma significativamente menores que los suelos fertilizados con enmiendas orgánicas. En cambio, los suelos fertilizados con enmiendas orgánicas de origen urbano (Capítulo 8) y animal (Capítulo 9) revelaron una mayor magnitud del resistoma y moviloma que los suelos que recibieron una fertilización mineral, poniendo de manifiesto el incremento de ARGs y MGEs como consecuencia de la aplicación de las enmiendas orgánicas. Aun así, un aumento del resistoma en el medioambiente debido a las actividades antrópicas no indica necesariamente una amenaza inmediata ni directa para la salud humana: los ARGs representan un riesgo para la salud humana sólo cuando se expresan en potenciales patógenos humanos.

Existen algunas discrepancias a la hora de evaluar el riesgo asociado a las AMR en el medioambiente, sobre todo por la falta de estandarización de los métodos y un marco legal específico (Bengtsson-Palme & Larsson, 2015). Sin embargo, hay cierto consenso a la hora de definir los objetivos de alta prioridad. La abundancia y diversidad de los ARGs detectados en una muestra determinada no son suficientes para evaluar el riesgo asociado a la resistencia a los antibióticos. Es de suma importancia considerar las vías de exposición entre el medioambiente y los seres humanos, así como la movilidad de los ARGs y la patogenicidad del huésped. En este sentido, se han propuesto varias metodologías para abordar la evaluación del riesgo. En el Capítulo 4 de este trabajo se ha empleado la herramienta bioinformática MetaCompare que proporciona un índice de riesgo basado en (i) la presencia de ARGs, (ii) su asociación con MGEs, y (iii) su asociación con patógenos humanos en los contigs (Oh et al., 2018). De forma similar, Zhang et al. (2021) proponen unos niveles de riesgo que se fundamentan en la evaluación de tres criterios: (i) enriquecimiento de ARGs en entornos asociados al ser humano, (ii) movilidad de los ARGs, y (iii) patogenicidad del huésped

(presencia/ausencia de patógenos ESKAPE). El cumplimiento de estos criterios define una jerarquía de cuatro niveles de riesgo, que van del riesgo I (el riesgo más alto, cumplen todos los criterios) al riesgo IV (el riesgo más bajo, no cumplen ningún criterio). Este planteamiento de riesgos se aplicó a 4.050 ARGs, clasificando 122, 23, 618 y 1816 en los niveles I, II, III y IV, respectivamente (un total de 1.471 ARGs no se detectaron en el conjunto de datos metagenómicos, por lo que no se pudieron clasificar). Por último, en un estudio reciente (Zhang et al., 2022) se evaluó cuantitativamente el riesgo de las resistencias a los antibióticos para la salud humana, teniendo en cuenta (i) la accesibilidad humana, (ii) la movilidad de los ARGs, (iii) la patogenicidad humana, y (iv) el uso clínico de los antibióticos.

En cualquier caso, es preciso definir un marco sistemático para el seguimiento de la AMR en el medioambiente, al objeto de determinar qué, dónde y cómo monitorizar esta creciente amenaza para la salud pública (Berendonk et al., 2015). Para una vigilancia a gran escala, la monitorización de los lodos de depuradora podría ser una actividad particularmente beneficiosa, ya que aportan una muestra compuesta e integradora de los microbiomas de muchas personas. Este sistema de monitorización actuaría como herramienta de alerta temprana ("early warning") sobre la emergencia y diseminación de resistencias importantes desde el punto de vista de la salud humana, para así poder actuar con la requerida celeridad. Recientemente, el seguimiento del SARS-CoV-2 RNA realizado en las estaciones depuradoras de aguas residuales por numerosos grupos de investigación ha potenciado el interés por este enfoque (Ahmed et al., 2020; Peccia et al., 2020; Agrawal et al., 2021). De forma similar, en las explotaciones agroganaderas se podrían muestrear regularmente las balsas de purín y las pilas de estiércol, las cuales, al contener las deyecciones de todos los animales en producción, proporcionan una información integradora de toda la explotación. La aplicación de estos enfoques a gran escala, combinados con la vigilancia tradicional de los aislados clínicos, proporcionarían una visión global de la problemática que nos ocupa.

En cuanto a los métodos analíticos, los enormes avances realizados en los últimos años, sobre todo en el campo de la biología molecular, están permitiendo ahondar en el conocimiento de la AMR. Tal y como hemos comentado en el apartado *Procedimientos Generales,* cada técnica tiene sus ventajas e inconvenientes, por lo que es preferible combinar varios métodos para obtener una visión más completa. Por ejemplo, la secuenciación metagenómica ofrece el potencial de secuenciar todo el material genético de una muestra determinada, obteniendo información valiosa sobre el contexto genético y funcional. Sin embargo, el resistoma y el moviloma representan una parte muy pequeña del

microbioma y los análisis metagenómicos tienen la limitación de mostrar una baja sensibilidad en su detección, a menos que se apliquen estrategias de secuenciación con una profundidad de lecturas suficientes a un elevado coste económico (Macedo et al., 2021). Estas limitaciones pueden superarse utilizando estrategias de secuenciación basadas en metagenómica dirigida. Este enfoque reduce el número de secuencias que hay que analizar y, por lo tanto, representan una alternativa rentable y de alto rendimiento. Aunque se utiliza sobre todo para el diagnóstico de enfermedades humanas hereditarias (Jones & Good, 2016), esta metodología ofrece un enorme potencial para impulsar los avances en los estudios medioambientales y ecológicos que requieren el aislamiento de secuencias de interés a partir de una mezcla de DNA de una comunidad compleja de organismos. Recientemente se ha desarrollado ResCap, la primera captura de secuencias dirigida desarrollada específicamente para analizar resistomas (Lanza et al., 2018). Incluye sondas para 7.963 genes de resistencia a antibióticos y 704 genes que confieren resistencia a metales o biocidas, además de 2.517 genes de relaxasa (marcadores de plásmidos). ResCap mejora en gran medida la sensibilidad y especificidad de los métodos metagenómicos disponibles y ofrece la posibilidad de analizar genes relacionados con la selección y transferencia de la AMR (biocidas, metales pesados, plásmidos). Es sin duda una estrategia muy interesante a testar en el futuro.

No obstante, la interpretación de los resultados obtenidos de distintos análisis moleculares de las comunidades bacterianas ambientales presenta limitaciones y sesgos asociados (i) al muestreo: la exposición a condiciones ambientales diferentes del hábitat natural, el tamaño muestral y número de réplicas que articulan que sea representativo el muestreo, el manejo y transporte de las muestras al laboratorio; (ii) a la extracción de DNA: los procesos físicos y químicos empleados para lisar la célula durante la extracción pueden disminuir la calidad del DNA; (iii) a las plataformas de secuenciación: las técnicas basadas en la PCR introducen sesgos a través de la elección de cebadores y en el proceso de amplificación del gen diana, los métodos metagenómicos dependen en gran medida de la profundidad de secuenciación; (iv) al análisis bioinformático: los pipeline y bases de datos empleados; y (v) al análisis estadístico: la transformación de datos y los criterios de selección, entre otros (Sipos et al., 2007; Hazen et al., 2013; Gil-Gil et al., 2021; Guseva et al., 2022).

Este tipo de técnicas generan un gran volumen de datos que requieren de herramientas de procesado adecuadas. En los últimos años, las técnicas de inteligencia artificial, entre ellas el “machine learning” y el “deep learning”, están revolucionado la mayoría de los campos del conocimiento (Jordan & Mitchell, 2015). A pesar de la creciente popularidad del uso de estos algoritmos, este ámbito sigue

siendo desconocido para la mayoría de los investigadores del campo de la ecología (microbiana) y la evolución biológica, hecho preocupante dado que estos avances están desafiando la supremacía de los enfoques estadísticos clásicos (Pichler & Hartig, 2022). Actualmente, se emplean enfoques de "deep learning" en los modelos predictivos para, por ejemplo, predecir la presencia de especies (Wilkinson et al., 2019). Las técnicas de "deep learning" pueden reconocer patrones a partir de una determinada información y entrenamiento, siendo el método de aprendizaje automático más poderoso para diversas aplicaciones (Arango-Argoty et al., 2018). En la línea de este trabajo, el modelo de "deep learning" DeepARG identifica los ARGs comparando las secuencias de DNA con las bases de datos de una forma que genera menos falsos negativos que los métodos tradicionales (Arango-Argoty et al., 2018). En este sentido, es fundamental completar las bases de datos con nuevas secuencias de ARGs.

Por último, el estrecho vínculo que hay entre la salud ambiental, humana y animal en la problemática de la AMR requiere imperiosamente la perspectiva *EcoHealth* y/o *One Health*. Siendo conscientes de los numerosos retos complejos que implica la lucha contra la AMR en el medioambiente, es importante subrayar la necesidad inaplazable de disponer de sistemas armonizados de vigilancia efectiva del riesgo del resistoma-moviloma en los diferentes compartimentos ambientales, a modo de redes de monitorización, desde escalas locales a globales. Los citados retos requieren inevitablemente un esfuerzo ímprobo en I+D+i, fuertes inversiones inaplazables y el fomento de la colaboración entre diferentes agentes. En relación con los trabajos aquí desarrollados, no debemos olvidar la criticidad de proteger y mejorar la salud de los suelos agrícolas, por ejemplo, mediante prácticas agroganaderas sostenibles y seguras que, además de la imprescindible productividad agrícola, promuevan y maximicen el suministro de servicios ecosistémicos por parte de los agroecosistemas, así como la conservación de la biodiversidad y la salud de las personas, animales y ecosistemas.

CONCLUSIONES Y TESIS

11.1. CONCLUSIONES

1. Los sistemas agroganaderos son importantes reservorios de genes de resistencia a antibióticos, elementos genéticos móviles y potenciales patógenos humanos. No obstante, en nuestro estudio, el riesgo del resistoma disminuyó a lo largo de la vía de exposición estudiada, *i.e.* "deyecciones animales - enmiendas orgánicas – suelos - cultivos". La cuantificación del riesgo aquí estimada para cada uno de los citados compartimentos ambientales es de utilidad para establecer una priorización en relación con los esfuerzos de mitigación del resistoma en entornos agroganaderos.

2. Los genes de resistencia a antibióticos *aad(6)*, *tetM* y *tetW* muestran potencial como biomarcadores de riesgo de resistencia a antibióticos en sistemas agroganaderos vinculados a explotaciones de vacuno de leche, como se deriva de su presencia en varios de los compartimentos ambientales de la citada vía de exposición así como su asociación con elementos genéticos móviles implicados en la diseminación de genes de resistencia.

3. La evaluación de la aptitud de las enmiendas orgánicas para su uso agrícola debe incorporar el análisis de los posibles riesgos asociados a la presencia de contaminantes emergentes potencialmente perjudiciales para la salud humana y/o el medioambiente, además de los beneficios vinculados a sus propiedades físico-químicas y biológicas.

4. La adición repetida de oxitetraciclina sobre suelos enmendados con estiércol de vacuno induce a corto plazo la resistencia de las comunidades bacterianas edáficas a dicho antibiótico. En este sentido, el gradiente de concentración de oxitetraciclina al que se exponen las comunidades bacterianas edáficas se tradujo en un gradiente de resistencia a esa oxitetraciclina.

5. El origen (ganadería convencional *versus* ganadería ecológica) de las enmiendas orgánicas no influyó en el resistoma ni en el moviloma del sistema "enmienda – suelo – cultivo". El purín de vacuno fue la enmienda orgánica que mostró mayores abundancias de genes que confieren resistencia a diferentes clases de antibióticos, en comparación con el estiércol fresco y el estiércol envejecido. Sin embargo, el resistoma de los cultivos enmendados (*i.e.*, lechuga, trigo) no se dedujo directamente del análisis del resistoma de las enmiendas, lo que indica que el perfil del resistoma de las enmiendas no se mantiene a lo largo de la vía de exposición estudiada.

6. La aplicación de lodos de depuradora urbana digeridos anaeróbicamente y deshidratados aumentó la magnitud del resistoma y moviloma en el suelo. La variación de las abundancias de los genes de resistencia a antibióticos se atribuyó principalmente (i) al tiempo transcurrido desde la última aplicación de lodos, (ii) a los genes asociados a elementos genéticos móviles, y (iii) a la composición de las comunidades bacterianas edáficas.

7. La optimización del tratamiento y manejo de las enmiendas orgánicas, previamente a su aplicación al suelo, es una opción idónea para mitigar el riesgo asociado al resistoma y, en particular, la entrada de resistencias a los antibióticos a los agroecosistemas. En este sentido, la digestión anaerobia y la adición de biochar redujeron significativamente la magnitud del resistoma y moviloma en las enmiendas orgánicas y suelos enmendados.

11.2. TESIS

La aplicación de enmiendas orgánicas de origen animal y urbano a los suelos agrícolas es una práctica recomendada dentro de los paradigmas de bioeconomía y economía circular. Sin embargo, su empleo conlleva riesgos asociados a la diseminación de genes de resistencia a los antibióticos a lo largo de la vía de exposición “enmienda orgánica – suelo – cultivo”, por lo que se recomienda encarecidamente aplicar previamente tratamientos y prácticas de manejo que reduzcan dichos riesgos. Las resistencias a los antibióticos son un problema global de enorme importancia cuyo abordaje requiere un enfoque *One Health* que integre conocimientos, medidas y políticas desde muy diversos ámbitos para, de esta forma, aunar esfuerzos en la compleja, a la vez que urgente y preocupante, lucha contra la emergencia y diseminación de resistencias a los antibióticos.

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