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FACULTY OF BIOTECHNOLOGY
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EVALUATION OF POSSIBLE RISKS OF ANTIBIOTIC RESISTANCE TRANSMISSION TO HUMANS BY WASTEWATER-IRRIGATED CROPS

Thesis submitted to the *Universidade Católica Portuguesa* to attain the degree of Ph.D. in Biotechnology with specialization in Microbiology

Nazareno Scaccia

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Nazareno Scaccia

Supervisor: ***Professor Célia Maria Manaia Rodrigues***

Co-supervisor: ***Doctor Ivone Cristina Vaz Moreira***

July 2020

To Rosaria, to my family,
and to all the people I crossed
during this journey.

Ad maiora!

Resumo

A reutilização de águas residuais tratadas tem sido incentivada em todo o mundo, em particular para irrigação agrícola, porque pode ser uma fonte de água confiável e importante, principalmente em países sob estresse hídrico. Ainda se sabe pouco sobre os riscos associados à utilização de águas residuais tratadas, designadamente no que se refere à disseminação de bactérias resistentes a antibióticos (ARB) e genes de resistência a antibióticos (ARGs). Uma das principais consequências da disseminação através da irrigação agrícola utilizando águas residuais tratadas é a contaminação da cadeia alimentar humana. Atualmente, o conhecimento sobre a transmissão de ARB e ARGs para seres humanos a partir de fontes ambientais é escasso. No entanto, a reutilização de águas residuais surge como uma fonte potencial de transmissão direta ou indireta, principalmente através da cadeia alimentar humana. Esta tese explorou estas questões com base em pesquisa bibliográfica e em abordagens experimentais.

O estudo foi planeado com o objetivo de inferir se as bactérias que sobrevivem nas águas residuais serão capazes de colonizar as plantas e se ARB isoladas de águas residuais e os respectivos ARGs poderiam persistir na presença da microbiota fecal autóctone de um indivíduo saudável. As hipóteses propostas para este trabalho foram as de que: 1) ARB e ARGs isoladas de águas residuais tratadas podem colonizar plantas e, eventualmente, estabelecer-se como endófitos; 2) ARGs de ARB isoladas de água residual tratada podem persistir na presença da microbiota fecal de um humano saudável. O argumento que suporta esta duas hipóteses é o de que, se ingeridas através de vegetais, as ARB e ARGs poderiam persistir no intestino humano. Utilizaram-se três abordagens principais para testar as hipóteses referidas: a) foi realizada pesquisa bibliográfica com termos de busca relacionados com bactérias endófitas, filtrada para variedades agrícolas edíveis e normalmente consumidas cruas, e que pertencessem a grupos bacterianos também reportados em águas residuais e no microbioma humano; b) analisou-se a presença de ARB e ARGs em isolados bacterianos em vegetais edíveis (agrião e morango prontos para consumo); e c) foi avaliada a persistência de ARGs transportados por ARB isoladas de efluentes na presença de microbiota fecal de uma criança saudável.

A investigação baseada na literatura teve como objetivo explorar a diversidade de bactérias endofíticas associadas a diferentes habitats (plantas, águas residuais e microbioma

humano) e inferir a probabilidade de que as ARB e ARGs possam ser transferidos das águas residuais para as plantas e depois para os seres humanos (Capítulo 3). Este estudo bibliográfico permitiu compilar uma lista de bactérias endofíticas distribuídas por mais de 20 filos reportadas em mais de 45 variedades de vegetais. Como o estudo procurava explorar as possíveis vias de transmissão para humanos, a lista de espécies agrícolas encontradas na literatura foi reduzida apenas a plantas que podem ser consumidas cruas, como sejam alface, cenoura, rabanete, pepino e tomate. Com o objetivo de avaliar a probabilidade dessas plantas captarem bactérias que podem atuar como vetores de resistência a antibióticos, a presença dos grupos bacterianos identificados como endófitos foi também investigada em comunidades microbianas de águas residuais e em microbiomas humanos. Para grupos bacterianos, cuja ocorrência foi relatada nos três tipos de ambiente, endófitos de plantas edíveis/cruas, águas residuais e microbioma humano, foi realizada uma análise exploratória com base em literatura e bases de dados públicas, de genes de resistência a antibióticos e de virulência. Este estudo sugere que bactérias relacionadas com géneros como *Enterobacter, Acinetobacter, Pseudomonas* e *Staphylococcus*, cuja relação taxonómica com agentes patogénicos oportunistas e portadores de resistência a antibióticos, é conhecida, podem ser encontrados como endófitos em variedades agrícolas comestíveis, sugerindo que podem ser veiculados por águas residuais e ser transmitidos para o microbioma humano. Esses resultados sustentam a hipótese de que as bactérias transportadas pelas águas residuais, com propriedades fisiológicas e ecológicas semelhantes a bactérias endófitas, podem ser captadas por variedades agrícolas edíveis e, assim, se ingeridas, podem eventualmente colonizar o corpo humano. Deste modo, sugere-se que a irrigação com águas residuais pode aumentar os riscos de transmissão de bactérias clinicamente relevantes do meio ambiente para os seres humanos, através da cadeia alimentar.

Com recurso a métodos dependentes da cultura, avaliou-se a diversidade de bactérias cultiváveis e resistentes a antibióticos que podem ser encontradas em associação com variedades edíveis, especificamente o agrião e morangos prontos para o consumo (capítulo 4). Os agriões prontos para consumo foram adquiridos em supermercados locais, enquanto as amostras de morangos foram colhidas ao longo de uma cadeia de processamento de frutas de uma empresa que produz preparados de frutas para a indústria alimentar. Estudou-se a fração microbiana das folhas de agrião e as bactérias associadas ao morango (comunidade epífita e endofítica). Os isolados de agrião (n = 68) e morango (n = 52) foram caracterizados fenotipicamente de acordo com seu perfil de resistência a antibióticos. Destes, selecionou-se uma coleção de isolados que exibia fenótipo de resistência a pelo menos três classes de

antibióticos, para identificação com base na análise da sequência do gene 16S rRNA e caracterização por reação em cadeia da polimerase (PCR) convencional, quanto à presença do gene da integrase de intregrões de classe 1, *intl*1, os ARGs *sul*1, bla_{TEM}, bla_{CTX-M}, bla_{OXA-A} e bla_{SHV}, e os grupos de incompatibilidade de plasmídeos com base nos tipos de origem de replicação FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, e FIIA. Além disso, testou-se se as bactérias isoladas de morango e agrião poderiam adquirir ARGs por transformação. Observou-se que o agrião pronto a consumir apresentava contagens de heterotróficos totais na ordem de $3,5 \times 10^7 \pm 4,9 \times 10^7$ CFU/mL e $5,4 \times 10^5 \pm 6,2 \times 10^4$ CFU/mL de solução de lavagem de agrião para os supermercados A e B, respectivamente, enquanto o morango processado apresentava contagens de bactérias cultiváveis na ordem de $9,1 \times 10^5 \pm 1,8 \times 10^5$ CFU/g de peso seco de morango, com alguns dos isolados a apresentarem fenótipos de resistência a antibióticos. Algumas destas bactérias foram identificadas como membros dos géneros *Pseudomonas, Stenotrophomonas, Erwinia, Rahnella, Methylobacterium* e *Chryseobacterium*. Alguns destes grupos bacterianos foram anteriormente identificados com endófitos e géneros como *Pseudomonas* e *Stenotrophomonas* estão proximamente relacionados com patogénicos oportunistas que podem ser transportadores de ARGs adquiridos. A pesquisa de ARGs, *intI*1 e grupos de incomatibilidade de plasmídeos revelou que estes elementos genéticos foram encontrados numa pequena fração das bactérias analisadas (2 isolados). Além disso, resultados preliminares sugeriram que os isolados de agrião e morango podem ser suscetíveis a adquirir resistência a antibióticos e, assim, estar envolvidos na disseminação da resistência a antibióticos.

O terceiro estudo baseou-se no argumento de que bactérias de águas residuais ou os seus ARGs poderiam ser rapidamente eliminados em presença da complexa comunidade da microbiota fecal. Este estudo foi realizado em ensaios de microcosmo com material fecal (FMAs) inoculado com bactérias isoladas de água residual, portadoras de genes de resistência a antibióticos conhecidos (Capítulo 5). Os isolados de águas residuais inoculados foram *Escherichia coli* (estirpe A2FCC14) e *Enterococcus faecium* (estirpe H1EV10), portadores dos ARGs bla_{TEM}, bla_{CTX}, bla_{OXA-A} e *van*A, respectivamente. O efeito de condições como a presença ou ausência de oxigénio ou os antibióticos cefotaxima ou vancomicina, na composição da comunidade microbiana do material fecal e na persistência dos ARGs exógenos foram investigados.

Os FMAs foram monitorizados nos tempos 0, 1, 3 e 7 dias de incubação, com recurso a métodos de cultivo, PCR quantitativo (qPCR) e análises da comunidade bacteriana com base

na sequência nucleotídica de amplicões do gene para o rRNA 16S. Na comunidade bacteriana do material fecal predominavam membros dos filos *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* e *Verrucomicrobia*. Esta comunidade sofreu aumento na abundância relativa de *Proteobacteria*, quando os FMA foram incubados na presença de oxigénio. A presença de doses sub-inibitórias de antibióticos não esteve associada a variações relevantes na composição da comunidade microbiana.

Os ARGs das ARB inoculadas persistiram durante o período de 7 dias em todos os ensaios e, ao fim de 30 dias, podiam ainda ser quantificados, tanto em condições de aerobiose como de anaerobiose. A adição de doses sub-inibitórias de antibióticos não se correlacionou com variações significativas na persistência das ARB ou ARGs testados, quando comparados os respetivos controlos em antibióticos. Este estudo sugeriu que ARB que vivem em águas residuais podem persistir pelo menos por uma semana na presença de microbiota fecal complexa e, mesmo quando essas ARB estão abaixo do limite de detecção cultivável, os seus ARGs eram quantificáveis por qPCR. Esses resultados apoiam a hipótese de que, se as bactérias das águas residuais, por acaso, atingirem o intestino humano, poderá haver uma colonização bem-sucedida, um tema que merece mais investigação.

No geral, a investigação realizada nesta tese sugere que as águas residuais tratadas usadas para irrigação podem conter bactérias com relevância clínica que, por essa via, podem estabelecer associação estável com culturas edíveis, como endófitos. Se, como consequência desse fato, essas bactérias atingirem o intestino humano, há indícios de que terão capacidade para ali persistir. Embora os presentes estudos evidenciem a complexidade desta questão, a tese apresentada sustenta que as águas residuais, as variedades agrícolas edíveis e os humanos podem estar interligados na cadeia de transmissão de ARB e ARGs. Assim, esta tese abre também caminhos para novas investigações neste domínio.

Palavras-chave: reutilização de águas residuais, bactérias resistentes a antibióticos, genes de resistência a antibióticos, bactérias endofíticas, vegetais edíveis/crus, ensaios de microcosmo fecal, microbiota fecal.

Abstract

The reuse of treated wastewater has been encouraged worldwide, in particular for agricultural irrigation, because it can be a reliable and important water source, mainly in countries under water stress. The recycling of wastewater poses still not evaluated risks concerning, among other, the dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) across distinct One-Health compartments. One of the main concerns associated with such dissemination is the contamination of the human food-chain through crops irrigation with treated wastewater. Currently, the knowledge about the transmission of ARB and ARGs to humans through environmental sources is sparse. However, wastewater reuse emerges as a potential source for direct or indirect transmission, mainly through the human food-chain. This project addressed these issues, through literature- and experiment-based investigation about potential risks of antibiotic resistance transmission to humans emerging from treated wastewater reuse.

The study was planned aiming to infer if bacteria surviving in wastewater would be able to colonize plants and if wastewater ARB and the respective ARGs would be able to persist in the presence of the autochthonous fecal microbiota of a healthy human. The research hypotheses of this work were: 1) ARB and ARGs present in treated wastewater are capable of colonizing plants, and establish as endophytes; 2) ARB and ARGs are able to persist in the presence of the autochthonous human gut microbiota. The rationale behind these two hypotheses was that if ingested through vegetables, ARB and ARGs could be able to persist in the human gut. To test the study hypotheses were used three major approaches: a) it was made a literature search seeking to investigate the diversity of endophytic bacteria that belong to groups that can also be found in wastewater and in the human microbiota as well as in edible crops, normally consumed raw - this approach offered a predictor of the likelihood of a crop behave as a possible mode of transmission of ARB to humans; b) it was analysed the presence of potential ARB and ARGs in isolates from ready-to-eat watercress and strawberry; and c) it was assessed the persistence of ARGs harboured by wastewater ARB in the presence of fecal microbiota of a healthy child.

The literature-based investigation aimed to explore the diversity of endophytic bacteria associated with different habitats (plant, wastewater, and human microbiota), and infer the probability that ARB and ARGs might be transferred from wastewater to plants and then to humans (Chapter 3). This literature survey permitted to shortlist of more than 20 phyla of

endophytic bacteria distributed over more than 45 crop varieties. Because the major focus was on potential links of transmission to humans, the array of crops found in the literature was narrowed to plants that can be consumed raw, such as lettuce, carrot, radish, cucumber and tomato. In order to assess the likelihood that these plants uptake bacteria that might be antibiotic resistance vectors, the bacterial groups identified as endophytes were also investigated as potential wastewater and human microbiome member communities. Finally, for bacterial groups, whose occurrence was reported in the three types of environments, the previous description of antibiotic resistance and virulence genes was surveyed from public databases and literature. The search revealed that members of genera such as *Enterobacter*, *Acinetobacter*, *Pseudomonas*, and *Staphylococcus,* with known taxonomic proximity to human opportunistic pathogens and harbour of acquired antibiotic resistance may be found as endophytes in edible crops as well as in wastewater or the human microbiota. These results support the hypothesis that bacteria transported by wastewater, with physiological and ecological properties similar to endophytes, may be uptake by edible crops and being ingested may eventually colonize the human body. It is suggested that wastewater irrigation may raise the risks of transmission of clinically relevant bacteria from the environment to humans, via the food-chain.

Plant associated bacteria were also searched on ready-to-eat watercress and strawberry, based on culture-dependent methods (Chapter 4). Ready-to-eat watercress were purchased at local supermarkets and strawberries samples were collected along a fruit processing chain of a company that produces fruits preparations for the food industry. Bacteria adsorbed onto watercress leaves as well as strawberry-associated bacteria (epiphytic and endophytic community) were isolated and analysed. The antibiotic resistance phenotype of watercress (n=68) and strawberry (n=52) isolates was characterized. Isolates exhibiting resistance to at least three antibiotics classes were selected for identification based on the 16S rRNA gene sequence analysis, and the presence of class 1 integron integrase gene *intl*1, the ARGs *sul*1, bla_{TEM}, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$ and bla_{SHV} and the plasmid incompatibility groups FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA by conventional Polymerase Chain Reaction (PCR) were screened. Some isolates were also tested for the capacity to acquire ARGs by transformation assay. Ready-to-eat watercress presented counts of total heterotrophs in the order of $3.5 \times 10^7 \pm 4.9 \times 10^7$ and $5.4 \times 10^5 \pm 6.2 \times 10^4$ CFU/mL of watercress washing solution for supermarket A and B, respectively, whilst processed strawberry presented counts of total heterotrophs in the order of $9.1 \times 10^5 \pm 1.8 \times 10^5$ CFU/g of strawberry dry weight, with some isolates exhibiting antibiotic resistance phenotypes. The

bacteria identified belong to the genera *Pseudomonas, Stenotrophomonas, Erwinia, Rahnella, Methylobacterium,* and *Chryseobacterium*. These bacterial taxa were previously identified as endophytes and some, such as members of the genera *Pseudomonas* and *Stenotrophomonas* are closely related to human opportunistic pathogens that can harbor acquired ARGs. The screening of ARGs, *int*1 and plasmid incompatibility groups revealed that these genetic elements were present within a minority group (2 isolates) of the examined plant-associated bacterial isolates. Additionally, preliminary results suggested that watercress and strawberry isolates could be susceptible to acquire antibiotic resistance and thus be involved in the dissemination of antibiotic resistance.

The third study was designed based on the arguments that wastewater bacteria as well as their harboured ARGs would be rapidly lost in the complex community of fecal microbiota. This study was performed using fecal microcosm assays (FMAs) inoculated with wastewater ARB isolates harbouring known ARGs (Chapter 5). The inoculated wastewater isolates were *Escherichia coli* (strain A2FCC14) and *Enterococcus faecium* (strain H1EV10), harbouring the ARGs bla_{TEM}, bla_{CTX}, $bla_{\text{OXA-A}}$ and *van*A, respectively. The effect of variables such as the presence or absence of oxygen or sub-inhibitory dose of the antibiotic cefotaxime or vancomycin on the feces microbial community composition and on the persistence of the exogenous ARGs were investigated.

FMAs were monitored at time 0, 1, 3 and 7 days of incubation based on cultivation methods, quantitative PCR (qPCR) and 16S rRNA gene sequence amplicon community analyses. The fecal bacterial community was characterized by the predominance of members of the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. The presence of oxygen was associated with an increase in the relative abundance of *Proteobacteria*, while antibiotics did not lead to consistent microbial community composition variations.

The ARGs harboured by the inoculated ARB persisted over the 7 days incubation period in all the assays and could still be quantified at least for one month, under both aerobic and anaerobic conditions. Sub-inhibitory concentrations of antibiotics were not correlated with significant variations on the persistence of the tested ARB or ARGs, when compared with antibiotic-free microcosms. This microcosm-based investigation suggested that wastewater ARB can persist at least for a week in the complex fecal microbiota and, even when these ARB are below the culture detection limit, their ARGs were quantifiable by qPCR. These results support the hypothesis that, if wastewater bacteria by chance can reach the human

gut, a successful colonization cannot be disregarded, a topic that deserves further investigation.

Overall, the investigation conducted in this thesis suggests that bacteria with potential clinical relevance that may occur in treated wastewater used for irrigation, may establish stable association with edible crops, as endophytes. If, as consequence of this fact, these bacteria reach the human gut, they may be capable of persisting there. Although this investigation put in evidence the complexity of this issue, showing that further studies are required, the thesis presented is that the wastewater, edible crops and humans may be part of the same transmission link chain of ARB and ARGs.

Keywords: wastewater reuse, antibiotic resistant bacteria, antibiotic resistance genes, endophytic bacteria, raw-eaten vegetables, fecal microcosm assays, fecal microbiota.

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This Ph.D. thesis marks the end of an extraordinary journey that enriched me of high scientific knowledge, academic skills as well as life experience. I learned to persist, persevere, and thanks to that, to accomplish an extremely interesting work! However, it would not have been possible without the help of those who, directly or indirectly, have contributed to making it a successful journey, to whom I want to express my gratitude:

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List of Abbreviations

ARB	Antibiotic Resistant Bacteria
ANI	Average Nucleotide Identity
ANSWER	ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions
AR	Antibiotic Resistance
ARDB	Antibiotic Resistance Genes Database
ARGs	Antibiotic Resistance Genes
AVBS	Abwasserverband Braunschweig
CARD	Comprehensive Antibiotic Resistance Database
CCA	Canonical Correspondence Analysis
ChCA	Chromocult Coliform Agar
CFUs	Colony Forming Units
DNA	Deoxyribonucleic acid
DP	Diaminopyrimidins
ESBL	Extended-Spectrum Beta-Lactamase
ESKAPE	***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* spp.
ESR	Early Stage Researchers
FMAs	Fecal Microcosm Assays
ESR	Early Stage Researchers
GFP	Green Fluorescence Protein
HGT	Horizontal Gene Transfer
ISS	Instituto Superiore di Sanità
ITN	Innovative Training Networks
JKI	Julius Kühn-Institut
LA	Luria-Bertani Agar
MDR	Multiple Drug Resistance
MIC	Minimum Inhibitory Concentration
mFC	Membrane Fecal Coliform Agar

MGEs	Mobile Genetic Elements
MRGs	Metal Resistance Genes
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PGPB	Plant growth-promoting bacteria
PMM-Lab	Predictive Microbial Modelling Lab
QIIME2	Quantitative Insights Into Microbial Ecology
QMRA	Quantitative microbiological risk assessment
qPCR	Quantitative Polymerase Chain Reaction
RAST	Rapid Annotation using Subsystem Technology
RND	Resistance Nodulation Division
R2A	Reasoner's 2A agar
TC-DNA	Total community DNA
TWW	Treated Wastewater
WHO	World Health Organization
WWTPs	Wastewater Treatment Plants

Publications

The content of this thesis is partially published in:

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Introductory note

The present doctoral dissertation was developed within the framework of the Innovative Training Networks (ITN) programme Marie Skłodowska-Curie grant, through the project ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions, ANSWER (http://www.answer-itn.eu/), funded by the European Union's Horizon 2020 research and innovation programme. This introductory note aims to present the context in which Early Stage Researchers (ESRs), which corresponds to a three-year contract, are integrated in ITN programme and have the opportunity to accomplish a doctoral degree in association with the project. ANSWER aimed to unravel the environmental occurrence of antibiotics, and propagation of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) as a consequence of the reuse of domestic treated wastewater. ANSWER hired 15 ESRs, hosted by institutions located in a country distinct from the ESR residence country, which corresponded to ESRs from ten countries hosted by nine institutions located in eight European and associated countries.

In ANSWER, my role as ESR focused on the assessment of the risks of antibiotic resistance transmission to humans from treated wastewater, including through crops irrigated with reused water. According to the Marie Curie ITN guidelines, ESR activity includes training courses, outreach and communication tasks (Annex I), as well as secondments in foreign partner institutions. Two secondments were performed (a detailed description is provided in Annex II and Annex III). The first secondment was planned to be hosted by the partner Abwasserverband Braunschweig (AVBS) with Dr. Bernhard Teiser. However, since microbiology and molecular biology works could not proceed with the conditions available in those facilities, the experimental work was performed at the Institute for Epidemiology and Pathogen Diagnostics at the Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants in Braunschweig, under the guidance of Prof. Kornelia Smalla (Annex II). This secondment run between 16 July and 30 November 2018, and integrated an ongoing project with the major objective of investigating the diversity of antibiotic resistant bacteria, antibiotic resistance genes and mobile genetic elements (MGEs) associated with the microbiome of hydroponic lettuce growing in the presence of treated wastewater effluents inside a greenhouse system located next to a wastewater treatment plant (WWTP). Briefly, lettuce and wastewater samples from hydroponic production

(http://www.hypowave.de/projekt/) were analysed by culture-dependent and -independent approaches for: i) isolation of ARB; ii) capture of antibiotic resistance plasmids; iii) ARGs and MGEs analysis. Coliform ARB were isolated from treated wastewater effluent as well as from hydroponic lettuce. *Escherichia coli* CV601 transconjugants with acquired tetracycline or cefotaxime resistance were obtained from hydroponic lettuce and treated wastewater samples by exogenous plasmid capture. Analysis of total community DNA extraction from both samples, showed the presence of MGEs and ARGs. The results obtained are being complemented with other ongoing experiments at the Julius Kühn-Institut, and will be published in the future, not as part of this thesis.

The second secondment run between 01 December 2018 and 01 February 2019 at the Instituto Superiore di Sanità located in Rome under the supervision of Dr. Maura Manganelli and Dr. Emanuela Testai (Annex III). The aim of this secondment was to explore the rationale for a possible mathematical model designed to predict the survival of ARB in the fecal human microbiome using the data obtained at ESB-UCP (described in the Chapter 5 of this thesis). For that, the Predictive Microbial Modelling Lab (PMM-Lab) (https://foodrisklabs.bfr.bund.de/), an open source software, was used. Briefly, PMM-Lab allows users to use proprietary or public data to create bacterial growth/survival/inactivation models as well as growth/no-growth boundary models. Following the same knowledge used in the predictive microbiology, we wanted to simulate the behaviour of wastewater ARB within the human fecal microbiome under different conditions such as the presence or absence of oxygen as well as presence or absence of antibiotics. Although preliminary results have shown the applicability of predictive microbial models for the above-mentioned purposes, a deeper knowledge of secondary models as well as software for predictive microbiology are required. Additionally, due to time constraints, it was not possible to conclude this research project which may represent a possible future area of collaboration and investigation.

In preparation of Chapter 4 experiments and under the scope of the "Programa de mentorado comendador Arménio Miranda", it was preliminary investigated an ARB plant internalization model. For that, bacteria uptake was tested in a sprout germination system at a laboratory-scale, in the presence of the wastewater bacterial strain *Escherichia coli* A2FCC14, an antibiotic resistant strain previously isolated from municipal raw influent (Chapter 5) and *Lactobacillus plantarum* NCFB 1752, a probiotic strain isolated from pickled cabbage (Couto and Hogg, 1994) (these assays are summarized in Annex IV). Thanks to a lab-scale sprouting germination system, it was possible to demonstrate the

capability of the wastewater multi-drug resistant *Escherichia coli*, strain A2FCC14, to grow onto surface and uptake into vascular system of adzuki bean sprouts. Although the interactions among bacteria and plants are highly species specific, this approach may be useful to further explore the possible role of plants, particularly raw-eaten vegetables, as vehicles of harmful bacteria transmission to humans.

1. Introduction

1.1. Antibiotic resistance in the environment

Compounds with antimicrobial activity are produced in nature by environmental bacteria, who display intrinsic antibiotic resistance that may be due to single or epistatic genes action (Adegoke *et al.*, 2018; D'Costa *et al.*, 2011; Davies and Davies, 2010; Martínez, 2008). Antibiotic resistance genes have been also detected in bacteria that do not produce antibiotics (Martínez *et al.*, 2015; Peterson and Kaur, 2018). It has been argued that the co-existence of antibiotic-producer and non-produce bacteria might have led to the co-evolution of mechanisms of antibiotic resistance in latter (Davies and Davies, 2010; Martínez, 2008; Peterson and Kaur, 2018). Acquired resistance in members of a bacterial species that initially was susceptible to that antibiotic may be due to gene mutation or acquisition of ARGs by horizontal gene transfer (HGT) (Munita and Arias, 2016; Peterson and Kaur, 2018).

Antibiotics at sub-inhibitory concentrations may also act as signalling molecules among bacteria regulating several biochemical processes such as HGT (conjugation), gene expression, biofilm or flagella formation (Andersson and Hughes, 2014; Meek *et al.*, 2015). It is generally accepted that the global overuse of antibiotics in animals and humans has generated effective selective pressures that have contributed to accelerate the spread of antibiotic resistance (Andersson and Hughes, 2014; Davies and Davies, 2010; WHO, 2018a). Nevertheless, pollution caused by environmental contaminants as a consequence of anthropogenic activities, such as metals or biocides (i.e. disinfectants) is also supposed to have contributed to the increment of the antibiotic resistance phenomenon (Bengtsson-Palme *et al.*, 2018; Singer *et al.*, 2016). Besides the use of antibiotics for the treatment of bacterial infections, antibiotics have also been used for disease prevention (prophylactic use) and as food additive for growth promotion in livestock, poultry and aquaculture (Kirchhelle, 2018; Lulijwa *et al.*, 2019; Xie *et al.*, 2018). It is estimated that antibiotic sales in 2014 across Europe reached around 3,800 and 8,900 tonnes of antibiotics, sold for use in humans and food-producing animals, respectively (ECDC/EFSA/EMA, 2017). Another study estimated that two-thirds of all of the total antibiotics annually produced (around 65,000 of 100,000 tonnes) worldwide are used in animal husbandry (Gelband *et al.*, 2015). The presence of these contaminants in the environment may have incremented the prevalence of ARB in

many ecosystems making antibiotic resistance not only confined in clinic settings (Allen *et al.*, 2010; Davies and Davies, 2010).

The multiple sources of antibiotic resistant bacteria and antibiotic resistance genes have been documented, particularly wastewater treatment plants (WWTP), aquaculture, manure and sewage sludge (**Figure 1.1**) (Manaia *et al.*, 2018; Murray *et al.*, 2019; Watts *et al.*, 2017; Xie *et al.*, 2018). These environmental reservoirs are also the main pathways by which antibiotic residues together with ARB disseminate in the environment (Singer *et al.*, 2016) (**Figure 1.2**). Due to these and other dispersal phenomena, antibiotic resistance is nowadays considered a major global public health threat (O'Neill, 2014; WHO, 2018b).

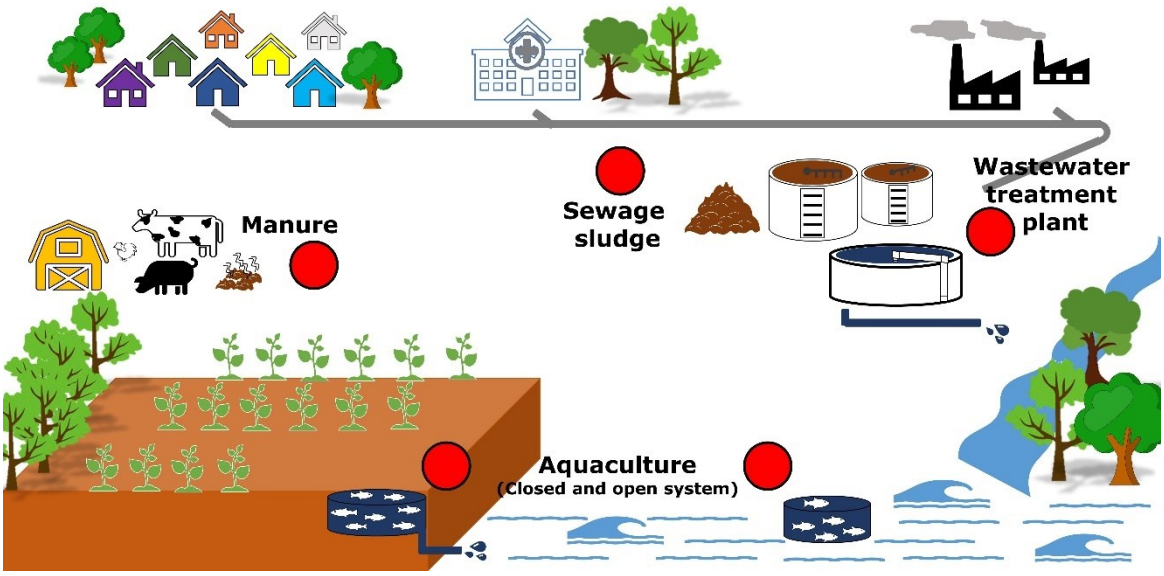


Figure 1.1. Examples of environmental sources (red dots) of antibiotic resistance in different ecosystems.

1.1.1. Antibiotic resistance acquisition and dissemination

In spite of the wide range of conserved essential functions and structures among the bacterial cells, antibiotics target few cellular processes, specifically related with nucleic acids, cell wall, or protein synthesis (Kohanski *et al.*, 2010; Lewis, 2013). Similarly, bacteria evolved a limited number of resistance mechanisms included into different categories: i) antibiotic modification/degradation, ii) antibiotic efflux, iii) antibiotic sequestration by special proteins, iv) antibiotic target modification, v) antibiotic target bypass and vi) antibiotic target protection (Lewis, 2013; Munita and Arias, 2016; Peterson and Kaur, 2018). Some of these mechanisms may co-exist in the same bacterial cell, conferring resistance to different drugs, one of the processes that may lead to multidrug resistance (Depardieu *et al.*, 2007; Mulani *et al.*, 2019). Other multidrug-resistance mechanisms may be associated with

efflux systems or processes that involve multi-substrate enzymes (Kaur and Peterson, 2018). These resistance mechanisms are phenotypically expressed by a wide heterogeneity of resistance genes, which may propagate vertically and horizontally, an event that eventually is exacerbated under selective pressure induced by the antibiotics (Depardieu *et al.*, 2007). When a given bacterial population is exposed to antibiotic concentrations below or above the MIC (Minimum Inhibitory Concentration) value - which is referred to as the lowest concentration of antibiotic that inhibits visible growth of a target bacteria, it may favour the enrichment for pre-existing ARB (Andersson and Hughes, 2014). Moreover, under the same condition, *de novo* mutations may also arise allowing some bacteria to survive, proliferate and spread by vertical transmission (Andersson and Hughes, 2014; Meek *et al.*, 2015).

Bacteria can also acquire exogenous DNA containing ARGs by HGT. The processes of HGT are: i) transformation where the cell can uptake external naked DNA, ii) transduction which is a phage-mediated process and, iii) conjugation which involves direct cell contact, also known as bacteria "sex" (Andersson and Hughes, 2014; Munita and Arias, 2016). The ability of the bacteria to transfer ARGs by vertical transmission and/or horizontally, allows bacteria to easily propagate ARGs. Today, many studies have shown that the prevalence and distributions of ARGs have increased over the last decades (Aslam *et al.*, 2018; Berendonk *et al.*, 2015; WHO, 2018b). In this respect, besides pathogenic bacteria, environmental bacteria are also important reservoirs of ARGs (Allen *et al.*, 2010; Peterson and Kaur, 2018), which due to the genetic stability of these determinants, the low fitness costs or the occurrence of selective pressures eventually present in the environment (i.e. antimicrobials, metals, surfactants, etc.) may contribute to the spread of antibiotic resistance among bacteria.

1.2. Wastewater as a reservoir of antibiotic resistance

WWTPs have been identified as sources of antibiotic resistance, potentially involved in the dissemination of antibiotic resistance from humans to the environment (Berendonk *et al.*, 2015; Pärnänen *et al.*, 2019; Rizzo *et al.*, 2013). Recently, it has been proposed that WWTPs influent might mirror the human microbiome resistome of the population connected with (Pärnänen *et al.*, 2019). In fact, severe cases of multi-drug resistant bacteria reported in clinical compartments such as vancomycin-resistant enterococci, extended-spectrum beta-lactamase (ESBL) *Enterobacteriaceae* have been found in WWTPs (Boopathy, 2017; Korzeniewska and Harnisz, 2013; Varela *et al.*, 2013).

It has been estimated that ARB in the sewage microbiota may reach more than 50% within a given bacterial group (i.e. enterobacteria or enterococci) (Rizzo *et al.*, 2013).

WWTPs receive antibiotic residues that result from the incomplete or partial metabolization, along with a broad diversity of human-derived bacteria, including some opportunistic pathogens, many holding ARGs (Krzeminski et al., 2019; Rizzo et al., 2013). The ARGs harboured by these bacteria can eventually be exchanged among neighbour cells by HGT processes and, bacteria with acquired resistance might be able to survive, persist, and spread in the environment (Berendonk *et al.*, 2015; Gillings, 2017; Rizzo *et al.*, 2013). A WWTP final effluent may dispose 10^9 - 10^{12} Colony Forming Units (CFUs) of ARB per day, per inhabitant equivalent (Manaia *et al.*, 2016; Vaz-Moreira *et al.*, 2014). These evidences show that although WWTPs act as pivotal barriers for environmental and human protection, they are not fully effective on the prevention of the accumulation, maintenance, and dissemination of antibiotic resistance into the environment. Therefore, the use of treated wastewater to irrigate crops may be a source of ARB and ARGs capable of contaminating the human food chain through irrigation practice, and therefore threat human health.

1.3. Wastewater reuse in agriculture

Irrigation refers to water application into soil to provide the essential moisture for plant growth (Pescod, 1992). In the arid, or semi-arid parts of the world, irrigation is essential for successful agricultural practices for human needs. Almost 60% of all the freshwater withdrawals in the world go to irrigation uses (EPA, 2012). However, in countries under water stress, due to freshwater scarcity or degradation of the existing water resources, for example resultant of pollution, the reuse of treated wastewater has been incentivized, mainly for irrigation in agriculture fields (Aquarec, 2006; Becerra-Castro *et al.*, 2015; Kalavrouziotis *et al.*, 2015). Wastewater is any water produced by anthropogenic activities resulting from a combination of domestic, commercial, institutional and industrial effluents, and depending on the sewerage system may include storm water, and other urban runoff (EPA, 2012; Mateo-Sagasta *et al.*, 2013). Treated wastewater refers to wastewater that has been processed within a WWTP through a physical, chemical and biological procedure to reduce its contents of organic matter (C, N, P); reclaimed water or recycled water instead, is treated wastewater that, under controlled conditions, can be recommended for reuse purposes (i.e. irrigation) (Mateo-Sagasta *et al.*, 2013). Wastewater reuse in agriculture is an ancient practice, as wastewater was disposed outside of the urban settlements for irrigation and as crop fertilizer in the ancient Greeks and Romans times (Cooper, 2001; Jaramillo and Restrepo, 2017). However, today it is well-known that the application of untreated wastewater directly into agriculture land may be a threat because it may contain pathogens,

antibiotic residues and other chemical pollutants that might have negative effects for the environment, humans and animals health (Jaramillo and Restrepo, 2017; Mateo-Sagasta *et al.*, 2013). The use of reclaimed water for irrigation (both agricultural and landscape) is implemented in countries like France, Italy, Spain, Cyprus, Malta, Israel or the USA (EMWIS, 2007; EPA, 2012; Kalavrouziotis *et al.*, 2015). In Israel for instance, over 80% of treated wastewater effluent is reused mainly for agricultural purposes (Inbar, 2007). Reclaimed water for agriculture irrigation may have benefits as well as risks that have been studied in previous studies (Hussain *et al.*, 2002; Mateo-Sagasta *et al.*, 2013).

For instance, wastewater can be a source of macro- and micro-nutrients that can improve crop yield and, reduce fertilizers use in agriculture (Jaramillo and Restrepo, 2017). Additionally, wastewater reuse may decrease freshwater depletion, recharge of groundwater reserves, and it may be available all year long to be used at any time (Mateo-Sagasta *et al.*, 2013). However, health risks due to recycled water may rise, for instance outbreaks of food- or water-borne diseases caused by bacteria, viruses, protozoa or helminths through occupational exposure, aerosols formation or ingestion of wastewater-irrigated crops (Adegoke *et al.*, 2018; Pescod, 1992). Nevertheless, it is worth mentioning that most of these health risks are mainly associated with untreated or partially treated wastewater. Yet, there is a growing concern that ARB and ARGs present in treated wastewater may reach the soil and further enter the plants (Becerra-Castro *et al.*, 2015). In spite of such concerns, the knowledge about human contamination by ARB and ARGs through wastewater-irrigated crops is still sparse.

The use of reclaimed water for irrigation seems to be a valid alternative to balance water demand and water supply, particularly for food needs due to the growing world populations. However, there are still serious health risks that should be taken into consideration for a safer implementation. Major advances have been made in order to produce safe treated effluents paying also attention to reduce the biological contaminants (Krzeminski *et al.*, 2019; Rizzo *et al.*, 2018). Nevertheless, as mentioned, there are still some open questions concerning the sustainability and safe wastewater reuse in regard to the removal efficiency of antibiotic residues, ARB and associated ARGs from effluents and sewage sludge as well as to the fate of these contaminants once released into the environment (i.e. soil, water, crops). Further efforts need to be performed to understand the behaviour of ARB and ARGs in the environment and whether these contaminants can reach humans or animals via wastewater-soil-plant, in order to develop risk-assessment guidelines to ensure the public health and promote the safe reuse of treated wastewater.

1.4. Humans and the contaminant antibiotic resistance

1.4.1. Humans as source and receptor of AR

Microenvironments composed of high-level structured microbial communities such as the oral biofilm or the human gut microbiome have been suggested as reservoirs of ARGs (Roberts and Mullany, 2010; Sommer *et al.*, 2009). The human gut microbiome resistome has been considered an important reservoir of antibiotic resistance, where pathogens can acquire ARGs (Sommer *et al.*, 2010; Sommer *et al.*, 2009). The bacterial cell density within the gut microbiota is extremely high and it can increase the chance of cell-to-cell contact and thus the possibility of HGT among the bacteria population. The same situation may occur in the animal gastrointestinal tract. Human commensal bacteria with acquired antibiotic resistance can be excreted by humans through urine and feces, entering the environment. Similarly, animals may emit ARB into the environment through manure.

The ubiquitous character of many ARB (whether pathogenic or not) is relevant for thriving in the environment and for acquiring ARGs that can become stable genetic elements, eventually transferred by vertical or HGT processes, due to permanent exchanges between bacterial flora in environmental, animal or human reservoir (Berendonk *et al.*, 2015; Huddleston, 2014; Summers, 2006). No real barriers exist for ARGs and ARB and they can easily move among different environments using different vehicles (i.e. water, animals) (Allen *et al.*, 2010; Manaia *et al.*, 2011). The likelihood of ARB transmission back to humans from the environment may occur by ingestion, inhalation or direct contact (**Figure 1.2**) (Manaia, 2017). Food products such as vegetables usually consumed without any processing (raw crops) have been suggested as part of the antibiotic resistance transmission pathway from the environment to humans (Blau *et al.*, 2018; Zhu *et al.*, 2017). In crops from lands amended with animal manure or sewage sludge or irrigated with wastewater, the probability of contamination with ARB and ARGs may be high (Adegoke *et al.*, 2018; Becerra-Castro *et al.*, 2015; Heuer *et al.*, 2011; Marti *et al.*, 2013).

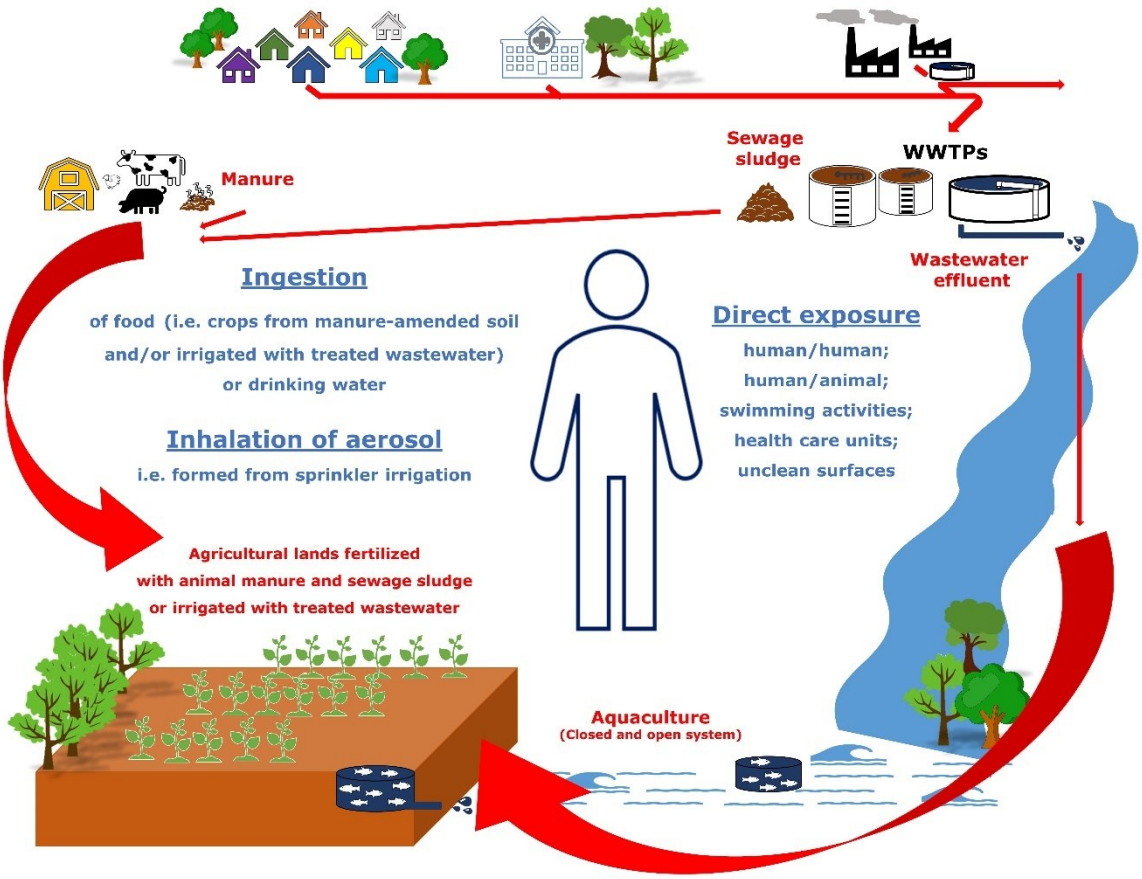


Figure 1.2. Cycle of antibiotic resistance in the environment and human exposure. A large fraction of antibiotics administered to humans in the community and hospital settings can be excreted in unchanged forms, along with ARB and ARGs, into urine and feces that will enter then the WWTPs through the sanitation system. The pharmaceutical industry can also discharge antibiotics residues by treated industrial wastewater. The WWTPs are not able to completely remove antibiotic residues as well as ARB and therefore, they represent sources of these contaminants into the environment, in particular to natural water bodies (rivers, lakes, sea) or through further applications of the sewage sludge in agriculture. As in humans, ARB and antibiotics used in livestock or poultry can pass into the environment through excreta, the antibiotics administered within aquaculture (open and closed systems) can also enter the water system by feces produced from the fishes, or directly disperse in the water ecosystem. Manure produced by livestock and poultry or aquaculture sludge originated by closed flow-through systems or municipal activated sludge can be applied in agriculture as crop fertilizer increasing the dissemination of antibiotic resistance in the environment.

1.4.2. Vegetables as a potential vehicle of AR transmission to humans

The consumption of raw vegetables irrigated with ARB contaminated water is hypothesised as one of the possible modes of transmission of ARB from the environment to humans. However, direct evidence of this transmission is difficult to establish because of the absence of a direct cause-effect connection (Pachepsky *et al.*, 2011). Nevertheless, different studies have demonstrated the occurrence of ARB or pathogenic bacteria in vegetables due

to wastewater irrigation practices (Abakpa *et al.*, 2015; Araújo *et al.*, 2017; Ibenyassine *et al.*, 2007; Pachepsky *et al.*, 2011). Studies on the efficiency of processes (from washing to packaging) on the elimination of potential microbial contaminants, mainly in what regards ARB, deserve further elucidation. However, the consumption of uncooked vegetables irrigated with ARB contaminated water, including non-complying treated wastewater, may play an important role in the transmission of antibiotic resistance and therefore represent a public health threat.

Wastewater irrigation can promote an increase of the soil microbial biomass and a disturbance on the autochthonous soil microbial community (Becerra-Castro *et al.*, 2015). Bacteria phyla such as *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* are the most abundant phyla in soil and wastewater habitats and have also been suggested as part of the complex plant microbiome (Jackson *et al.*, 2013; Leff and Fierer, 2013). Soil bacteria are beneficial for plants with complex and dynamic bacterial interactions within the rhizosphere playing important roles for plant health (Hayat *et al.*, 2010). Soil represents a reservoir of bacteria that can enter the plant vascular system (Bai *et al.*, 2015). Therefore, it can be hypothesized that wastewater ARB may outcompete and survive with the autochthonous soil microbiome and become associated with the plant. Microorganisms can colonize plants tissue through the root system or become predominant on the plant surface by aerosol formation during agriculture practices (Karmakar *et al.*, 2018; Pachepsky *et al.*, 2011). Besides numerous studies that show the occurrence of ARB on plant surface (Blau *et al.*, 2018; Hölzel *et al.*, 2018; Zhu *et al.*, 2017), there are also evidences suggesting that the endophytic bacterial community within the plant may be involved in the dissemination of antibiotic resistance (Zhang *et al.*, 2017). These evidences support the hypothesis that ARB with origin in wastewater have the potential to colonize plants and play a role in the dissemination of antibiotic resistance in the environment by wastewater irrigation processes.

1.4.3. The human gut microbiome

The expression human gut microbiota refers to the intricate community of microorganisms that colonize the gastrointestinal tract of humans since birth and that plays crucial roles in human health and disease (Donaldson *et al.*, 2016; Sekirov *et al.*, 2010). The microbial population of the human gut is different among individuals (Hillman *et al.*, 2017) and it is composed mainly by members of the bacterial phyla *Bacteroidetes* and *Firmicutes*, the most abundant, followed by *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Fusobacteria* (Hillman *et al.*, 2017; Tap *et al.*, 2009). The gastrointestinal tract is an open

system exposed to exogenous bacteria through the ingestion of food and water (Derrien and van Hylckama Vlieg, 2015). Some of these bacteria may also harbour ARGs (Salyers *et al.*, 2004). Exogenous bacteria entering humans by the ingestion route, might be able to survive the competition within the complex intestinal microbiome (Forsberg *et al.*, 2012; Vaz-Moreira *et al.*, 2014). The gut microbiota and their associated chemical products maintain the "colonization resistance", in other words, the gut microbiota protect itself against allochthon organisms, preventing exogenous colonization (Buffie and Pamer, 2013; Ribet and Cossart, 2015). Despite the ability of the indigenous gut bacterial community to hamper the colonization of external bacteria, exogenous organisms might still actively complement the gut microbiota (Derrien and van Hylckama Vlieg, 2015).

Bacteria such as some members of the families *Enterobacteriaceae* and *Enterococcaceae* are recognized colonizers of the human gastrointestinal tract. Although these groups are not among the most abundant bacteria in that habitat, they are considered typical gut inhabitants that include fecal contamination indicators. For this reason, these bacterial groups, in particular *Escherichia coli*, have been widely studied as environmental surrogates of ARB of human and animal origin. The ubiquity of these bacteria and the capability to thrive in the environment and human gut place them among the group of possible antibiotic resistance vectors, capable of interchanging ARGs with the native bacterial community (Manaia, 2017; Salyers *et al.*, 2004).

1.5. Hypotheses and objectives of the study

Treated wastewater used for agriculture irrigation may be a source of ARB and ARGs to humans, in particular through the food-chain, with particular emphasis on vegetables that can be consumed raw. The objectives of this work were based on two major hypotheses that proposed that: 1) ARB and ARGs present in treated wastewater are capable of colonizing plants, and establish as endophytes; 2) ARB and ARGs are able to persist in the presence of the autochthonous human microbiota. In order to test these hypotheses, the study was designed aiming to: a) identify bacterial groups that might represent the best candidates as possible vectors of transmission of antibiotic resistance from the environment to humans, through crops and identify the plants that may have an increased capacity to uptake and further transmit to humans environmental bacteria; b) explore the presence of ARB within vegetable-based food products, and c) assess the survival and persistence of ARB and ARGs in the presence of human fecal microbiome material.

2. Thesis roadmap

The recycling of wastewater is increasing worldwide, however, its use may pose still not evaluated risks concerning the dissemination of ARB and their associated resistance determinants in the environment. One of the main concerns of such dissemination is the contamination of the human food chain through crops irrigated with wastewater. Currently, evidences of transmission of ARB and ARGs from the environment to humans are scarce. Such uncertainty contributes to raising the concerns about wastewater reuse as it is admitted that direct or indirect transmission to humans may occur. This concern was the major motivation of this project, which focused on the assessment of the risks of antibiotic resistance transmission from treated wastewater to humans.

The core hypothesis of this study was that antibiotic resistant bacteria thriving in wastewater have the potential to reach humans and survive the human microbiome competition. In a water reuse scenario, crops were considered among the most probable paths of transmission from wastewater to humans. Therefore, a literature review was conducted aiming at investigating about the likelihood of wastewater bacteria become part of plant endophytic bacterial community, and among these community members which could be considered potential human colonizers. Based on this framework, a comprehensive overview of bacterial endophytes occurring in edible plants was obtained from literature published after 1995. Members of these bacterial communities were selected based on the relevance as possible carriers of antibiotic resistance genes and also as capable of colonizing humans.

From this information, it was possible to suggest some plants as potential sources of ARB from the environment to humans. This review is presented in Chapter 3 and discusses the bacterial diversity associated with edible vegetables that are normally consumed raw, the likelihood to colonize humans, and of transmitting ARGs. Under this rationale, as major conclusion, endophytic bacteria belonging to genera such as *Enterobacter*, *Acinetobacter*, *Pseudomonas, Staphylococcus, Burkholderia*, *Serratia*, *Stenotrophomonas* and *Bacillus* were found. It is argued that if bacteria of these groups are supplied in the irrigation water, they can be uptake by the plant, as well as their ARGs, making of these endophytes potential vectors in the transmission of antibiotic resistance from the wastewater to humans. Edible vegetables normally consumed raw, such as lettuce, tomato, cucumber, radish and carrot, were identified as potential paths of transmission of environmental ARB to humans. This

review work suggested that plants may play a role as vehicles of antibiotic resistance between the environment and humans and, if used for irrigation, treated wastewater might be a likely source of human-derived bacteria.

Besides the fact that vegetables and fruits might internalize bacteria from the surrounding environment (e.g. water and soil), the surface of vegetables or fruits may also be contaminated during the whole production system, allowing these bacteria to enter the food chain and be ingested by humans. To test this hypothesis, culturable bacteria thriving on the surface of watercress and within processed strawberry were examined. The bacterial fraction displaying resistance phenotypes to at least three antibiotics classes were identified and characterized for their antibiotic resistance phenotypes (Chapter 4). ARGs, class 1 integron integrase gene and plasmid incompatibility groups were also screened. Additionally, the possibility that plant-associated isolates might be susceptible to acquire ARGs, was also tested. The main findings were that plant-based products contain high counts of cultivable bacteria, identified as members of the genera *Pseudomonas, Stenotrophomonas, Erwinia, Rahnella, Methylobacterium,* and *Chryseobacterium,* some of which were previously identified as endophytic organisms and hosts of ARGs. However, only a minor fraction of isolates was ARB, mostly with intrinsic antibiotic resistance profiles. Also, preliminary results suggested that plant-associated bacteria may be susceptible to acquire antibiotic resistance and therefore, be involved in the dissemination of antibiotic resistance.

Considering the possibility that wastewater ARB may reach the human gut, a pertinent hypothesis is that these bacteria and their ARGs can be outcompeted by the complex human gut microbiome. This hypothesis was tested using feces-based microcosm assays inoculated with the wastewater isolates *E. coli* strain A2FCC14 and *Enterococcus faecium* H1EV10, harbouring the ARGs bla_{TEM}, bla_{CTX}, $bla_{\text{OXA-A}}$, and *van*A, respectively. The effect of aerobic or anaerobic conditions and of the presence of sub-inhibitory concentrations of cefotaxime and vancomycin under anaerobic conditions, were evaluated. Microcosm assays were monitored for at least 7 days, based on bacterial community analyses, cultivation and quantitative PCR. As discussed in Chapter 5, this work supported the hypothesis stated above by showing that the spiked ARGs of the inoculated ARB persisted in fecal microbial community for a week and the they could be quantified at least for one month, under both aerobic and anaerobic conditions. The presence of sub-inhibitory concentrations of antibiotics did not led to significant differences on the persistence of the tested ARGs when compared with antibiotic-free microcosms. This study demonstrated that ARGs of

wastewater ARB can persist at least for a week in the presence of the complex fecal microbiome and, even when the spiked ARB were below the culturable detection limit, their ARGs are quantifiable by qPCR. In contrast, cell-free DNA was readily degraded by the fecal material microbiota, suggesting that the bacterial host is determinant for ARGs persistence. The potential of wastewater ARB to survive the human gut microbiome and of their ARGs to persist for longer periods support the hypothesis of a potential successful transmission to humans.

This thesis includes three scientific peer-reviewed articles:

Chapter 3 – "Clues to investigate antibiotic-resistance transmission through endophytic bacteria", submitted;

Chapter 4 – "Bacterial diversity of culturable and potentially antibiotic resistant bacteria thriving on the surface of edible fruit and vegetables" submitted;

Chapter 5 – "Persistence of wastewater antibiotic resistant bacteria and their genes in human fecal material" (Scaccia *et al.*, 2020).

3. Clues to investigate antibiotic-resistance transmission through endophytic bacteria

Submitted for publication:

Authors: Nazareno Scaccia, Ivone Vaz-Moreira and Célia M. Manaia

Title: “Clues to investigate antibiotic-resistance transmission through endophytic bacteria”.

3.1. Abstract

Antibiotic resistance is a major human health threat with global distribution across humans, animals, plants and the environment. Under the One-Health concept, the contamination of water bodies and soil by antibiotic resistant bacteria cannot be dissociated from its potential transmission to humans, with edible crops representing likely paths. This literature review summarizes some ubiquitous bacterial groups, hosted by edible crops and that can also colonize humans and eventually cause opportunistic infections. Examples of these are members of genera that integrate important opportunistic pathogens often multidrug resistant (e.g. *Pseudomonas* spp., *Enterobacter* spp. or *Acinetobacter* spp.). Edible crops that host ubiquitous bacteria that combine the capacity to colonize humans, opportunistic pathogenicity, and acquired antibiotic resistance prefigure a potential human health threat.

3.2. Vegetables as potential vectors of antibiotic resistance

The use of antimicrobials, along with other chemicals, in veterinary and human medicine, as well as in agriculture, has contributed to generate environmental reservoirs of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) which represent a threat for the human health (Martens and Demain, 2017; Singer *et al.*, 2016). High levels of antibiotic resistance in several infections caused by bacteria have been found, making antibiotic resistance on of the major threats to the public health system, at a global level (WHO, 2018b).

Bacteria resistant to antibiotics and their associated ARGs are distributed across the distinct sectors comprehended under the One Health concept: humans, animals, plants, and the natural environment (McEwen and Collignon, 2018). Therefore, the human food chain is under strong pressure of contamination with ARB and ARGs (Bengtsson-Palme *et al.*, 2018; Chen *et al.*, 2019; Founou *et al.*, 2016). Although the modes of human exposure to environmental ARB and ARGs are still poorly understood, ingestion is probably a major entry portal (Manaia, 2017). Hence, food products such as vegetables usually consumed without any processing (e.g. raw vegetable salads) have been suggested as part of the antibiotic resistance transmission route from the environment to humans (Araújo *et al.*, 2017; Blau *et al.*, 2018; Hölzel *et al.*, 2018; Zhu *et al.*, 2017). While plants are naturally colonized by bacteria thriving in the surrounding water and soil environment (**Box 3.1**), the risks of plant association with ARB are enhanced with the enrichment of the environmental resistome with contaminant ARGs. This enrichment may result from practices such as soil amendment with manure or reuse of wastewater for irrigation (**Box 3.2**), mainly if they do not follow adequate safety recommendations (Becerra-Castro *et al.*, 2015; Zhang *et al.*, 2019). Based on these considerations, it is hypothesized that ARB and ARGs present in irrigation water or manure-based fertilizers can be transferred to plants (Tien *et al.*, 2017; Yang *et al.*, 2016; Zhang *et al.*, 2019) and from this source can reach humans through ingestion (**Figure 3.1**). This hypothesis has particular relevance for vegetables that are usually consumed uncooked or minimally processed (De Corato, 2019). Although bacteria thriving the surface of the plant can be removed by adequate washing procedures, this does not apply to endophytic bacteria, whose persistence after washing or disinfection and potential transmission to humans is recognized (Jackson *et al.*, 2013; Rosenblueth and Martinez-Romero, 2006).

Box 3.1. Plant-associated bacteria

Plants hold a large microbial diversity in different compartments of the vascular system and on the surface structures, known as endosphere and ectosphere, respectively (Berg *et al.*, 2014; Vorholt, 2012). The microbial community on the above-ground part of the plant is generally inhabited by phyllosphere bacteria that live on the phylloplane (leaves) or carposphere (fruits) microenvironments, which are exposed to the air microbiota (**Figure 3.1**). Phyllosphere bacteria may also originate from irrigation and agriculture practices, with origin in soil or water. Endophytic bacteria refer to bacteria that does not injury the plant, at least visibly, and can be isolated from the internal part of the plant (**Figure 3.1**) (Santoyo *et al.*, 2016). Endophytic bacteria can enter the plant mainly from the underground environment, specifically from the soil microbiota and root-associated bacteria (rhizosphere bacteria) (**Figure 3.1**) (Compant *et al.*, 2019; Frank *et al.*, 2017). Particularly, soil bacteria can enter the plant through plant root system suggesting the role of soil as the main reservoir of plant-associated bacteria (Chen *et al.*, 2019; Hallmann *et al.*, 1997). In addition, agriculture practices (irrigation procedure or manure application) may impact both soil and plant microbiota (Becerra-Castro *et al.*, 2015)

Box 3.2. A possible link between wastewater and agriculture

Water scarcity and droughts have been increasing and, it has been estimated that 40% of the population around the globe will suffer water stress in the next 50 years (Becerra-Castro *et al.*, 2015). Food needs due to the growing world population may also contribute to raise the water needs around the globe. Water scarcity is a reality worldwide and the use of treated wastewater for agriculture irrigation is becoming a reality. Treated wastewater refers to wastewater that has been processed through a physical, chemical and biological procedure to reduce its organic matter (C, carbon; N, nitrogen; P, phosphorus) content and should meet the quality criteria to be discharged in the environment (Becerra-Castro *et al.*, 2015; Mateo-Sagasta *et al.*, 2013). The reuse of treated wastewater has been incentivized in some world regions, mainly those under water stress, specifically for agriculture irrigation, because it can supply nutrients and contribute for natural resources recycling (Aleisa and Al-Zubari, 2017; Mateo-Sagasta *et al.*, 2013). Also, it may represent a valid alternative to overcome the unbalance between water demand and water supply. Treated wastewater is reused in different countries. For instance, Israel, Cyprus, Malta, Gulf Cooperation Council (GCC) countries (e.g. Qatar) destinate treated wastewater effluent mainly for agricultural purposes having a high reuse rate (Aleisa and Al-Zubari, 2017; Becerra-Castro *et al.*, 2015). Despite all the benefits that may arise from this practice, if adequate practices are not adopted, the reuse of treated wastewater pose environmental as well as human health risks. For instance, the high content of salts in wastewater may be responsible for soil salinization, which would be deleterious for agricultural crops (Becerra-Castro *et al.*, 2015; Mateo-Sagasta *et al.*, 2013). With respect to the human health, if wastewater contains antibiotic resistant bacteria or pathogens, such as bacteria, viruses, protozoa or helminths that can reach humans via food chain supply, the reuse of wastewater can represent a threat, even if the consequences are not perceived in the short time (Mateo-Sagasta *et al.*, 2013). Wastewater treatment plants have been recognized as potential sources of antibiotic resistance (Piña *et al.*, 2018; Rizzo *et al.*, 2013). The broad diversity of human- and animal-derived bacteria present in treated wastewater harbour ARGs that might be associated with MGEs and may disseminate among bacteria (Rizzo *et al.*, 2013).

Although the impacts of environmental ARB on humans are still poorly characterized, the likelihood of transmission from treated wastewater to humans, via food-products irrigated with reused water cannot be discarded.

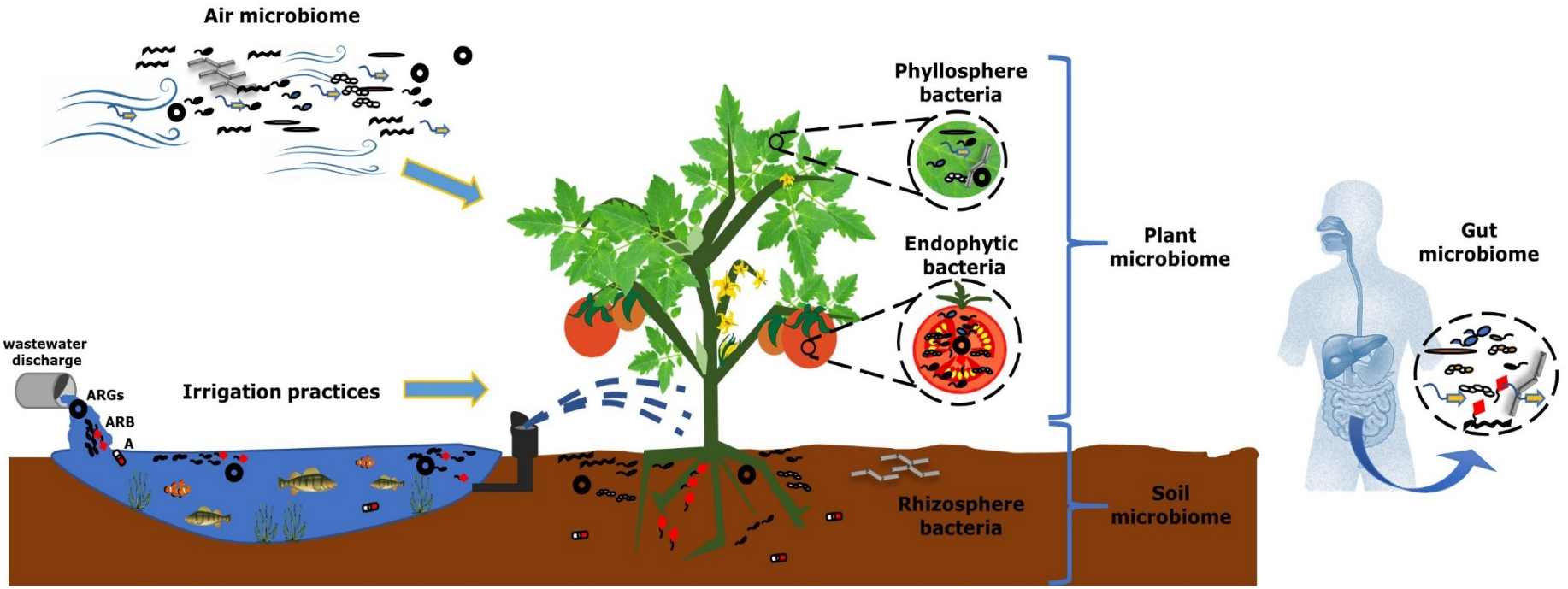


Figure 3.1. Possible routes that bacteria may use to become associated with plants and possible paths of transmission to humans through the consumption of uncooked vegetables. The consumption of raw vegetables, purchased at the retail market or produced at home, can be a source of bacteria, some of which may be antibiotic resistant and/or opportunistic pathogens capable of reaching the human gastrointestinal tract. Additionally, the ingestion of fresh products may have a higher concentration of free ARGs (black circles) than cooked vegetables and therefore, these genes may enter the human body where they can be uptake by native gut bacteria becoming stable genetic elements. Legend: A, antibiotics; ARB, antibiotic resistant bacteria; ARGs, antibiotic resistance genes.

Once ingested exogenous ARB may become resident in the gut microbiome community or, even during transient colonization, these bacteria may be able to transfer ARGs to the native gut microbiome (**Box 3.3**) (Chen *et al.*, 2019; Hölzel *et al.*, 2018). Considering this hypothesis, this work sought to explore the diversity of endophytic bacteria that have been found in edible crops, with a special focus on bacterial groups that have been found associated with the human microbiome and are recognized harbours of acquired ARGs. Since intended and unintended water reuse for irrigation is increasingly a reality, mainly in world regions under water stress (Aleisa and Al-Zubari, 2017), it was investigated if some of those bacterial groups can be found also in wastewater. With this rationale, bacterial groups and associated ARGs occurring simultaneously in wastewater, as plant endophytes and in the human microbiome, were searched in the literature and databases. The final goal was to identify bacterial groups that might represent the best candidates as possible vectors of transmission of antibiotic resistance from the environment to humans, through crops. Simultaneously, it was aimed at the shortlisting of plants that may have the capacity to uptake and further transmit to humans environmental ubiquitous bacteria.

3.3. Context and approach

Scientific publications published between 1995 and 2017 and meeting the keywords "endophytic bacteria", "bacterial endophytes", "communities of endophytes", and "bacterial communities and vegetables" were searched on PubMed and Google Scholar. From these publications were also retrieved some other sources of information based on the respective references list. Because the survey focused exclusively on endophytic bacteria, only research papers reporting data for bacteria identified in plants that were subjected to surface-sterilization procedures prior to the microbiota analyses were considered. A total of 67 publications were explored for the diversity of endophytic bacteria in plants. Most of the publications relied on culture-based methods and bacterial identification based on 16S rRNA gene sequence analysis. Only six out of 67 publications used pyrosequencing to explore the endophytic microbial community (for example, in lettuce, spinach, tomato, grapevine, soybean, wheat or populus). The data collected comprised endophytic bacteria reported in a total of 49 crop varieties, represented by 22 phyla.

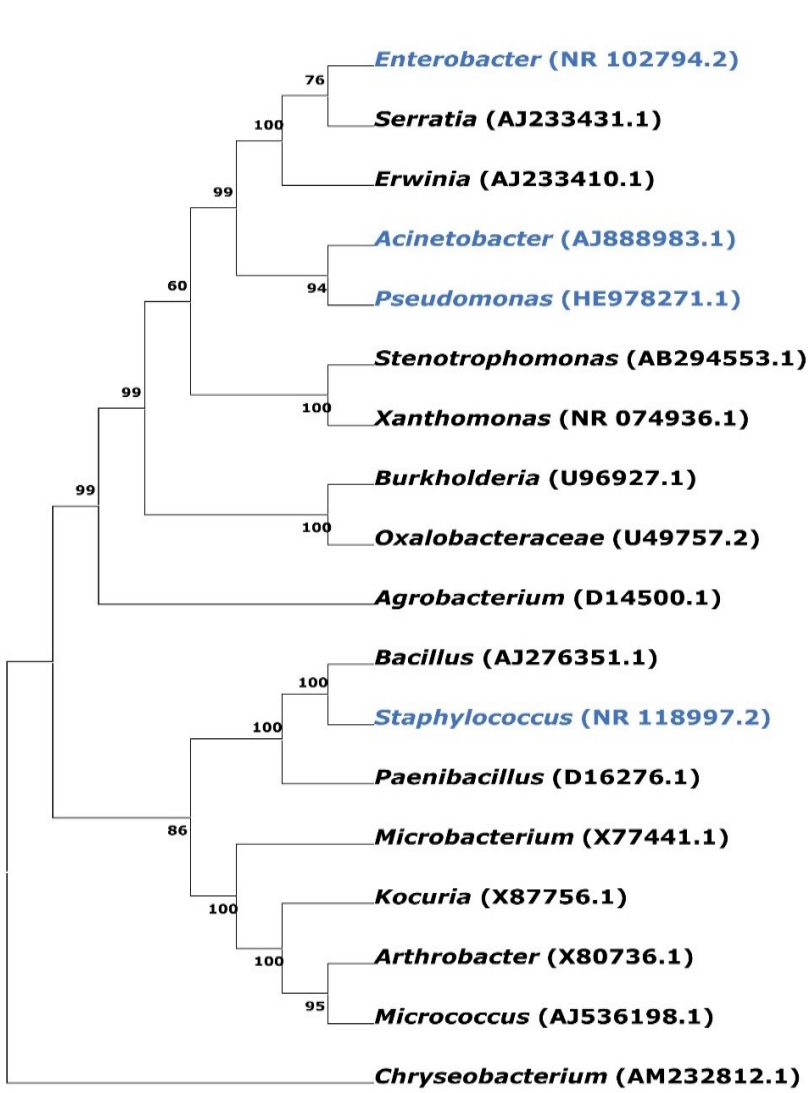
Box 3.3. A possible link between crops and the human resistome

"Gut microbiota" refers to all microorganisms living within the gastrointestinal tract. This microbial community plays a pivotal role in human health and disease (Wang *et al.*, 2017). The gut microbiota bacterial community is complex and rich in members of phyla such as *Bacteroidetes* and *Firmicutes,* the most abundant, followed by *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Fusobacteria* (Cani, 2018; Carding *et al.*, 2015). The gut microbiota compromises the native (indigenous or autochthonous) microbial flora which resides permanently in the gastrointestinal tract and the exogenous (or allochthonous) microorganisms that can colonize transiently the intestine (Derrien and van Hylckama Vlieg, 2015). Diet is an important source of exogenous bacteria and, it can also influence the microbial flora of the gut. For instance, plant-based food products may promote a greater microbial diversity of the gut or, stimulate the prevalence of beneficial gut-associated bacteria (Tomova *et al.*, 2019). Nevertheless, an important trait of the autochthonous gut microbial community is the "colonization resistance" or "barrier effect", which refers to the ability of the indigenous resident organisms to prevent the colonization of the gut by transient or exogenous bacteria. However, ingested bacteria can be temporarily integrated within the autochthonous microbiota and be part of the so-called transient microbiome (Derrien and van Hylckama Vlieg, 2015). Exogenous bacteria may impact the autochthonous gut microbes directly or indirectly, particularly exogenous bacteria can i) stimulate growth of the resident bacteria by production of specific metabolites, ii) impact pathogens by secondary processes (decrease of pH, niche competition or bacteriocins production) and iii) impact the gut microbiota by host response stimulation (Derrien and van Hylckama Vlieg, 2015). The gut microbiota corresponds to a dense and diverse community of bacteria, where horizontal gene transfer may occur with the spread of ARGs. If transient bacteria harbour acquired ARGs, in the presence of antibiotics, or even in its absence, may transfer those determinants to the native community (Salyers and Shoemaker, 2006).

To investigate the endophytic bacteria diversity only in edible vegetables, the data were filtered for crop varieties based on the fact that at least part of these are edible, and the endophytes were reported in two or more publications. Using these criteria, were identified in total 11 edible plants (banana, bell pepper, black pepper, carrot, cucumber, ginseng, lettuce, onion, radish, sugar beet, and tomato). For this group of edible plants were screened the most representative endophytic bacterial groups, refining the search based on the criteria of being reported in at least 5 publications (**Table S3.2**). Eighteen taxa fulfilled those criteria and were used as watchwords to characterize endophytic bacteria distribution in plants, namely vegetables that can be consumed raw, wastewater and human microbiome, characterizing their potential for pathogenicity and antibiotic resistance (**Table S3.2**; **Figure 3.2**). The dataset was later narrowed, to lettuce, carrot, radish, cucumber and tomato, because these are the edible plants frequently consumed raw, previously described with increased capacity to uptake contaminants of emerging concern (Christou *et al.*, 2018) (**Figure 3.2**).

The bacterial endophytes reported in those plants were searched in the wastewater microbiome, herein considered to be raw and treated wastewater and activated sludge. The same bacterial groups were searched in the Human Microbiome Project Catalog (https://www.hmpdacc.org/catalog/). The antibiotic resistance profiles of bacteria belonging to the same groups as the bacterial endophytes were searched in the databases CARD (comprehensive antibiotic resistance database - https://card.mcmaster.ca/analyze/rgi) and ARDB (antibiotic resistance genes database - https://ardb.cbcb.umd.edu/index.html). The potential virulence of bacteria belonging to the same groups of the bacterial endophytes was characterized inferred from the literature, in particular regarding reports of bacterial infectious diseases (**Figure 3.2**; **Table S3.2**).

Additionally, whole genomes sequences of endophytic bacteria closely related to human pathogens such as *Enterobacter*, *Acinetobacter*, *Pseudomonas* and *Staphylococcus* herein found associated with lettuce, carrot, radish, cucumber and tomato (**Figure 3.2**), were searched on the genome database of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Two genomes for each genus were selected, based on their endophytic origin, and analysed for the presence of ARGs using the databases CARD and ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/). Genomes annotation was done using RAST (http://rast.theseed.org/FIG/rast.cgi) as a screening for other relevant genes such as metal resistance genes (MRGs).



Family and genus	Raw eaten vegetables	Habitat	AR and pathogenicity	References
Enterobacter (NR 102794.2)		HM, WM	ARGs, HP	(Amador *et al.*, 2015; Pati *et al.*, 2018)
Serratia (AJ233431.1)		HM, WM	ARGs, HP	(Gupta and Thakur, 2015; Khanna *et al.*, 2013)
Erwinia (AJ233410.1)		WM	ARGs	(Li *et al.*, 2010)
Acinetobacter (AJ888983.1)		HM, WM	ARGs, HP	(Da Silva *et al.*, 2007)
Pseudomonas (HE978271.1)		HM, WM	ARGs, HP	(Azam and Khan, 2019; Luczkiewicz *et al.*, 2015)
Stenotrophomonas (AB294553.1)		HM, WM	ARGs, HP	(Brooke, 2012; Kalidasan *et al.*, 2018)
Xanthomonas (NR 074936.1)		n.i.	ARGs	n.i.
Burkholderia (U96927.1)		HM, WM	ARGs, HP	(Eberl and Vandamme, 2016; Liu *et al.*, 2014)
Oxalobacteraceae (U49757.2)		HM, WM	ARGs	(Baldani *et al.*, 2014)
Agrobacterium (D14500.1)		WM	ARGs	(Christakis *et al.*, 2006; Yan *et al.*, 2017)
Bacillus (AJ276351.1)		HM, WM	ARGs, HP	(Bottone, 2010; Vaz-Moreira *et al.*, 2014)
Staphylococcus (NR 118997.2)		HM, WM	ARGs, HP	(Gómez *et al.*, 2016; Lakhundi and Zhang, 2018)
Paenibacillus (D16276.1)		HM, WM	ARGs, HP	(Grady *et al.*, 2016; Mohammed *et al.*, 2017)
Microbacterium (X77441.1)		HM, WM	n.i.	(Kim *et al.*, 2011)
Kocuria (X87756.1)		HM, WM	HP	(Benmalek and Fardeau, 2016; Dotis *et al.*, 2015)
Arthrobacter (X80736.1)		HM, WM	ARGs, HP	(Agunbiade *et al.*, 2017; Bernasconi *et al.*, 2004)
Micrococcus (AJ536198.1)		HM, WM	HP	(Liu *et al.*, 2007; Miltiadous and Elisaf, 2011)
Chryseobacterium (AM232812.1)		HM, WM	ARGs, HP	(Kämpfer *et al.*, 2003; Mehta and Pathak, 2018)

Figure 3.2. Most representative endophytic bacterial family and genera (cited in ≥ 5 publications) found in carrot, cucumber, lettuce, radish, and tomato and, their occurrence in wastewater (WM) and human (HM) microbiome. For the different taxa are indicated the occurrence of antibiotic resistance genes (ARGs) or of members described as human pathogens (HP). In blue are indicated bacteria closely related to human pathogens. n.i., no information was found.
Note: For the construction of the dendrogram were used sequences of the type species or type genus as representatives of each genus or family, respectively.

3.4. Wastewater, plants and human gut: different habitats for the same bacteria?

Endophytic bacteria reported in the literature comprised an impressive diversity, being the most commonly found members of the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, also described as prevailing community members in soil and wastewater habitats (Becerra-Castro *et al.*, 2015; Vaz-Moreira *et al.*, 2014).

At the class level, *Gamma*-, *Alpha*- and *Betaproteobacteria* were the predominant *Proteobacteria*, together with *Bacilli* and *Clostridia* from *Firmicutes*. The phylum *Actinobacteria* was represented by the *Actinobacteria* class, whilst the most abundant classes of *Bacteroidetes* were *Flavobacteriia* and *Chitinophagia*.

3.4.1. Endophytic bacteria in edible plants

The endophytic bacteria observed in edible plants usually consumed raw and most commonly reported in the literature (cited at least in 5 publications) were observed to belong to 17 bacterial genera and one bacterial family (**Figure 3.2**; **Table S3.2**). These endophytic bacteria belonged to the classes *Flavobacteriia* (phylum *Bacteroidetes*), *Alpha*-, *Beta*- and *Gammaproteobacteria* (phylum *Proteobacteria*), *Bacilli* (phylum *Firmicutes*), and order *Micrococcales* (phylum *Actinobacteria*). These bacteria were the genera *Enterobacter*, *Serratia*, *Erwinia*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Burkholderia*, *Agrobacterium*, *Bacillus*, *Staphylococcus*, *Paenibacillus*, *Microbacterium*, *Kocuria*, *Arthrobacter*, *Micrococcus, Chryseobacterium* and the family *Oxalobacteraceae* (**Figure 3.2**; **Table S3.2**). Most of these bacterial groups comprise ubiquitous mesophilic bacteria, facultative aerobes, non-fastidious, with fast growth, wide metabolic versatility and recognized genome plasticity (Asif *et al.*, 2018; Azam and Khan, 2019; Khanna *et al.*, 2013; Li *et al.*, 2010; Santajit and Indrawattana, 2016). Such characteristics justify that members of those genera can also thrive in humans, animals, soil, plants, waters, or sewage (Asif *et al.*, 2018; Khanna *et al.*, 2013; Li *et al.*, 2010; Santajit and Indrawattana, 2016).

The role of these bacteria as plant symbionts, growth promotors, xenobiotic degraders, or as human and animal pathogens is also of note (Asif *et al.*, 2018; Gkorezis *et al.*, 2015; Khanna *et al.*, 2013; Li *et al.*, 2010; Liu *et al.*, 2012; Luczkiewicz *et al.*, 2015; Pati *et al.*, 2018). These different roles can indeed be observed in a same genus, whose members in general share eco-physiological properties and therefore the range of habitats where they can thrive. For example, some *Gamma-proteobacteria,* such as *Stenotrophomonas* spp. or *Xanthomonas* spp. are obligate aerobes, non-fermentative bacteria ubiquitously found in soil and plants (An *et al.*, 2019; Kalidasan *et al.*, 2018). In the genus *Stenotrophomonas* it is

possible to find plant growth-promoting bacteria (PGPB) with bioremediation potential, while species S. *maltophilia* is an important human pathogen (Brooke, 2012). Other examples of recognized humans and animals opportunistic pathogens of *Gamma-proteobacteria* are included in ubiquitous genera of *Pseudomonas*, *Acinetobacter* or *Enterobacter*, specifically the species *Enterobacter cloacae*, *Acinetobacter baumannii* or *Pseudomonas aeruginosa* (Santajit and Indrawattana, 2016). This multiple beneficial and harmful effects can also be observed in the class *Beta-proteobacteria,* in which, for example, members of the genus *Burkholderia* comprising obligate aerobic bacteria that are distributed in the soil, plants, animals and humans (Eberl and Vandamme, 2016), and includes also important pathogens, such as the members of *Burkholderia cepacia* complex (Eberl and Vandamme, 2016; Liu *et al.*, 2014). Members of the phylum *Firmicutes,* many of which are facultative anaerobes, non-fastidious, endospore-forming bacteria are also ubiquitously distributed among humans, animals, plants, soil, and water environments, being of these the *Bacillus, Paenibacillus* and *Staphylococcus* good examples (Bottone, 2010; Grady *et al.*, 2016; Lakhundi and Zhang, 2018). As for the Gram-negative *Proteobacteria,* these genera comprise plant growth promoters, pollutant degraders, and human pathogens (Chaudhry and Patil, 2016; Grady *et al.*, 2016; Nithya and Babu, 2017). For instance, the species *Bacillus cereus* is responsible for foodborne illness (Bottone, 2010) whilst, the species *Staphylococcus aureus* is an important human pathogen (Mulani *et al.*, 2019; Santajit and Indrawattana, 2016). The same can be illustrated for members of the phylum *Actinobacteria* also widely reported in the environment. Members of genera such as *Microbacterium*, *Kocuria*, *Arthrobacter* or *Micrococcus* comprise plant beneficial bacteria, biocontrol agents and, simultaneously, opportunistic pathogens causing endocarditis (e.g. *Arthrobacter woluwensis* or *Micrococcus luteus*) (Bernasconi *et al.*, 2004; Dotis *et al.*, 2015; Miltiadous and Elisaf, 2011; Nithya and Babu, 2017). Members of the phylum *Bacteroidetes* are also typical ubiquitous bacteria, with members of the genus *Chryseobacterium* being reported in humans, animals, soil, water, wastewater, and the species *C. indologenes* is considered an emerging human pathogen (Mehta and Pathak, 2018). Based on the diversity of endophytic bacteria reported in edible vegetables the data was refined in order to restrict to those groups that could be found also in wastewater and in the human microbiome, as well as have potential clinical relevance.

3.4.2. Potential overlaps between endophytic and wastewater bacterial communities

Wastewater results from human uses of water in domestic, commercial, and industrial activities, and may include storm water and other urban runoff (Mateo-Sagasta *et al.*, 2013).

The recycling of wastewater, especially for agriculture, has important economic and environmental benefits. The reuse of treated wastewater may have important benefits, as the supply for agriculture production of organic and inorganic matter and micronutrients, aligned with sustainable practices, with reduced fertilizers use. However, water reuse may also comprise some environmental and human health risks, mainly due to contamination of water bodies, soil or plants by contaminants of emerging concern, in which antibiotic resistant bacteria and genes are included (**Box 3.2**) (Becerra-Castro *et al.*, 2015). Treated wastewater contains a wide phylogenetic and ecological diversity of environmental-, human- and animal-derived bacteria. *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Acidobacteria* and *Verrucomicrobia* are reported as predominant community members (Becerra-Castro *et al.*, 2015; Vaz-Moreira *et al.*, 2014). Indeed, these phyla are predominant in different types of natural and human-impacted environments and, not surprisingly, members of the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* were also among the most cited endophytic bacteria observed in the publications surveyed for this review (**Figure 3.2**). At lower taxonomic ranks, it was observed that almost all of the most common endophytic bacteria were observed also in wastewater (Agunbiade *et al.*, 2017; Amador *et al.*, 2015; Baldani *et al.*, 2014; Benmalek and Fardeau, 2016; Da Silva *et al.*, 2007; Gómez *et al.*, 2016; Gupta and Thakur, 2015; Kalidasan *et al.*, 2018; Kämpfer *et al.*, 2003; Kim *et al.*, 2011; Li *et al.*, 2010; Liu *et al.*, 2014; Liu *et al.*, 2007; Luczkiewicz *et al.*, 2015; Mohammed *et al.*, 2017; Vaz-Moreira *et al.*, 2014; Yan *et al.*, 2017) (**Figure 3.2**; **Table S3.2**). This observation supports the hypothesis that bacteria belonging to the same groups can thrive in both wastewater and as plant endophytes, suggesting that plants may uptake bacteria supplied in irrigation water.

3.4.3. Potential overlaps between endophytic and human microbiota

Microbe-host interactions have essential roles in the proper functioning and health of both animal and plant systems (Berg *et al.*, 2017; Wang *et al.*, 2017). In humans, the gut microbiota is the most diverse compared to other body parts, with essential roles in the host metabolism and the emergence of disease episodes in situations of dysbiosis or of microbial imbalance (Cani, 2018; Carding *et al.*, 2015). The human gut microbiota is fed mainly

through diet, with products containing live microorganisms, like water, fermented foods or vegetables consumed raw, which, therefore, represent important sources of bacteria that can colonize the gastrointestinal tract (**Box 3.3**) (Derrien and van Hylckama Vlieg, 2015; Lang *et al.*, 2014). Among endophytic bacteria that were reported in the five edible plants usually consumed raw (lettuce, carrot, radish, cucumber and tomato), 15 bacterial groups were also described in the human microbiome (i.e. gastrointestinal and urogenital tract, oral cavity, skin and airways) (**Figure 3.2**; **Table S3.2**). Specifically, the genera of endophytic bacteria *Enterobacter*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Burkholderia*, *Bacillus*, *Staphylococcus*, *Paenibacillus*, *Microbacterium*, *Kocuria*, *Arthrobacter*, *Micrococcus* and *Chryseobacterium* and the family *Oxalobacteraceae* were also reported in the human microbiome (https://www.hmpdacc.org/catalog/). This observation supports the hypothesis that bacteria belonging to these groups can thrive in both plants and humans, suggesting that endophytic bacteria observed in plants have the potential to colonize the human body. This hypothesis is supported by discussion of Lang and co-authors (2014), who estimated that during a vegan meal (fruits, vegetables and whole grains), 10^6 bacterial cells can be ingested. Among the 50 most abundant bacterial families present in those vegan meals belonged to the same groups as those reported in the current literature survey of plant endophytes, with members of the bacterial families *Enterobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Bacillaceae*, *Staphylococcaceae*, *Paenibacillaceae*, *Micrococcaceae* and *Flavobacteriaceae* being part of that list (Lang *et al.*, 2014).

3.5. Acquired antibiotic resistance in edible plants microbiome: how likely is it?

The antibiotic resistome of the plant microbiome is described as the set of ARGs associated with pathogenic and non-pathogenic microbes present in endophytic and phyllosphere bacteria (Chen *et al.*, 2019). Recently, the role of plant microbiome in the spread of antibiotic resistance has been suggested (Blau *et al.*, 2018; Chen *et al.*, 2019; Sundin and Wang, 2018). For example, the surface microbiome of leaf vegetables such as lettuce has been reported as a source of multidrug resistant bacteria, of transferable plasmids conferring antibiotic resistance and/or ARGs (Blau *et al.*, 2018; Hölzel *et al.*, 2018; Zhang *et al.*, 2019; Zhu *et al.*, 2017). The use of antibiotics in agriculture especially for controlling food crops disease has also been suggested as a driver for the occurrence of acquired antibiotic resistance in plant-associated bacteria (Sundin and Wang, 2018; Zhang *et al.*, 2017). For instance, the long-term application of streptomycin in agriculture is supposed to

have contributed to the selection of plant-associated pathogenic bacteria resistant to this antibiotic. Examples of these pathogens are members of the species *Erwinia amylovora*, *Pseudomonas syringae* or *Xanthomonas campestris,* harbouring distinct streptomycin resistance mechanisms either due to chromosome mutations or associated with the transposon Tn*5393,* presumably through HGT acquisition (Sundin and Wang, 2018). In environmental bacteria, it is common to observe resistance against clinically relevant antibiotics, which, nevertheless, is an intrinsic property of those microorganisms.

Intrinsic antibiotic resistance is found in most or all members of a species and refers to the innate ability of bacteria to resist the antibiotics by mechanisms as diverse low-affinity targets, low cell permeability and presence of efflux system, or other (Peterson and Kaur, 2018). This kind of resistance differs from the acquired type, which risk of dissemination and propagation by HGT is of major concern (Peterson and Kaur, 2018).

Acquired antibiotic resistance comprises mutation of pre-existing genes or HGT processes such as conjugation, transformation or transduction which are involved in the dissemination of ARGs among the bacterial community (Davies and Davies, 2010; Peterson and Kaur, 2018). Bacteria that acquire antibiotic resistance traits may have an enhanced fitness in the presence of antibiotics, meaning a better capacity of survival and proliferation in comparison with bacteria that did not acquire resistance and thus, may become more prevalent in a given environment (Davies and Davies, 2010; Peterson and Kaur, 2018). In some cases, the acquired resistance traits may become stable parts of the microbial genome, without representing additional costs for the cell, even in the absence of any selective pressures. The plant antibiotic resistome may comprise acquired resistance determinants, whose involvement in HGT to opportunistic pathogenic bacteria cannot be neglected (Chen *et al.*, 2019; Dantas and Sommer, 2012; Peterson and Kaur, 2018). The array of antibiotic resistance genes that can be found in endophytic bacterial genera suggests that these bacteria may act as vectors of ARGs in the transmission of ARB from plant to humans (**Table S3.2**). Indeed, genera of the phylum *Proteobacteria* such as *Enterobacter*, *Acinetobacter* or *Pseudomonas*, harbour ARGs that encode resistance against different classes of antibiotics, specifically aminoglycosides, beta-lactams, macrolides, quinolone, sulfonamides and tetracyclines (**Table S3.2**).

While an important part of ARGs described in endophytic bacteria may be part of their intrinsic resistome, some represent interesting examples of ARGs associated with MGEs (e.g. *amp*C, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC}, bla_{OXA48} (Ragupathi *et al.*, 2019)). Moreover, if the range of reported endophytic bacteria comprise

ubiquitous species, some of which with highly dynamic genomes, where acquired traits can be integrated and stabilized, it cannot be discarded the possibility that ARB with acquired ARGs cannot be successful endophytes and therefore be transmitted to humans, once the conditions permit such transmission. These considerations support the hypothesis that endophytic bacteria present in raw-eaten vegetables may harbour ARGs and therefore, when consumed raw, those vegetables may play a role in antibiotic resistance transmission to humans.

3.5.1. ARGs in endophytic bacteria closely related to human pathogens

Members of genera *Enterobacter*, *Acinetobacter*, *Pseudomonas* and *Staphylococcus* were included in the 17 bacterial genera most commonly reported in edible vegetables that can be consumed raw and simultaneously reported in wastewater and in the human microbiome (**Figure 3.2**; **Table S3.2**). These genera include important human opportunistic pathogenic bacterial species (section 3.6), with a wide array of clinically relevant ARGs (**Table S3.2**) and, therefore, deserved a deeper insight. The whole genome sequences of endophytic bacteria of those genera, specifically the strains *Staphylococcus hominis* RIT-PI-k, *Staphylococcus epidermidis* SE2.9, *Enterobacter kobei* ENHKU01, *Enterobacter* sp. 638, *Acinetobacter oleivorans* PF1, *Acinetobacter ursingii* M3, *Pseudomonas syringae* DC3000 and *Pseudomonas syringae* B301D, were available in public databases and were screened for the presence of ARGs and metal resistance genes (MRGs), as described in section 3.3 (**Table 3.1**). This analysis showed that these endophytic bacteria yield a broad diversity of genes encoding antibiotic and metal efflux pumps that may be responsible for multidrug resistance profiles. Efflux pumps display important functions in the host cells, vital bacteria/plant interactions such as plant colonization, plant toxin resistance or plant bacterial virulence (Blanco *et al.*, 2016). Examples of efflux pumps observed in endophytic bacteria were the MexAB-OprM or the MdtABC/MdtUVW, both under the resistance–nodulation–cell division (RND) superfamily, in *Pseudomonas syringae* or in *Erwinia amylovora*, respectively (Blanco *et al.*, 2016). The ARGs bla_z, *erm*C, *fos*B and *mph*C that encode resistance to beta-lactams, macrolides, and fosfomycins were identified in the endophytic *Staphylococcus hominis* RIT-PI-k (**Table 3.1)**. These genes were previously described as plasmid-associated in the genus *Staphylococcus* (Fessler *et al.*, 2018; Ragupathi *et al.*, 2019).

Additionally, *Staphylococcus*, *Enterobacter* and *Acinetobacter* strains displayed the presence of several MRGs for arsenic and chromium resistance (**Table 3.1**).

Table 3.1. Antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) surveyed from whole genome sequences of endophytic bacteria closely related to pathogenic microorganisms. In bold, efflux pump mediated resistance genes.

Endophytic bacterial species closely related to pathogenic	Plant origin	Accession no.	Reference	Assembly level	ARGs[1]	Antibiotic	% Identity	MRGs[2]	Metal	% Identity
Staphylococcus hominis RIT-PI-k	Poison Ivy	LHPB00000000.1	(Tran *et al.*, 2015)	Contig	*bla*$_I$***; *bla*$_Z$**	β-L	99.2; 99.9	*ars*D	As	98.3
					*erm*C**	M	100			
					*gyr*A***; *gyr*B**	Q	99.9; 99.7			
					*par*C***; *par*E***	Q	99.7; 99.7			
					qac*A	**T, M**	**99.8**	***mer*R**	**Co-Zn-Cd**	**99.2**
					tca*A**	**GP**	**99.8**			
					tet*R**	**T**	**98.4**			
					ydh*E/ *nor*M**	**AG**	**98.0**			
Staphylococcus epidermidis SE2.9	Rice seed	JRVN00000000.1	(Chaudhry and Patil, 2016)	Contig	*fos*B**	F	100	*ars*D	As	100
					*gyr*A***; *gyr*B***	Q	99.9; 99.8			
					*mph*C**	M	100			
					*par*C***; *par*E***	Q	100; 99.9			
					dfr*C	**DP**	**99.4**	***czc*D**	**Co-Zn-Cd**	**99.7**
					mgr*A	**T, M**	**95.2**	***mer*R**	**Co-Zn-Cd**	**98.8**
					msr*A	**G**	**98.4**			
					nor*A	**M, Q**	**98.7**			
					qac*A	**T, M**	**99.8**			
					tca*A; *tca*R*****	**GP**	**99.3; 97.3**			
					tet*R**	**T**	**98.2**			
Enterobacter kobei ENHKU01	Bell pepper	CP003737.1	(Liu *et al.*, 2012)	Complete genome	*amp*C***	β-L	99.7	*ars*D	As	99.1
					*fos*A***	F	95.0			
					*gyr*A***; *gyr*B***	Q	99.9; 99.9			
					*par*C***; *par*E***	Q	99.9; 99.8			
					*uhp*T*	F	93.9			

Endophytic bacterial species closely related to pathogenic	Plant origin	Accession no.	Reference	Assembly level	ARGs[1]	Antibiotic	% Identity	MRGs[2]	Metal	% Identity
					oqx*A	**T, M**	**90.8**	***cor*C**	**Cu**	**99.0**
					acr*A	**T, M**	**98.7**	***cus*B; *cus*C**	**Co-Zn-Cd**	**100; 98.7**
					tet*R	**T**	**99.5**	***cus*F; *cus*R**	**Co-Zn-Cd**	**99.1; 99.6**
					bae*R*; *bae*S	**T, β-L, M**	**95.4; 100**	***cut*F**	**Cu**	**99.1**
					emr*B*; *emr*R	**M**	**92.4; 93.1**	***mer*R**	**Co-Zn-Cd**	**100**
					mac*A; ***mac*B***	**MAR**	**99.7; 100**	***scs*C; *scs*D**	**Cu**	**99.0; 99.4**
					mar*C; ***mar*R***	**MAR**	**99.1; 99.1**	***zit*B**	**Co-Zn-Cd**	**100**
					msb*A	**T, M**	**94.7**			
					tol*C	**MAR**	**99.8**			
Enterobacter sp. 638	Poplar	CP000653.1	(Taghavi *et al.*, 2009)	Complete genome	*glp*T*	F	92.9	*ars*H	As	100
					*gyr*A***; *gyr*B***	Q	100; 100			
					*par*C***; *par*E***	Q	100; 100			
					tet*R	**T**	**100**	***cor*C**	**Cu**	**98.9**
					bae*R*; *bae*S	**T, β-L, M**	**94.1; 100**	***cus*A; *cus*B**	**Co-Zn-Cd**	**100; 100**
					crp*	**T, M,**	**97.6**	***cut*A; *cut*C; *cut*F**	**Cu**	**100; 100 100**
					emr*R	**M**	**94.3**	***ltn***	**Cu**	**100**
					kpn*H	**T, β-L**	**92.2**	***mer*R**	**Co-Zn-Cd**	**100**
					mac*A; ***mac*B***	**MAR**	**100; 100**	***zit*B**	**Co-Zn-Cd**	**100**
					mar*A; ***mar*B***	**MAR**	**100; 94.2**			
					mar*C; ***mar*R***	**MAR**	**100; 95.8**			
					mdt*B; ***mdt*C***	**MAR**	**100; 100**			
					msb*A	**T, M**	**94.0**			
					nod*T**	**MAR**	**100**			
					tol*C	**MAR**	**100**			
Acinetobacter oleivorans PF1	Poplar	JHQK00000000.1	(Gkorezis *et al.*, 2015)	Contig	*amp*C*	β-L	93.7	*ars*B; *ars*H	As	100; 100
					bla_{OXA}***; $bla_{OXA-359}$*	β-L	99.6; 96.3	*chr*A	Cr	100
					*gyr*A***; *gyr*B***	Q	97.8; 96.8			
					*par*C***; *par*E***	Q	97.2; 97.4			

Endophytic bacterial species closely related to pathogenic	Plant origin	Accession no.	Reference	Assembly level	ARGs[1]	Antibiotic	% Identity	MRGs[2]	Metal	% Identity
					***aba*Q**[*]	**T, M**	**98.1**	***cor*C**	**Cu**	**99.3**
					***abe*S**[*]	**T, AG**	**96.3**	***czc*A; *czc*B; *czc*D**	**Co-Zn-Cd**	**97.5; 92.6; 100**
					***ade*F**[*]**; *ade*G**[***]**; *ade*L**[*]**; *ade*K**[*]	**MAR**	**98.0; 98.1; 97.3; 96.5**	***ltn***	**Cu**	**96.5**
					***amv*A**[*]	**T, M**	**95.1**	***mer*R**	**Co-Zn-Cd**	**95.5**
					***cme*B**[***]**; *cme*C**[***]	**MAR**	**95.6; 100**			
					***mac*A**[***]**; *mac*B**[***]	**MAR**	**98.2; 100**			
					***tol*C**[***]	**MAR**	**100**			
Acinetobacter ursingii M3	Duckweed	AP018824.1	(Ishizawa *et al.*, 2018)	Complete genome	*amp*C[***]	β-L	98.1	*ars*B; *ars*H	As	100; 100
					*gyr*A[***]; *gyr*B[***]	Q	100; 99.8	*chr*A; *chr*B	Cr	99.4; 100
					*par*C[***]; *par*E[***]	Q	99.7; 99.8			
					***cme*B**[***]**; *cme*C**[***]	**MAR**	**100; 100**	***cor*C**	**Cu**	**91.7**
					***mac*A**[***]**; *mac*B**[***]	**MAR**	**100; 100**	***czc*A; *czc*D**	**Co-Zn-Cd**	**100; 98.7**
					***tol*C**[***]	**MAR**	**100**	***cus*R**	**Co-Zn-Cd**	**99.5**
								ltn	**Cu**	**99.2**
								***mer*R**	**Co-Zn-Cd**	**100**
Pseudomonas syringae DC3000	Tomato	AE016853.1	(Buell *et al.*, 2003)	Complete genome	*gyr*A[***]; *gyr*B[***]	Q	99.9; 100			
					*par*C[***]; *par*E[***]	Q	99.9; 99.8			
					***cme*B**[***]**; *cme*C**[***]	**MAR**	**99.7; 100**	***acr*3**	**As**	**91.7**
					***mac*A**[***]**; *mac*B**[***]	**MAR**	**100; 100**	***cor*C**	**Cu**	**100**
					***mex*E**[***]**; *mex*F**[***]	**MAR**	**99.8; 99.9**	***czc*A; *czc*C**	**Co-Zn-Cd**	**100; 99.0**
					***mex*T**[***]	**MAR**	**99.8**	***ltn***	**Cu**	**99.4**
					***opr*N**[***]	**MAR**	**99.9**	***mer*R**	**Co-Zn-Cd**	**98.4**
					***tet*R**[***]	**MAR**	**99.6**			
					***tol*C**[***]	**MAR**	**99.8**			
					***ydh*E/*nor*M**[***]	**Q, AG**	**89.4**			
Pseudomonas syringae B301D	Sugarcane	CP005969.1	(Ravindran *et al.*, 2015)	Complete genome	*amp*C[***]	β-L	100	*ars*H	As	100
					*gyr*A[***]; *gyr*B[***]	Q	99.9; 99.9			
					*par*C[***]; *par*E[***]	Q	99.9; 100			

Endophytic bacterial species closely related to pathogenic	Plant origin	Accession no.	Reference	Assembly level	ARGs[1]	Antibiotic	% Identity	MRGs[2]	Metal	% Identity
					***cme*B**[***]	**MAR**	**99.8**	***acr*3**	**As**	**99.7**
					***mac*B**[***]	**MAR**	**100**	***cor*C**	**Cu**	**100**
					***mex*E**[***]**; *mex*F**[***]**; *mex*T**[***]	**MAR**	**99.8; 99.8; 100**	***czc*A; *czc*C**	**Co-Zn-Cd**	**99.8; 94.9**
					***opr*N**[***]	**MAR**	**99.9**	***ltn***	**Cu**	**100**
					***tet*R**[***]	**MAR**	**99.5**	***mer*R**	**Co-Zn-Cd**	**99.5**
					***tol*C**[***]	**MAR**	**100**			
					***ydh*E/*nor*M**[***]	**Q, AG**	**89.9**			

[1] Endophytic bacterial species closely related to pathogenic microorganisms were screened for the presence of ARGs using the following database: RGI (Resistance Gene Identifier) from CARD (https://card.mcmaster.ca/analyze/rgi)[*]; ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder)[**] and RAST (Rapid Annotation using Subsystem Technology) (http://rast.theseed.org/FIG/rast.cgi)[***].

[2] Endophytic bacterial species closely related to pathogenic microorganisms were also surveyed for the presence of metal resistance genes using RAST (http://rast.theseed.org/FIG/rast.cgi).

Legend for the classes of antibiotics: Aminoglycosides (AG), Beta-lactams (β-L), Diaminopyrimidins (DP), Fosfomycins (F), Glycopeptide (GP), Macrolides (M), Oxazolidinones (OL), Quinolone (Q), Streptogramins (SG), Tetracyclines (T), Multiple Antibiotic Resistance (MAR). Legend for metals: Arsenic (As), Cadmium (Cd), Chromium (Cr), Cobalt (Co), Copper (Cu) and Zinc (Zn). The threshold value used for all databases was > 90% similarity.

Although these MRGs might be responsible for intrinsic resistance, these findings should deserve further investigation in the future. Indeed, the co-localization of ARGs and MRGs on the same MGEs might promote horizontal transmission of antibiotic resistance among bacteria under the selective pressure exert of environmental pollutants such as antibiotics and metals (Fessler *et al.*, 2018; Li *et al.*, 2017; Pal *et al.*, 2015).

3.6. Assessment of human health risk related to ARB and ARGs transmission to humans through edible plant

Some of the groups of endophytic bacteria, in particular, *Enterobacter*, *Acinetobacter*, *Pseudomonas*-and *Staphylococcus* belong to genera that comprise human pathogens with the recognized capability of acquiring ARGs. Those genera are phylogenetically closely related with recognized human pathogens of the ESKAPE (acronym for ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* spp.) group which comprises difficult-to-treat infections, frequently due to multidrug resistance (Mulani *et al.*, 2019; Santajit and Indrawattana, 2016). The risk posed to humans by crop-borne ARB is influenced by a complex interplay of factors such as the ARB fitness in the crop and in the human body, the infective dose, and the health condition of the host, among other (Becerra-Castro *et al.*, 2015; Hölzel *et al.*, 2018). Risk assessment procedures evaluate the risk resulting from a hazard based on four major steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (Hölzel *et al.*, 2018; Manaia, 2017). The hazard identification refers to possible effects on human health, while hazard characterization expresses qualitatively or quantitatively the severity of such effects (Hölzel *et al.*, 2018). Exposure assessment expresses the probability of exposure to a specific hazard (Hölzel *et al.*, 2018). Therefore, it is the combination of the severity and the probability of occurrence that determines the risk associated with a given hazard. Risk assessment of foodborne human pathogens due to raw-eaten vegetable consumption has been established (Hölzel *et al.*, 2018; Pang *et al.*, 2017).

Different risk assessment approaches have been proposed for quantitative microbial risk assessment (QMRA). These can involve models to assess human risks associated with the transmission of foodborne microorganisms, or include also the food chain supply with the identification of intervention points where risk can be mitigated (Pang *et al.*, 2017). When the values of critical exposure doses are available for specific pathogens, it may be possible to calculate the probability of infection (Brouwer *et al.*, 2018). Although it can be argued that these models could be adapted to ARB, an important difference is that foodborne

pathogens are associated with acute infection or intoxication episodes, while ARB can colonize and proliferate asymptomatically in humans for long periods of time, eventually with never being responsible for any diseases or symptoms (Hölzel *et al.*, 2018; Manaia, 2017). This is one of the limitations to develop a reliable risk assessment. Another one refers to the quantitative analysis of exposure, since risk-relevant exposure doses can be far below those that common microbiology and molecular biology tools can offer. Also a limitation to assess hazard exposure is due to the difficulty in determining the infectious dose of ARB, specifically the minimal number of cells that may be needed to develop a successful colonization and infection in the human body (Ben *et al.*, 2018; Bengtsson-Palme *et al.*, 2018; Hölzel *et al.*, 2018; Manaia, 2017). This is also related with limited understanding that the scientific community have regarding the mechanisms of colonization and infection by ARB and the processes that underlie the transfer of ARGs to commensal and pathogenic bacteria (Ben *et al.*, 2018; Bengtsson-Palme *et al.*, 2018; Hölzel *et al.*, 2018; Manaia, 2017). In spite of the uncertainty on the quantification of risks posed by environmental and crop-born ARB, different evidence recommends that barriers must exist to minimize the transmission to humans via food-chain. Since raw-eaten vegetable can be a source of antibiotic resistance, the quality and safety of these fresh products is a priority to mitigate transmission to humans. International guidelines recommending specific actions such as agriculture practice (i.e. irrigation water quality parameters or application of fertilizers in a specific manner) and trade rules, may be determinant to reduce the human exposure to antibiotic resistance. An example is the irrigation water which has been considered a major source of ARB vegetable contamination (Adegoke *et al.*, 2018; Araújo *et al.*, 2017; Hölzel *et al.*, 2018; Pang *et al.*, 2017) and, albeit European guidelines that monitor antibiotic resistance in the irrigation water exist, mandatory national laws are missing (Gekenidis *et al.*, 2018). Therefore, at the legislative level, each country should start to take into consideration antibiotic resistance parameters in the irrigation water as well as proper management of the manure before soil application in order to limit these contaminants at the sources (Piña *et al.*, 2018; Pruden *et al.*, 2013).

3.7. Concluding Remarks and Future Perspectives

Endophytic bacteria associated with edible vegetables that may be eaten raw may act as possible vectors for the dissemination of antibiotic resistance from the environment to humans. Thus, plant-based products such as leafy and root vegetables (e.g. lettuce, carrot, radish) and fruit vegetables (e.g. tomato and cucumber), might host ARB which may

represent a potential risk for human health. The literature survey revealed a high diversity of endophytic bacteria associated with crops. The phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* were the most frequently reported in edible plants. Bacterial genera listed in this literature survey such as *Enterobacter*, *Acinetobacter*, *Pseudomonas* (which belong to the phylum *Proteobacteria*) and *Staphylococcus* (belonging to the phylum *Firmicutes*) are closely related to human pathogens, recognized ARGs hosts, and were also reported in wastewater, plants and human microbiomes. Other bacterial taxa with similar characteristics were the members of the genera *Burkholderia*, *Serratia*, *Stenotrophomonas* and *Bacillus*. It is argued and demonstrated that plant endophytic bacteria include members of groups that may move across and survive in these different habitats within the One-Health compartments, and specifically act as potential antibiotic resistance vectors across treated wastewater, plant endophytes and human microbiome. Specifically, it was shown that bacteria belonging to the genera *Enterobacter, Pseudomonas*, *Stenotrophomonas* or *Bacillus* were endophytes in edible crops that are normally consumed raw (lettuce, carrot, radish, cucumber or tomato). Members of those three genera of *Gamma-proteobacteria (Pseudomonas*, *Enterobacter* and *Stenotrophomonas)* have been recognized as harbours of acquired antibiotic resistance. The key message is that as long as bacteria belonging to these groups will get access to the vascular system of the plant, they may become endophytes. If these bacteria harbour antibiotic resistance, multidrug resistance or virulence genes because they are transported by sources of animal or human origin, e.g. through the reuse of wastewater or manure soil fertilization with inadequate quality, they can find in the crops a silent path of transmission to humans. In such a case, the regular consumption of raw vegetables may expose the human gut microbiome to bacteria hosting antibiotic resistance genes. The knowledge on plant microbiome resistance and its interaction with human microbiome is still scarce. This review suggests that antibiotic-resistant endophytic bacteria is an issue deserving further research, including the perspective of risks of transmission to humans.

Supplementary Information

Chapter 3. Clues to investigate antibiotic-resistance transmission through endophytic bacteria

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Authors: Nazareno Scaccia, Ivone Vaz-Moreira and Célia M. Manaia

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Table S3.1. Endophytic bacterial diversity in crop varieties.

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Alphaproteobacteria				Greapwine, populus, soybean, **tomato**, wheat	Leaf, cane, root, trunk	AR, AT, USA	CI	(Gottel *et al.*, 2011; Rascovan *et al.*, 2016; Romero *et al.*, 2014; West *et al.*, 2010)
Alphaproteobacteria	*Acetobacteraceae*	*Acetobacter*		Grapevine	Leaf, cane, trunk	AT	CD	(West *et al.*, 2010)
Alphaproteobacteria	*Acetobacteraceae*	*Acidiphilium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Acetobacteraceae*	*Gluconacetobacter*	*Gluconacetobacter diazotrophicus*	Coffea, rice, sugarcane, tea	Root, stem	MX	CD	(Bertalan *et al.*, 2009; Jimenez-Salgado *et al.*, 1997; Rosenblueth and Martinez-Romero, 2006; Santoyo et al., 2016)
Alphaproteobacteria	*Acetobacteraceae*	*Rhodopila*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Acetobacteraceae*	*Roseomonas*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Acetobacteraceae*	*Roseomonas*	*Roseomonas aerolata*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Alphaproteobacteria	*Aurantimonadaceae*	*Aureimonas*	*Aureimonas altamirensis*	Rice	Leaf	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Aurantimonadaceae*	*Martelella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Beijerinckiaceae*	*Methylocapsa*	*Methylocapsa acidiphila*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Beijerinckiaceae*	*Methylorosula*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Afipia*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Bosea*		Bell pepper, soybean, wheat	Root, shoot	AT, AR	CI, CD	(Rasche *et al.*, 2006; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Bradyrhizobium*		Grapevine, soybean, wheat	Leaf, cane, root, trunk	AT, AR	CI, CD	(West *et al.*, 2010)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Bradyrhizobium*	*Bradyrhizobium elkanii*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Bradyrhizobium*	*Bradyrhizobium japonicum*	Rice, wild rice	Root, stem	GN, JP, NP, KE, SN	CD	(Chaintreuil *et al.*, 2000; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Rhodopseudomonas*	*Rhodopseudomonas palustris*	Rice	Stem	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Bradyrhizobiaceae*	*Tardiphaga*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Brucellaceae*	*Brucella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Brucellaceae*	*Ochrobactrum*		Rice, soybean, wheat	Root	AR	CI, CD	(Mano and Morisaki, 2008; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Brucellaceae*	*Ochrobactrum*	*Ochrobactrum anthropi*	Cotton, maize, rice	Root, seed, stem	USA	CD	(Mano and Morisaki, 2008; McInroy and Kloepper, 1995)
Alphaproteobacteria	*Brucellaceae*	*Ochrobactrum*	*Ochrobactrum haematophilum*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Alphaproteobacteria	*Brucellaceae*	*Pseudochrobactrum*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Caulobacteraceae*	*Asticcacaulis*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Caulobacteraceae*	*Brevundimonas*		Bell pepper, soybean	Root, shoot	AT, AR	CI, CD	(Rasche *et al.*, 2006; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas diminuta*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas mediterranea*	Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Alphaproteobacteria	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas vesicularis*	**Carrot**, common bean, maize	Leaf, root, shoot	BR, CA, PT	CD	(de Oliveira Costa *et al.*, 2012; Pereira and Castro, 2014; Surette *et al.*, 2003)
Alphaproteobacteria	*Caulobacteraceae*	*Caulobacter*		Betula, rice, soybean, wheat	Root	AR, ES	CD, CI	(Mano and Morisaki, 2008; Mesa *et al.*, 2017; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Caulobacteraceae*	*Caulobacter*	*Caulobacter crescentus*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Caulobacteraceae*	*Phenylobacterium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Hyphomicrobiaceae*	*Devosia*		**Lettuce**, soybean	Leaf, root, shoot	AR, USA	CD, CI	(Jackson *et al.*, 2013; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Hyphomicrobiaceae*	*Hyphomicrobium*	*Hyphomicrobium sulfonivorans*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Hyphomicrobiaceae*	*Rhizomicrobium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Hyphomicrobiaceae*	*Rhodoplanes*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*		Bell pepper, cotton, maize, grapevine, red clover, rice, potato	Cane, leaf, nodule, root, seed, shoot, stem, taproot, tuber, trunk	AR, AT, CA, USA	CD, CI	(Hallmann *et al.*, 1997; Mano and Morisaki, 2008; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rasche *et al.*, 2006; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998; West *et al.*, 2010)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium adhaesivum*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium aquaticum*	Rice	Leaf, seed	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium extorquens*	Bell pepper, citrus plant	Branche, shoot, n.i.	AT, BR	CD	(Araújo *et al.*, 2002; Rasche *et al.*, 2006; Rosenblueth and Martinez-Romero, 2006)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium gregans*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium mesophilicum*	Citrus plant	Branche	BR	CD	(Araújo *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium platani*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Alphaproteobacteria	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium populi*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Alphaproteobacteria	*Methylocystaceae*	*Pleomorphomonas*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Methylocystaceae*	*Pleomorphomonas*	*Pleomorphomonas koreensis*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Phyllobacteriaceae*	*Mesorhizobium*		Winged pea, soybean, wheat	Nodule, root	AR, IT	CI, CD	(Muresu *et al.*, 2008; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Phyllobacteriaceae*	*Phyllobacterium*		Betula, commun bean, cotton, maize, red clover	Nodule, root, stem, taproot	CO, CA, ES, USA	CD	(Lopez-Lopez *et al.*, 2010; McInroy and Kloepper, 1995; Mesa *et al.*, 2017; Miliute *et al.*, 2015; Sturz *et al.*, 1998)

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Alphaproteobacteria	*Phyllobacteriaceae*	*Phyllobacterium*	*Phyllobacterium myrsinacearum*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Alphaproteobacteria	*Phyllobacteriaceae*	*Phyllobacterium*	*Phyllobacterium rubiacearum*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Alphaproteobacteria	*Rhizobiaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*		Banana, cotton, **cucumber**, winged pea, **lettuce**, potato, rice, soybean, wheat, wild rice	Leaf, nodule, root, shoot, stem, tuber	AR, AT, BR, CN, CA, IT, IN, JP, KE, NP, USA	CD, CI	(Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Jackson *et al.*, 2013; Kuklinsky-Sobral *et al.*, 2004; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Muresu *et al.*, 2008; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998; Thomas and Soly, 2009; Zinniel *et al.*, 2002)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*	*Agrobacterium larrymoorei*	Maize	Shoot	PT	CD	(Pereira and Castro, 2014)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*	*Agrobacterium radiobacter*	Cotton, maize, rice	Root, stem	USA	CD	(McInroy and Kloepper, 1995)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*	*Agrobacterium rhizogenes*	Greapvine, red clover	Nodule, root, taproot	CA	CD	(Compant *et al.*, 2011; Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*	*Agrobacterium rubi*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*	*Agrobacterium tumefaciens*	Ginseng, maize, **radish**, red clover	Nodule, root, stem, taproot	CA, KO, PT	CD	(Miliute *et al.*, 2015; Pereira and Castro, 2014; Seo *et al.*, 2010; Sturz *et al.*, 1998; Vendan *et al.*, 2010)
Alphaproteobacteria	*Rhizobiaceae*	*Agrobacterium*	*Agrobacterium vitis*	Rice	Stem	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Rhizobiaceae*	*Ensifer*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhizobiaceae*	*Kaistia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhizobiaceae*	*Neorhizobium*	*Neorhizobium alkalisoli*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Alphaproteobacteria	*Rhizobiaceae*	*Paracoccus*		Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*		Bell pepper, betula, common bean, grapevine, winged pea, red clover, soybean, wheat	Nodule, root, shoot, taproot	AT, AR, CO, CA, IT, ES	CD, CI	(Compant *et al.*, 2011; Lopez-Lopez *et al.*, 2010; Mesa *et al.*, 2017; Miliute *et al.*, 2015; Muresu *et al.*, 2008; Rasche *et al.*, 2006; Rascovan *et al.*, 2016; Sturz *et al.*, 1998)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium etli*	Maize	Root, stem	IN, USA	CD	(Miliute *et al.*, 2015; Rai *et al.*, 2007; Zinniel *et al.*, 2002)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium herbae*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium japonicum*	Cotton, maize	Root	USA	CD	(McInroy and Kloepper, 1995)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium larrymoorei*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium leguminosarum*	Rice, wild rice	Root, stem	JP, NP, KE	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Yanni *et al.*, 1997)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium loti*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium radiobacter*	**Carrot**, rice	Root	CA	CD	(Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium taibaishanense*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	*Rhizobium terangae*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Rhodobacteraceae*	*Paracoccus*		Banana, common bean	Seed, shoot	CO, IN	CD	(Lopez-Lopez *et al.*, 2010; Thomas and Soly, 2009)
Alphaproteobacteria	*Rhodobacteraceae*	*Paracoccus*	*Paracoccus halophilus*	Bell pepper, maize, **tomato**, watermelon	Root, seed, shoot	USA	CD	(Xia *et al.*, 2015)
Alphaproteobacteria	*Rhodobacteraceae*	*Paracoccus*	*Paracoccus yeeii*	Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Alphaproteobacteria	*Rhodobiaceae*	*Shinella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhodobiaceae*	*Sinorhizobium*	*Sinorhizobium meliloti*	Sweet potato	Stem, tuber	UG, KE	CD	(Reiter *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006)
Alphaproteobacteria	*Rhodospirillaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhodospirillaceae*	*Azospirillum*		Rice, soybean, wild rice, wheat	Root, stem	AR, IT, JP, KE, NP	CD, CI	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhodospirillaceae*	*Azospirillum*	*Azospirillum brasilense*	Banana, rice, wild rice	Leaf, root, stem	BR, JP, KE, NP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Weber *et al.*, 1999)
Alphaproteobacteria	*Rhodospirillaceae*	*Azospirillum*	*Azospirillum irakense*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Rhodospirillaceae*	*Azospirillum*	*Azospirillum lipoferum*	Rice, wild rice	Root, stem	IT, NP	CD	(Engelhard *et al.*, 2000; Wisniewski-Dye *et al.*, 2011)
Alphaproteobacteria	*Rhodospirillaceae*	*Dongia*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhodospirillaceae*	*Enhydrobacter*	*Enhydrobacter aerosaccus*	Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Alphaproteobacteria	*Rhodospirillaceae*	*Inquilinus*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Rhodospirillaceae*	*Nitrospirillum*	*Nitrospirillum amazonense*	Banana, pineapple, rice	Leaf, root, stem	BR	CD	(Mano and Morisaki, 2008; Rosenblueth and Martinez-Romero, 2006; Weber *et al.*, 1999)
Alphaproteobacteria	*Rhodospirillaceae*	*Niveispirillum*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Sphingomonadaceae*			Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Sphingomonadaceae*	*Novosphingobium*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Sphingomonadaceae*	*Novosphingobium*	*Novosphingobium resinovorum*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Alphaproteobacteria	*Sphingomonadaceae*	*Novosphingobium*	*Novosphingobium tardaugens*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingobium*		**Lettuce**, soybean, wheat	Leaf, toor	AR, USA	CD, CI	(Jackson *et al.*, 2013; Rascovan *et al.*, 2016)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingobium*	*Sphingobium fuliginis*	Maize	Root	PT	CD	(Pereira and Castro, 2014)

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Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*		Bell pepper, common bean, maize, potato, red clover, soybean, **tomato**, watermelon, wheat	Nodule, root, shoot, stem, taproot, tuber	AR, AT, CO, CA, USA	CD, CI	(Lopez-Lopez *et al.*, 2010; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998; Xia *et al.*, 2015)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas adheasiva*	Rice	Stem	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas dokdonensis*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas dokdonensis*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas echinoides*	Rice	Leaf, seed	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas melonis*	Rice	Leaf, seed	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas parapaucimobilis*	Rice	Seed	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas paucimobilis*	Cotton, maize, rice	Root, shoot, stem	NP, PT, USA	CD	(Engelhard *et al.*, 2000; Mano and Morisaki, 2008; McInroy and Kloepper, 1995; Pereira and Castro, 2014; Rosenblueth and Martinez-Romero, 2006)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sanguinis*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas yabuuchiae*	Rice	Leaf, seed	n.i.	CD	(Mano and Morisaki, 2008)
Alphaproteobacteria	*Sphingomonadaceae*	*Sphingopyxis*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Unclassified Rhizobiales*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Unclassified Rhizobiales*	*Nordella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Unclassified Rhizobiales*	*Vasilyevaea*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Xanthobacteraceae*	*Ancylobacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Xanthobacteraceae*	*Azorhizobium*		Wild rice	Root, stem	JP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015)
Alphaproteobacteria	*Xanthobacteraceae*	*Azorhizobium*	*Azorhizobium caulinodans*	Rice, wild rice	Root, stem	JP, NP, KE	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015)
Alphaproteobacteria	*Xanthobacteraceae*	*Labrys*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Xanthobacteraceae*	*Pseudolabrys*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Alphaproteobacteria	*Xanthobacteraceae*	*Xanthobacter*		**Cucumber**	Root	USA	CD	(Mahaffee and Kloepper, 1997)
Betaproteobacteria				Bell pepper, populus, **tomato**	Leaf, root, shoot	AR, AT, USA	CD, CI	(Gottel *et al.*, 2011; Rasche *et al.*, 2006; Romero *et al.*, 2014)
Betaproteobacteria	*Alcaligenaceae*	*Achromobacter*		Maize, soybean, wheat	Root, stem	AR, IN, USA	CD, CI	(Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Zinniel *et al.*, 2002)
Betaproteobacteria	*Alcaligenaceae*	*Achromobacter*	*Achromobacter xylosoxidans*	**Cucumber**, rice	Crude extract, root	USA	CD, CI	(Mano and Morisaki, 2008; Nithya and Babu, 2017)
Betaproteobacteria	*Alcaligenaceae*	*Alcaligenes*		Citrus plant, potato	Stem, tuber	AT, CA	CD	(Lodewyckx *et al.*, 2002; Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Betaproteobacteria	*Alcaligenaceae*	*Alcaligenes*	*Alcaligenes piechaudii*	Cotton	Root	USA	CD	(McInroy and Kloepper, 1995)
Betaproteobacteria	*Alcaligenaceae*	*Bordetella*		Red clover, soybean	Nodule, root, taproot	AR, CA	CD, CI	(Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Sturz *et al.*, 1998)
Betaproteobacteria	*Alcaligenaceae*	*Bordetella*	*Bordetella bronchiseptica*	**Carrot**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*		Banana, betula, cotton, **cucumber**, maize, pineapple, rice, soybean, wheat	Leaf, nodule, root, stem	AR, CN, IN, NE, NP, SP, USA	CD, CI	(Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Kuklinsky-Sobral *et al.*, 2004; Mano and Morisaki, 2008; McInroy and Kloepper, 1995; Mesa *et al.*, 2017; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Weber *et al.*, 1999; Zinniel *et al.*, 2002)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia cenocepacia*	Bell pepper, maize, **tomato**, watermelon	Root, shoot	USA	CD	(Xia *et al.*, 2015)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia cepacia*	Citrus plant, maize, rice, wheat, wild rice, yellow lupine	Leaf, root, seed, stem	JP, IN, KE, NP, ES, USA	CD	(Araujo *et al.*, 2001; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rosenblueth and Martinez-Romero, 2006; Zinniel *et al.*, 2002)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia fungorum*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia gladioli*	Bell pepper, maize, **tomato**, watermelon	Root, seed, shoot	USA	CD	(Xia *et al.*, 2015)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia glathei*	Grapevine	Leaf, cane, trunk	AT	CD	(West *et al.*, 2010)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia graminis*	Wild rice	Root, stem	JP, NP, KE	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia kururiensis*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia phytofirmans*	Barley, betula, canola, grapevine, maize, onion, potato, **tomato**	Root	ES	CD	(Mesa *et al.*, 2017; Santoyo et al., 2016; Weilharter *et al.*, 2011)
Betaproteobacteria	*Burkholderiaceae*	*Burkholderia*	*Burkholderia stabilis*	Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Betaproteobacteria	*Burkholderiaceae*	*Cupriavidus*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Burkholderiaceae*	*Ralstonia*		Winged pea, **lettuce**, soybean, wheat	Leaf, nodule, root	AR, IT, USA	CD, CI	(Jackson *et al.*, 2013; Muresu *et al.*, 2008; Rascovan *et al.*, 2016)
Betaproteobacteria	*Burkholderiaceae*	*Ralstonia*	*Ralstonia eutropha*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Betaproteobacteria	*Burkholderiaceae*	*Ralstonia*	*Ralstonia pickettii*	Maize, pea	Stem, root	NL, USA	CD	(Elvira-Recuenco and van Vuurde, 2000; McInroy and Kloepper, 1995; Rosenblueth and Martinez-Romero, 2006)

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Betaproteobacteria	*Burkholderiales genera incertae sedis*	*Aquabacterium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Burkholderiales genera incertae sedis*	*Ideonella*	*Ideonella dechloratans*	Rice, wild rice	Root, stem	JP, KE, NP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015)
Betaproteobacteria	*Chromobacteriaceae*	*Chromobacterium*	*Chromobacterium violaceum*	**Carrot**, rice, wild rice	Root, stem	CA, JP, KE, NP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003)
Betaproteobacteria	*Chromobacteriaceae*	*Leeia*		**Lettuce**	Leaf	USA	CI	(Jackson *et al.*, 2013)
Betaproteobacteria	*Chromobacteriaceae*	*Paludibacterium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Acidovorax*		Potato, red clover, rice, soybean, wheat	Nodule, root, seed, stem, taproot, tuber	AR, AT, CA	CD, CI	(Mano and Morisaki, 2008; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Betaproteobacteria	*Comamonadaceae*	*Acidovorax*	*Acidovorax facilis*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Comamonadaceae*	*Acidovorax*	*Acidovorax oryzae*	Maize	Shoot	PT	CD	(Pereira and Castro, 2014)
Betaproteobacteria	*Comamonadaceae*	*Caenimonas*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Comamonas*		Grapevine, potato, red clover, soybean, wheat	Nodule, root, stem, taproot, tuber	AR, AT, CA, AU	CD, CI	(Bell *et al.*, 1995; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998; West *et al.*, 2010)
Betaproteobacteria	*Comamonadaceae*	*Comamonas*	*Comamonas terrigena*	Grapevine	Xylem sap	CA	CD	(Bell *et al.*, 1995)
Betaproteobacteria	*Comamonadaceae*	*Comamonas*	*Comamonas testosteroni*	Cotton, rice	Root	USA	CD, CI	(Mano and Morisaki, 2008; McInroy and Kloepper, 1995)
Betaproteobacteria	*Comamonadaceae*	*Curvibacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Curvibacter*	*Curvibacter gracilis*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Comamonadaceae*	*Delftia*		Soybean, wheat	Root	AR	CD, CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Delftia*	*Delftia acidovorans*	**Carrot**, rice	Root	CA	CD, CI	(Mano and Morisaki, 2008; Surette *et al.*, 2003)
Betaproteobacteria	*Comamonadaceae*	*Delftia*	*Delftia tsuruhatensis*	Common bean, rice	Leaf, root	BR	CD, CI	(de Oliveira Costa *et al.*, 2012; Mano and Morisaki, 2008)
Betaproteobacteria	*Comamonadaceae*	*Diaphorobacter*	*Diaphorobacter nitroreducens*	Rice	Leaf	n.i.	CD	(Mano and Morisaki, 2008)
Betaproteobacteria	*Comamonadaceae*	*Hydrogenophaga*		Cotton, maize	Root, stem	USA	CD	(McInroy and Kloepper, 1995)
Betaproteobacteria	*Comamonadaceae*	*Hydrogenophaga*	*Hydrogenophaga taeniospiralis*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Comamonadaceae*	*Limnohabitans*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Pelomonas*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Polaromonas*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Pseudacidovorax*		Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Betaproteobacteria	*Comamonadaceae*	*Pseudorhodoferax*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Ramlibacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Comamonadaceae*	*Variovorax*		Red clover, rice, soybean, wheat	Nodule, root, taproot	AR, CA	CD, CI	(Mano and Morisaki, 2008; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Sturz *et al.*, 1998)
Betaproteobacteria	*Comamonadaceae*	*Variovorax*	*Variovorax boronicumulans*	Betula, maize	Root	PT, ES	CD	(Mesa *et al.*, 2017; Pereira and Castro, 2014)
Betaproteobacteria	*Comamonadaceae*	*Variovorax*	*Variovorax paradoxus*	Betula, **carrot**, cotton, grapevine, maize	Cane, leaf, root, stem, trunk	AT, CA, PT, ES, USA	CD	(McInroy and Kloepper, 1995; Mesa *et al.*, 2017; Pereira and Castro, 2014; Surette *et al.*, 2003; West *et al.*, 2010)
Betaproteobacteria	*Gallionellaceae*	*Gallionella*		Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Betaproteobacteria	*Gallionellaceae*	*Gallionella*	*Gallionella ferruginea*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Methylophilaceae*	*Methylophilus*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Collimonas*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Duganella*		Bell pepper, soybean, wheat	Root, shoot	AR, AT	CD, CI	(Rasche *et al.*, 2006; Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Duganella*	*Duganella violaceinigra*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Oxalobacteraceae*	*Herbaspirillum*		Rice, soybean, wheat	Root	AR, NP	CD, CI	(Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Herbaspirillum*	*Herbaspirillum frisingense*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Oxalobacteraceae*	*Herbaspirillum*	*Herbaspirillum rubrisubalbicans*	Sugarcane	Leaf, stem, root	n.i.	CD	(Olivares *et al.*, 1996; Rosenblueth and Martinez-Romero, 2006)
Betaproteobacteria	*Oxalobacteraceae*	*Herbaspirillum*	*Herbaspirillum seropedicae*	Banana, maize, rice, sorghum, sugarcane, wild rice	Leaf, root, seed, stem	BR, JP, IN, KE, NP, USA	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Rai *et al.*, 2007; Rosenblueth and Martinez-Romero, 2006; Rosenblueth *et al.*, 2004; Weber *et al.*, 1999; Zinniel *et al.*, 2002)
Betaproteobacteria	*Oxalobacteraceae*	*Janthinobacterium*		Bell pepper, **lettuce**	Leaf, shoot	AT, USA	CD, CI	(Jackson *et al.*, 2013; Rasche *et al.*, 2006)
Betaproteobacteria	*Oxalobacteraceae*	*Massilia*		Bell pepper, **lettuce**, soybean, wheat	Leaf, root, shoot	AT, AR, USA	CD, CI	(Jackson *et al.*, 2013; Rasche *et al.*, 2006; Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Oxalicibacterium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Pseudoduganella*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Oxalobacteraceae*	*Undibacterium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Sterolibacteriaceae*	*Georgfuchsia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Sterolibacteriaceae*	*Methyloversatilis*	*Methyloversatilis universalis*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Betaproteobacteria	*Sterolibacteriaceae*	*Sterolibacterium*	*Sterolibacterium denitrificans*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Betaproteobacteria	*Thiobacillaceae*	*Thiobacillus*		Winged pea	Nodule	IT	CD	(Muresu *et al.*, 2008)
Betaproteobacteria	*Unclassified Burkholderiales*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Unc. Burkholderiales*	*Burkholderiales genera incertae sedis*	*Roseateles depolymerans*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Betaproteobacteria	*Unc. Burkholderiales*	*Leptothrix*	*Leptothrix ginsengisoli*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Betaproteobacteria	*Unc. Burkholderiales*	*Mitsuaria*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Unc. Burkholderiales*	*Sphaerotilus*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Unc. Burkholderiales*	*Thiobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Betaproteobacteria	*Zoogloeaceae*	*Azoarcus*		Kallar grass, rice, soybean, wild rice	Root, stem	AR, JP, KE, NL, NP, PH, PK	CD, CI	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Krause *et al.*, 2006; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Santoyo et al., 2016)
Betaproteobacteria	*Zoogloeaceae*	*Azoarcus*	*Azoarcus indigens*	Rice	Root	NP	CD	(Engelhard *et al.*, 2000; Mano and Morisaki, 2008)
Deltaproteobacteria				Populus, **tomato**, wheat	Leaf, Root	AR, USA	CI	(Gottel *et al.*, 2011; Rascovan *et al.*, 2016; Romero *et al.*, 2014)
Deltaproteobacteria	*Bdellovibrionaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Bdellovibrionaceae*	*Bdellovibrio*	*Bdellovibrio bacteriovorus*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Deltaproteobacteria	*Cystobacteraceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Desulfobacteraceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Geobacteraceae*	*Geobacter*		Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Deltaproteobacteria	*Labilitrichaceae*	*Labilithrix*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Pelobacteraceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Polyangiaceae*	*Byssovorax*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Polyangiaceae*	*Chondromyces*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Syntrophaceae*	*Desulfomonile*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deltaproteobacteria	*Vulgatibacteraceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Epsilonproteobacteria				Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Epsilonproteobacteria	*Campylobacteraceae*	*Sulfurospirillum*	*Sulfurospirillum multivorans*	Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria				Populus, **radish**, soybean, **tomato**, watermelon, wheat	Leaf, nodule, root	AR, IT, KO, USA	CI, CD	(Gottel *et al.*, 2011; Rascovan *et al.*, 2016; Seo *et al.*, 2010; Xia *et al.*, 2015)
Gammaproteobacteria	*Aeromonadaceae*	*Aeromonas*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Aeromonadaceae*	*Aeromonas*	*Aeromonas hydrophila*	**Cucumber**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Gammaproteobacteria	*Alteromonadaceae*	*Marinobacterium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Chromatiaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Coxiellaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Ectothiorhodospiraceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*			**Cucumber**, winged pea, wheat	Fruit, nodule, root	AR, IT	CD, CI	(Hallmann *et al.*, 1997; Muresu *et al.*, 2008; Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Citrobacter*		Banana	Leaf, stem, root	MX	CD	(Martínez *et al.*, 2003; Rosenblueth and Martinez-Romero, 2006)
Gammaproteobacteria	*Enterobacteriaceae*	*Citrobacter*	*Citrobacter freundii*	**Radish**	Leaf	KO	CD	(Seo *et al.*, 2010)
Gammaproteobacteria	*Enterobacteriaceae*	*Citrobacter*	*Citrobacter koseri*	Maize	Root, stem	USA	CD	(McInroy and Kloepper, 1995)
Gammaproteobacteria	*Enterobacteriaceae*	*Cronobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*		Cotton, **cucumber**, grapevine, lemon, **lettuce**, maize, potato, **radish**, red clover, rice, soybean, spinach	Leaf, nodule, root, stem, tuber	AR, AT, BR, CA, KO, IN, AU, USA	CD, CI	(Bell *et al.*, 1995; Compant *et al.*, 2011; Hallmann *et al.*, 1997; Hou *et al.*, 2013; Jackson *et al.*, 2013; Kuklinsky-Sobral *et al.*, 2004; Mano and Morisaki, 2008; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Seo *et al.*, 2010; Sturz *et al.*, 1998; West *et al.*, 2010; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter aerogenes*	**Carrot**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter agglomerans*	Grapevine, soybean	Leaf, stem, root, xylem sap	BR, CA	CD	(Bell *et al.*, 1995; Kuklinsky-Sobral *et al.*, 2004; Rosenblueth and Martinez-Romero, 2006)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter asburiae*	Common bean, potato, sweet patato	Leaf, stem, tuber	AT, BR, CA	CD	(de Oliveira Costa *et al.*, 2012; Miliute *et al.*, 2015; Reiter *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006; Sturz *et al.*, 1998)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter cancerogenus*	Rice, wild rice	Root, stem	JP, KE, NP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015)

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Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter cloacae*	Black pepper, citrus plant, grapevine, maize, rice, red clover, strawberry	Branche, cane, leaf, nodule, root, stem, taproot, trunk, xylem sap	AT, BR, CA, HK, IN, USA	CD	(Araújo *et al.*, 2002; Bell *et al.*, 1995; Mano and Morisaki, 2008; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rosenblueth and Martinez-Romero, 2006; Santoyo et al., 2016; West *et al.*, 2010; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter hormaechei*	**Carrot**, common bean	Crude extract, leaf	BR, USA	CD	(de Oliveira Costa *et al.*, 2012; Nithya and Babu, 2017)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter ludwigii*	Maize, rice	Root	PT	CD	(Mano and Morisaki, 2008; Pereira and Castro, 2014)
Gammaproteobacteria	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter sakazakiia*	Soybean	Leaf, stem, root	BR	CD	(Kuklinsky-Sobral *et al.*, 2004; Rosenblueth and Martinez-Romero, 2006)
Gammaproteobacteria	*Enterobacteriaceae*	*Escherichia*		Cotton, maize, soybean, wheat	Nodule, root, stem, taproot	AR, CA, USA	CD, CI	(McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Sturz *et al.*, 1998)
Gammaproteobacteria	*Enterobacteriaceae*	*Escherichia*	*Escherichia coli*	**Tomato**	Stem, fruit	n.i.	CD	(Miliute *et al.*, 2015)
Gammaproteobacteria	*Enterobacteriaceae*	*Klebsiella*		Bell pepper, cotton, maize, potato, red clover, rice, soybean, sweet potato, wheat, wild rice	Nodule, root, stem, taproot, tuber	AR, AT, CA, JP, KE, IN, KE, NP, UG, USA	CD, CI	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Mano and Morisaki, 2008; Mbai *et al.*, 2013; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rasche *et al.*, 2006; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006; Sturz *et al.*, 1998; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella oxytoca*	Soybean, rice	Leaf, nodule, root, seed, stem	BR, USA	CD	(Kuklinsky-Sobral *et al.*, 2004; Mano and Morisaki, 2008; Miliute *et al.*, 2015; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella ozaenae*	Grapevine	Stem, xylem sap	CA, AU	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; West *et al.*, 2010)
Gammaproteobacteria	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella pneumoniae*	Banana, grapevine, maize, rice, soybean, wheat	Leaf, nodule, root, shoot, stem, xylem sap	CA, IN, NP, AU, BR, USA	CD	(Bell *et al.*, 1995; Engelhard *et al.*, 2000; Fouts *et al.*, 2008; Kuklinsky-Sobral *et al.*, 2004; Mano and Morisaki, 2008; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rosenblueth and Martinez-Romero, 2006; Santoyo et al., 2016; Thomas and Soly, 2009; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella terrigena*	**Carrot**, maize, grapevine	Root, stem	CA, IN, AU, USA	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003; West *et al.*, 2010; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella variicola*	Banana, maize, rice, sugarcane, wild rice	Root, stem	JP, MX, KE, NP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Rosenblueth *et al.*, 2004)
Gammaproteobacteria	*Enterobacteriaceae*	*Kluyvera*		Coton, maize, soybean	Root, stem	AR, USA	CD, CI	(McInroy and Kloepper, 1995; Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Kluyvera*	*Kluyvera ascorbata*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Enterobacteriaceae*	*Kluyvera*	*Kluyvera cochleae*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Enterobacteriaceae*	*Kosakonia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Lelliottia*	*Lelliottia aigenus*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Enterobacteriaceae*	*Phaseolibacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Providencia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Pseudocitrobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Raoultella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Raoultella*	*Raoultella planticola*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Enterobacteriaceae*	*Raoultella*	*Raoultella terrigena*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Enterobacteriaceae*	*Rosenbergiella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Salmonella*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Enterobacteriaceae*	*Salmonella*	*Salmonella enterica*	**Carrot**, **radish**, **tomato**	Crude extract, root, seed	USA	CD	(Guo *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006)
Gammaproteobacteria	*Erwiniaceae*	*Erwinia*		Alfalfa, cotton, **lettuce**, maize, potato, soybean, spinach, sugarbeet, woody plants	Boll, leaf, nodule, radicle, root, seed, stem, tuber, unopened flowers	AT, BR, CA, IT, IN, USA	CD, CI	(Hallmann *et al.*, 1997; Hou *et al.*, 2013; Jackson *et al.*, 2013; Kuklinsky-Sobral *et al.*, 2004; Miliute *et al.*, 2015; Rai *et al.*, 2007; Reiter *et al.*, 2002; Sturz *et al.*, 1998; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Erwiniaceae*	*Erwinia*	*Erwinia carotovora*	Cotton	Root, stem	USA	CD	(McInroy and Kloepper, 1995)
Gammaproteobacteria	*Erwiniaceae*	*Erwinia*	*Erwinia persicinus*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Gammaproteobacteria	*Erwiniaceae*	*Erwinia*	*Erwinia rhapontici*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Erwiniaceae*	*Pantoea*		Cotton, grapevine, **lettuce**, maize, potato, rice, soybean, spinach, wheat, wild rice	Leaf, nodule, root, seed, stem, tuber	AR, AT, CN, CA, JP, KE, NP, AU, ES, USA	CD, CI	(Bell *et al.*, 1995; Compant *et al.*, 2011; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Hou *et al.*, 2013; Jackson *et al.*, 2013; Kuklinsky-Sobral *et al.*, 2004; Mano and Morisaki, 2008; Mbai *et al.*, 2013; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006; Sturz *et al.*, 1998; Verma *et al.*, 2004; West *et al.*, 2010; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Erwiniaceae*	*Pantoea*	*Pantoea agglomerans*	**Carrot**, citrus plant, ginseng, grapevine, pea, red clover, rice, sweet potato	Branches, leaf, nodule, root, seed, stem, taproot, xylem sap	AT, CA, IT, JP, KO, NL, ES	CD	(Araujo *et al.*, 2001; Araújo *et al.*, 2002; Asis and Adachi, 2004; Bell *et al.*, 1995; Bulgari *et al.*, 2009; Cho *et al.*, 2007; Compant *et al.*, 2011; Elvira-Recuenco and van Vuurde, 2000; Mano and Morisaki, 2008; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Sturz *et al.*, 1998; Surette *et al.*, 2003)
Gammaproteobacteria	*Erwiniaceae*	*Pantoea*	*Pantoea allii*	Maize	Root, shoot	PT	CD	(Pereira and Castro, 2014)

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Gammaproteobacteria	*Erwiniaceae*	*Pantoea*	*Pantoea ananatis*	Ginseng, grapevine, rice	Leaf, root, seed	IT, KO	CD	(Bulgari *et al.*, 2009; Cho *et al.*, 2007; Mano and Morisaki, 2008)
Gammaproteobacteria	*Legionellaceae*	*Legionella*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Moraxellaceae*			Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Moraxellaceae*	*Acinetobacter*		Bell pepper, common bean, potato, soybean, wheat	Leaf, nodule, shoot, seem, tuber	AR, AT, CO, CN, CA	CD, CI	(de Oliveira Costa *et al.*, 2012; Kuklinsky-Sobral *et al.*, 2004; Lopez-Lopez *et al.*, 2010; Miliute *et al.*, 2015; Rasche *et al.*, 2006; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter baumannii*	Cotton, rice	Root, stem	USA	CD, CI	(Mano and Morisaki, 2008; McInroy and Kloepper, 1995)
Gammaproteobacteria	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter calcoaceticus*	**Carrot**, common bean	Root	CO, CA	CD	(Lopez-Lopez *et al.*, 2010; Surette *et al.*, 2003)
Gammaproteobacteria	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter lwoffii*	Banana	Shoot	AT, IN	CD	(Thomas and Soly, 2009)
Gammaproteobacteria	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter radioresistens*	Common bean	Leaf, root	CO, BR	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010)
Gammaproteobacteria	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter schindleri*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Gammaproteobacteria	*Moraxellaceae*	*Alkanindiges*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Moraxellaceae*	*Alkanindiges*	*Alkanindiges illinoisensis*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Gammaproteobacteria	*Moraxellaceae*	*Moraxella*	*Moraxella bovis*	Grapevine	Stem, xylem sap	CA, AU	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; West *et al.*, 2010)
Gammaproteobacteria	*Moraxellaceae*	*Psychrobacter*		Patato, red clover	Nodule, root, stem, taproot, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Moraxellaceae*	*Psychrobacter*	*Psychrobacter immobilis*	Red clover	Nodule, root, taproot	CA	CD	(Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Gammaproteobacteria	*Morganellaceae*	*Morganella*		**Lettuce**	Leaf	USA	CI	(Jackson *et al.*, 2013)
Gammaproteobacteria	*Morganellaceae*	*Proteus*		**Radish**	Root	KO	CD	(Seo *et al.*, 2010)
Gammaproteobacteria	*Morganellaceae*	*Providencia*		**Lettuce**, **radish**	Leaf	KO, USA	CD, CI	(Jackson *et al.*, 2013; Seo *et al.*, 2010)
Gammaproteobacteria	*Orbaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Pasteurellaceae*	*Pasteurella*		Potato, red clover	Nodule, root, stem, taproot, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Pectobacteriaceae*	*Dickeya*	*Dickeya chrysanthemi*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Pectobacteriaceae*	*Pectobacterium*	*Pectobacterium carotovorum*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Gammaproteobacteria	*Piscirickettsiaceae*			Bell pepper, ginseng	Root, shoot	AT, KO	CD	(Cho *et al.*, 2007; Rasche *et al.*, 2006)
Gammaproteobacteria	*Pseudomonadaceae*	*Acidibacter*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Pseudomonadaceae*	*Dokdonella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Pseudomonadaceae*	*Dyella*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Pseudomonadaceae*	*Flavimonas*	*Flavimonas oryzihabita*	Maize	Root	USA	CD	(McInroy and Kloepper, 1995)
Gammaproteobacteria	*Pseudomonadaceae*	*Luteibacter*		Betula, wheat	Root	AR, ES	CD, CI	(Mesa *et al.*, 2017; Rascovan *et al.*, 2016)
Gammaproteobacteria	*Pseudomonadaceae*	*Luteibacter*	*Luteibacter yeojuensis*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Gammaproteobacteria	*Pseudomonadaceae*	*Methylophaga*	*Methylophaga marina*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Gammaproteobacteria	*Pseudomonadaceae*	*Photobacterium*		Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*		Alfalfa, bell pepper, black pepper, cereals, cotton, **cucumber**, grapevine, lemon, **lettuce**, maize, potato, **radish** soybean, strawberry, sugarbeet, **tomato**, watermelon, wheat, wild rice, woody plants	Flower, fruit, leaf, root, seed, shoot, stem, tuber, xylem sap	AR, AT, CA, JP, IN, KE, NP, SR, USA	CD, CI	(Aravind *et al.*, 2009; Bell *et al.*, 1995; Compant *et al.*, 2011; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Hou *et al.*, 2013; Lodewyckx *et al.*, 2002; Mbai *et al.*, 2013; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Seo *et al.*, 2010; Stajković *et al.*, 2009; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas aeruginosa*	**Carrot**, common bean, maize, **tomato**	Crude extract, fruit, leaf, root, stem	BR, CA, IN, USA	CD	(de Oliveira Costa *et al.*, 2012; Miliute *et al.*, 2015; Nithya and Babu, 2017; Rai *et al.*, 2007; Surette *et al.*, 2003; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas aptata*	Pea	Stem	NL	CD	(Elvira-Recuenco and van Vuurde, 2000)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas boreopolis*	Rice	Seed	n.i.	CD	(Mano and Morisaki, 2008)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas brassicacearum*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas chlororaphis*	**Carrot**, maize	Root	CA, PT	CD	(Miliute *et al.*, 2015; Pereira and Castro, 2014; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas cichorii*	Grapevine, red clover	Nodule, root, stem, taproot, xylem sap	CA, AU	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; Sturz *et al.*, 1998; West *et al.*, 2010)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas citronellolis*	Soybean	Leaf, nodule, root, stem	BR, USA	CD	(Kuklinsky-Sobral *et al.*, 2004; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas corrugata*	Grapevine, pea, red clover, strawberry	Nodule, root, stem, taproot, xylem sap	CA, NL	CD	(Bell *et al.*, 1995; Elvira-Recuenco and van Vuurde, 2000; Lodewyckx *et al.*, 2002; Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	**Carrot**, **cucumber**, grapevine, **lettuce**, maize, pea	Cane, crude extract, leaf, root, stem, trunk	AT, CA, IN, NL, USA	CD	(Elvira-Recuenco and van Vuurde, 2000; Rai *et al.*, 2007; West *et al.*, 2010; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fulgida*	Grapevine	Flower	AT	CD	(Compant *et al.*, 2011)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fulva*	Red clover, rice	Nodule, root, seed, taproot	CA	CD	(Mano and Morisaki, 2008; Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas indoloxydans*	**Cucumber**	Crude extract	USA	CD	(Nithya and Babu, 2017)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas jessenii*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas marginalis*	Ginseng, grapevine, rice	Stem, xylem sap	CA, KO	CD	(Bell *et al.*, 1995; Vendan *et al.*, 2010)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas mendocina*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas moraviensis*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas mucidolens*	Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas oryzihabitans*	Soybean	Leaf, nodule, root, stem	BR, USA	CD	(Kuklinsky-Sobral *et al.*, 2004; Miliute *et al.*, 2015; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas paucimobilis*	Strawberry	n.i.	n.i.	CD	(Lodewyckx *et al.*, 2002)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas poae*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas putida*	Bell pepper, **carrot**, grapevine, maize, poplar, **tomato**, watermelon	Root, shoot, xylem sap	CA, USA	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; Rosenblueth and Martinez-Romero, 2006; Santoyo et al., 2016; Surette *et al.*, 2003; Taghavi *et al.*, 2009; Xia *et al.*, 2015)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas staminea*	Soybean	Leaf, nodule, root, stem	BR, USA	CD	(Kuklinsky-Sobral *et al.*, 2004; Miliute *et al.*, 2015; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas stutzeri*	Banana, **cucumber**, onion, rice	Crude extract, root, shoot	IN, USA	CD, CI	(Mano and Morisaki, 2008; Nithya and Babu, 2017; Santoyo et al., 2016; Thomas and Soly, 2009; Yan *et al.*, 2008)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas syringae*	**Carrot**, grapevine, **lettuce**, red clover, **tomato**	Cane, fruit, leaf, nodule, root, stem, taproot, trunk	AT, CA, USA	CD	(Jackson et al., 2013; Miliute *et al.*, 2015; Sturz *et al.*, 1998; Surette *et al.*, 2003; West *et al.*, 2010)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas tolaasii*	Potato, red clover, strawberry	Nodule, root, stem, taproot, tuber	AT, CA	CD	(Lodewyckx *et al.*, 2002; Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas viridiflava*	Pea	Stem	NL	CD	(Elvira-Recuenco and van Vuurde, 2000)
Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas xanthomarina*	**Cucumber**, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Gammaproteobacteria	*Shewanellaceae*	*Shewanella*		Potato	Tuber, stem	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Unclassified Enterobacterales*	*Plesiomonas*	*Plesiomonas shigelloides*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Gammaproteobacteria	*Unclassified family*			**Radish**	Root	KO	CD	(Seo *et al.*, 2010)
Gammaproteobacteria	*Unclassified family*	*Pseudoxanthomonas*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Vibrionaceae*	*Rhodanobacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Vibrionaceae*	*Rugamonas*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Xanthomonadaceae*			**Tomato**, soybean, wheat	Fruit, root	AR	CD, CI	(Hallmann *et al.*, 1997; Rascovan *et al.*, 2016)
Gammaproteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*		Bell pepper, common bean, **cucumber**, dune grass, grapevine, **lettuce**, maize, **radish**, rice, soybean, spinach, **tomato**, watermelon, wheat, wild rice	Cane, leaf, root, seed, shoot, stem, trunk	AR, AT, BR, JP, KO, IN, KE, NE, NP, USA	CD, CI	(Dalton *et al.*, 2004; de Oliveira Costa *et al.*, 2012; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Jackson et al., 2013; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Seo *et al.*, 2010; West *et al.*, 2010; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Gammaproteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas chelatiphaga*	Bell pepper, maize, **tomato**, watermelon	Root, shoot	PT, USA	CD	(Pereira and Castro, 2014; Xia *et al.*, 2015)
Gammaproteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas maltophilia*	Bell pepper, **carrot**, common bean, ginseng, maize, onion, poplar, rice, **tomato**, watermelon	Crude extract, leaf, root, seed, shoot, stem	AT, BR, CA, KO, USA, PT, USA	CD, CI	(de Oliveira Costa *et al.*, 2012; Mano and Morisaki, 2008; McInroy and Kloepper, 1995; Nithya and Babu, 2017; Pereira and Castro, 2014; Rasche *et al.*, 2006; Santoyo et al., 2016; Surette *et al.*, 2003; Taghavi *et al.*, 2009; Vendan *et al.*, 2010; Xia *et al.*, 2015)
Gammaproteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas rhizophila*	**Cucumber**, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Gammaproteobacteria	*Xanthomonadaceae*	*Thiohalobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Xanthomonadaceae*	*Thiomicrospira*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Gammaproteobacteria	*Xanthomonadaceae*	*Vibrio*		Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*		Cotton, grapevine, winged pea, **lettuce**, potato, soybean, strawberry, sugarbeet, wheat	Boll, leaf, nodule, radicle, root, stem, tuber, unopened flower, xylem sap	AR, AT, CA, IT, AU, USA	CD, CI	(Bell *et al.*, 1995; Compant *et al.*, 2011; Hallmann *et al.*, 1997; Jackson et al., 2013; Lodewyckx *et al.*, 2002; Miliute *et al.*, 2015; Muresu *et al.*, 2008; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998; West *et al.*, 2010)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*	*Xanthomonas arboricola*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*	*Xanthomonas axonopodis*	**Cucumber**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*	*Xanthomonas compestris*	Cotton, maize, red clover	Nodule, root, stem, taproot	CA, USA	CD	(McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*	*Xanthomonas fuscans*	Onion	Crude extract	USA	CD	(Nithya and Babu, 2017)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*	*Xanthomonas oryzae*	Red clover	Nodule, root, taproot	CA	CD	(Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Gammaproteobacteria	*Xanthomonadaceae*	*Xanthomonas*	*Xanthomonas translucens*	Rice	Seed	n.i.	CD	(Mano and Morisaki, 2008)
Gammaproteobacteria	*Yersiniaceae*	*Ewingella*	*Ewingella americana*	Grapevine	Leaf	IT	CD	(Bulgari *et al.*, 2009)
Gammaproteobacteria	*Yersiniaceae*	*Ewingella*	*Ewingella persicina*	Grapevine	Leaf	IT	CD	(Bulgari *et al.*, 2009)
Gammaproteobacteria	*Yersiniaceae*	*Rahnella*		Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Gammaproteobacteria	*Yersiniaceae*	*Rahnella*	*Rahnella aquatilis*	Grapevine, pea	Stem, xylem sap	CA, NL, AU	CD	(Bell *et al.*, 1995; Elvira-Recuenco and van Vuurde, 2000; Miliute *et al.*, 2015; West *et al.*, 2010)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Gammaproteobacteria	*Yersiniaceae*	*Serratia*		Betula, black pepper, cotton, lemon, **lettuce**, maize, red clover, rice, soybean, spinach, wheat, wild rice	Leaf, nodule, root, sprout, stem, taproot	AR, CN, CA, JP, IN, KE, NP, ES, USA	CD, CI	(Aravind *et al.*, 2009; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Jackson et al., 2013; Kuklinsky-Sobral *et al.*, 2004; Mbai *et al.*, 2013; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Sturz *et al.*, 1998)
Gammaproteobacteria	*Yersiniaceae*	*Serratia*	*Serratia liquefaciens*	Potato	Stem, tuber	CA, AT	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Gammaproteobacteria	*Yersiniaceae*	*Serratia*	*Serratia marcescens*	Bell pepper, rice, wild rice	Root, shoot, stem	AT, JP, KE, NP	CD	(Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Mano and Morisaki, 2008; Mbai *et al.*, 2013; Rasche *et al.*, 2006; Rosenblueth and Martinez-Romero, 2006)
Gammaproteobacteria	*Yersiniaceae*	*Serratia*	*Serratia plymuthica*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Gammaproteobacteria	*Yersiniaceae*	*Serratia*	*Serratia proteamaculans*	Betula, soybean	Root, shoot	ES, USA	CD	(Mesa *et al.*, 2017; Santoyo et al., 2016; Taghavi *et al.*, 2009)
Gammaproteobacteria	*Yersiniaceae*	*Yersinia*	*Yersinia frederiksenii*	**Carrot**, cotton, maize	Root, stem	CA, USA	CD	(McInroy and Kloepper, 1995; Surette *et al.*, 2003)
Firmicutes	*Bacillaceae*	*Bacillus*		Bell pepper, black pepper, cereals, citrus plant, common bean, cotton, **cucumber**, grapevine, winged pea, lemon, **lettuce**, maize, potato, soybean, sugarbeet, **tomato**, watermelon, wheat, wild rice, woody plants	Boll, branche, leaf, nodule, radicle, root, seed, shoot, stem, tuber, unopened flowers	AR, AT, BR, CO, CN, CA, IT, JP, IN, KE, NP, USA	CD, CI	(Araujo *et al.*, 2001; Araújo *et al.*, 2002; Aravind *et al.*, 2009; Bulgari *et al.*, 2009; Compant *et al.*, 2011; de Oliveira Costa *et al.*, 2012; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Hou *et al.*, 2013; Jackson et al., 2013; Kuklinsky-Sobral *et al.*, 2004; Lopez-Lopez *et al.*, 2010; Mbai *et al.*, 2013; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Muresu *et al.*, 2008; Rai *et al.*, 2007; Rasche *et al.*, 2006; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus acidiceler*	Ginseng	Stem	KO	CD	(Vendan *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus aerius*	**Cucumber**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus aerophilus*	**Carrot**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus alclophialus*	Potato	Stem, tuber	AT, BR	CD	(Miliute *et al.*, 2015) (Reiter *et al.*, 2002; Sturz *et al.*, 1998; Vendan *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus amyloliquefaciens*	Common bean, ginseng	Leaf, root, stem	CO, CA, KO	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010; Vendan *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus anthracis*	Onion	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus aquimaris*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus arbutinivorans*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus aryabhattai*	Onion	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus asahii*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus bataviensis*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus boroniphilus*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus brevis*	Red clover	Nodule, root, taproot	CA	CD	(Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus carboniphilus*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus casei*	Grapevine	Leaf, cane, trunk	AT	CD	(West *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus cereus*	Banana, bell pepper, **carrot**, citrus plant, common bean, ginseng, grapevine, maize, rice, **tomato**, watermelon	Cane, crude extract, flower, leaf, root, seed, shoot, stem, trunk	AT, CO, CA, IN, KO, USA	CD	(Cho *et al.*, 2007; Compant *et al.*, 2011; Lopez-Lopez *et al.*, 2010; Mano and Morisaki, 2008; Nithya and Babu, 2017; Surette *et al.*, 2003; Thomas and Soly, 2009; Vendan *et al.*, 2010; West *et al.*, 2010; Xia *et al.*, 2015)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus chosinensis*	Alfalfa	Root	SR	CD	(Miliute *et al.*, 2015; Stajković *et al.*, 2009)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus circulans*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus coryneforms*	Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus endophyticus*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus fastidiosus*	Grapevine, soybean	Leaf, nodule, root, stem, xylem sap	NE, CA, AU, BR	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; West *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus firmus*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus flexus*	**Carrot**, common bean, ginseng, **tomato**	Crude extract, root, seed, stem	CO, KO, USA	CD	(Cho *et al.*, 2007; Lopez-Lopez *et al.*, 2010; Nithya and Babu, 2017; Vendan *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus foraminis*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus gibsonii*	Common bean, rice	Leaf, root	CO	CD	(Lopez-Lopez *et al.*, 2010; Mano and Morisaki, 2008)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus insolitus*	Grapevine	Stem, xylem sap	CA, AU	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; West *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus korlensis*	Common bean	Root, seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus lentimorbus*	Grapevine	Leaf, cane, trunk	AT	CD	(West *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus lentus*	Citrus plant	n.i.	n.i.	CD	(Lodewyckx *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus licheniformis*	Bell pepper, maize, **radish**, **tomato**, watermelon	Leaf, root, seed, shoot	KO, USA	CD	(Seo *et al.*, 2010; Xia *et al.*, 2015)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus luciferensis*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus megaterium*	Alfalfa, **carrot**, citrus plant, common bean, **cucumber**, ginseng, grapevine, maize, pea, red clover, rice, **tomato**	Cane, crude extract, leaf, nodule, root, seed, stem, taproot, trunk	AT, BR, CO, CA, IN, KO, NL, SR, USA	CD	(Lopez-Lopez *et al.*, 2010; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Nithya and Babu, 2017; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003) (Araujo *et al.*, 2001; Cho *et al.*, 2007; Elvira-Recuenco and van Vuurde, 2000; Mano and Morisaki, 2008; Rai *et al.*, 2007; Stajković *et al.*, 2009; Sturz *et al.*, 1998; Vendan *et al.*, 2010; West *et al.*, 2010; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus mojavensis*	Maize	Root, stem	IN, USA	CD	(Miliute *et al.*, 2015; Rai *et al.*, 2007; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus muralis*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus mycoides*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus nealsonii*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus niacini*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus pasteurii*	Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus polymyxa*	Wheat	Root	n.i.	CD	(Miliute *et al.*, 2015; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus pseudomycoides*	Ginseng	Stem	KO	CD	(Vendan *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus pumilus*	Bell pepper, betula, **carrot**, citrus plant, common bean, ginseng, maize, onion, rice, **tomato**, watermelon	Crude extract, leaf, root, seed, shoot, stem	CO, IN, KO, ES, USA	CD	(Lopez-Lopez *et al.*, 2010; Mano and Morisaki, 2008; Mesa *et al.*, 2017; Miliute *et al.*, 2015; Nithya and Babu, 2017; Rai *et al.*, 2007; Vendan *et al.*, 2010; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus sphaericus*	Ginseng, potato	Root, stem, tuber	AT, CA, KO	CD	(Cho *et al.*, 2007; Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	Bell pepper, **carrot**, citrus plant, common bean, ginseng, maize, onion, **radish**, rice, **tomato**, watermelon	Crude extract, leaf, root, seed, shoot, stem	BR, CO, CA, IN, KO, USA	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010; Mano and Morisaki, 2008; Miliute *et al.*, 2015; Nithya and Babu, 2017; Rai *et al.*, 2007; Seo *et al.*, 2010; Surette *et al.*, 2003; Vendan *et al.*, 2010; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus tequilensis*	**Tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus thioparans*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus thuringiensis*	Bell pepper, common bean, ginseng, grapevine, maize, **tomato**, watermelon	Cane, leaf, root, seed, shoot, stem, trunk	AT, BR, KO, IN, USA	CD	(de Oliveira Costa *et al.*, 2012; Miliute *et al.*, 2015; Rai *et al.*, 2007; Vendan *et al.*, 2010; West *et al.*, 2010; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Bacillus*	*Bacillus weihenstephanensis*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Firmicutes	*Bacillaceae*	*Exiguobacterium*		Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Firmicutes	*Bacillaceae*	*Exiguobacterium*	*Exiguobacterium acetylicum*	**Tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Fictibacillus*	*Fictibacillus barbaricus*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Geobacillus*	*Geobacillus stearothermophilus*	**Cucumber**, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Lysinibacillus*		Maize, wheat	Root, stem	AR, IN, USA	CD, CI	(Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Zinniel *et al.*, 2002)
Firmicutes	*Bacillaceae*	*Lysinibacillus*	*Lysinibacillus fusiformis*	Bell pepper, common bean, ginseng, maize, **tomato**, watermelon	Root, shoot, stem	CO, KO, USA	CD	(Lopez-Lopez *et al.*, 2010; Vendan *et al.*, 2010; Xia *et al.*, 2015)
Firmicutes	*Bacillaceae*	*Lysinibacillus*	*Lysinibacillus massiliensis*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Bacillaceae*	*Lysinibacillus*	*Lysinibacillus sphaericus*	Betula, common bean, ginseng	Leaf, root, stem	BR, KO, ES	CD	(de Oliveira Costa *et al.*, 2012; Mesa *et al.*, 2017; Vendan *et al.*, 2010)
Firmicutes	*Bacillaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Bacillaceae*	*Terribacillus*	*Terribacillus saccharophilus*	**Tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Bacillaceae*	*Virgibacillus*	*Virgibacillus proomii*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Firmicutes	*Listeriaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Listeriaceae*	*Listeria*	*Listeria monocytogenes*	Onion	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Paenibacillaceae*	*Brevibacillus*	*Brevibacillus agri*	Common bean, rice	Leaf, root	BR	CD	(de Oliveira Costa *et al.*, 2012; Mano and Morisaki, 2008)
Firmicutes	*Paenibacillaceae*	*Brevibacillus*	*Brevibacillus brevis*	**Tomato**	Stem, fruit	n.i.	CD	(Miliute *et al.*, 2015)
Firmicutes	*Paenibacillaceae*	*Brevibacillus*	*Brevibacillus parabrevis*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Firmicutes	*Paenibacillaceae*	*Brevibacillus*	*Brevibacillus reuszeri*	Bell pepper, maize, **tomato**, watermelon	Root, shoot	USA	CD	(Xia *et al.*, 2015)
Firmicutes	*Paenibacillaceae*	*Cohnella*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Paenibacillaceae*	*Cohnella*	*Cohnella thermotolerans*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Paenibacillaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*		Banana, bell pepper, betula, common bean, maize, **radish**, soybean, **tomato**, watermelon, wheat	Leaf, root, seed, shoot, stem	AR, BR, CO, IN, KO, ES, USA	CD, CI	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010; Mesa *et al.*, 2017; Miliute *et al.*, 2015; Rai *et al.*, 2007; Rascovan *et al.*, 2016; Seo *et al.*, 2010; Thomas and Soly, 2009; Xia *et al.*, 2015; Zinniel *et al.*, 2002)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus alvei*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus amylolyticus*	Rice	Seed	n.i.	CD	(Mano and Morisaki, 2008)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus barengoltzii*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)

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Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus cineris*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus glucanolyticus*	Ginseng	Stem	KO	CD	(Vendan *et al.*, 2010)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus humicus*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus illinoisensis*	**Carrot**, common bean	Crude extract, root	CO, USA	CD	(Lopez-Lopez *et al.*, 2010; Nithya and Babu, 2017)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus lautus*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus nanensis*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus odorifer*	Potato, sweet patato	Stem, tuber	AT, CA, KE, UG	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006; Sturz *et al.*, 1998)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus pabuli*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Firmicutes	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus polymyxa*	Bell pepper, **carrot**, ginseng, maize, **tomato**, watermelon	Crude extract, root, shoot	CA, KO, USA	CD	(Cho *et al.*, 2007; Nithya and Babu, 2017; Surette *et al.*, 2003; Xia *et al.*, 2015)
Firmicutes	*Planococcaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Planococcaceae*	*Sporosarcina*		Common bean, wheat	Leaf, root	AR, BR	CD, CI	(de Oliveira Costa *et al.*, 2012; Rascovan *et al.*, 2016)
Firmicutes	*Planococcaceae*	*Sporosarcina*	*Sporosarcina aquimarina*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Planococcaceae*	*Planomicrobium*	*Planomicrobium mcmeekinii*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Firmicutes	*Planococcaceae*	*Planomicrobium*	*Planomicrobium okeanokoites*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Firmicutes	*Planococcaceae*	*Solibacillus*	*Solibacillus silvestris*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Planococcaceae*	*Sporosarcina*		Common bean, wheat	Leaf, root	AR, BR	CD, CI	(de Oliveira Costa *et al.*, 2012; Rascovan *et al.*, 2016)
Firmicutes	*Planococcaceae*	*Sporosarcina*	*Sporosarcina aquimarina*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Staphylococcaceae*	*Macrococcus*	*Macrococcus equipercicus*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*		Bell pepper, common bean, cotton, grapevine, winged pea, maize, **tomato**, watermelon	Leaf, nodule, root, shoot, stem, xylem sap	AT, BR, CA, IT, USA	CD	(Bell *et al.*, 1995; de Oliveira Costa *et al.*, 2012; McInroy and Kloepper, 1995; Muresu *et al.*, 2008; Rasche *et al.*, 2006; Xia *et al.*, 2015)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus aureus*	Bell pepper, **carrot**, grapevine, onion	Crude extract, seed, shoot	AT, USA	CD	(Compant *et al.*, 2011; Nithya and Babu, 2017; Rasche *et al.*, 2006)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus caprae*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus cohnii*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus epidermidis*	Banana, bell pepper, **carrot**, common bean, ginseng, onion	Crude extract, leaf, root, seed, shoot, stem	AT, BR, CO, CA, IN, KO, USA	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010; Nithya and Babu, 2017; Rasche *et al.*, 2006; Surette *et al.*, 2003; Thomas and Soly, 2009; Vendan *et al.*, 2010)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus gallinarum*	Onion, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus haemolyticus*	Banana, common bean, **cucumber**	Crude extract, root, seed, shoot	CO, IN, USA	CD	(Lopez-Lopez *et al.*, 2010; Nithya and Babu, 2017; Thomas and Soly, 2009)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus hominis*	**Carrot**, common bean	Root	CO, CA	CD	(Lopez-Lopez *et al.*, 2010; Surette *et al.*, 2003)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus kloosii*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus pasteuri*	Common bean, ginseng	Root, seed, stem	CO, KO	CD	(Lopez-Lopez *et al.*, 2010; Vendan *et al.*, 2010)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus saprophyticus*	**Carrot**, common bean	Leaf, root	BR, CA	CD	(de Oliveira Costa *et al.*, 2012; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus sciuri*	**Carrot**, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus warneri*	**Carrot**, common bean	Leaf, root	BR, CO, CA	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010; Surette *et al.*, 2003)
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus xylosus*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Firmicutes	*Thermoactinomycetaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Carnobacteriaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Enterococcaceae*			Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Enterococcaceae*	*Enterococcus*		Common bean, grapevine	Leaf, seed	CO, IT	CD	(Bulgari *et al.*, 2009; Lopez-Lopez *et al.*, 2010)
Firmicutes	*Enterococcaceae*	*Enterococcus*	*Enterococcus faecium*	Onion	Crude extract	USA	CD	(Nithya and Babu, 2017)
Firmicutes	*Lactobacillaceae*	*Lactobacillus*		Sugarbeet	Root	n.i.	CD	(Hallmann *et al.*, 1997; Miliute *et al.*, 2015)
Firmicutes	*Leuconostocaceae*	*Leuconostoc*		Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Firmicutes	*Leuconostocaceae*	*Leuconostoc*	*Leuconostoc mesenteroides*	Betula	Root	ES	CD	(Mesa *et al.*, 2017)
Firmicutes	*Streptococcaceae*	*Streptococcus*		Bell pepper, **lettuce**, wheat	Leaf, root, shoot	AR, AT, USA	CD, CI	(Jackson et al., 2013; Rasche *et al.*, 2006; Rascovan *et al.*, 2016)
Firmicutes	*Clostridiaceae*	*Clostridium*		Maiden silvergrass, rice, soybean, wheat	Leaf, root, stem	AR, JP	CD, CI	(Mano and Morisaki, 2008; Miyamoto *et al.*, 2004; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006)
Firmicutes	*Clostridiales Family XII Incertae Sedis*	*Acidaminobacter*	*Acidaminobacter hydrogenoformans*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Firmicutes	*Clostridiales Family XVII Incertae Sedis*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Clostridiales Family XVII Incertae Sedis*	*Anaerovorax*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)

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Firmicutes	*Clostridiales Family XVII Incertae Sedis*	*Thermaerobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Gracilibacteraceae*	*Gracilibacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Lachnospiraceae*	*Lachnoclostridium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Lachnospiraceae*	*Lachnospiraceae*	*Lachnospiraceae bacterium*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Firmicutes	*Peptococcaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Ruminococcaceae*	*Ruminiclostridium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Symbiobacteriaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Syntrophomonadaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Halanaerobiaceae*	*Halanaerobium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Halobacteroidaceae*	*Natroniella*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Veillonellaceae*	*Anaerospora*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Veillonellaceae*	*Sporomusa*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Veillonellaceae*	*Pelosinus*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Veillonellaceae*	*Dendrosporobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Veillonellaceae*	*Megasphaera*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Firmicutes	*Thermolithobacteraceae*			Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Actinomycetaceae*	*Actinomyces*		Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Actinobacteria	*Cellulomonadaceae*	*Actinotalea*	*Actinotalea fermentans*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Actinobacteria	*Cellulomonadaceae*	*Cellulomonas*		Red clover, cotton	Nodule, root, stem, taproot	CA, USA	CD	(McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*		Banana, lemon, maize, potato, sugarbeet	Root, shoot, stem, tuber	AT, CA, IN, USA	CD	(Hallmann *et al.*, 1997; Miliute *et al.*, 2015; Rai *et al.*, 2007; Reiter *et al.*, 2002; Sturz *et al.*, 1998; Thomas and Soly, 2009; Zinniel *et al.*, 2002)
Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*	*Corynebacterium ammoniigenes*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Actinobacteria	*Dermabacteraceae*	*Brachybacterium*		**Radish**	Leaf	KO	CD	(Seo *et al.*, 2010)
Actinobacteria	*Dermabacteraceae*	*Brachybacterium*	*Brachybacterium zhongshanense*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Dietziaceae*	*Dietzia*	*Dietzia cinnamea*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Actinobacteria	*Geodermatophilaceae*	*Modestobacter*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Intrasporangiaceae*	*Janibacter*	*Janibacter melonis*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Intrasporangiaceae*	*Knoellia*		Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Kineosporiineae*	*Kineosporia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Microbacteriaceae*	*Agromyces*	*Agromyces mediolanus*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Actinobacteria	*Microbacteriaceae*	*Aureobacterium*		Maize, cotton	Root, stem	USA	CD	(McInroy and Kloepper, 1995)
Actinobacteria	*Microbacteriaceae*	*Clavibacter*		Grapevine, cotton	Boll, unopened flowers, radicle, root, stem	CA, AU	CD	(Bell *et al.*, 1995; Hallmann *et al.*, 1997; Miliute *et al.*, 2015; West *et al.*, 2010)
Actinobacteria	*Microbacteriaceae*	*Clavibacter*	*Clavibacter michiganense*	**Carrot**, cotton, maize, grapvine	Root, stem, xylem sap	CA, USA	CD	(Bell *et al.*, 1995; McInroy and Kloepper, 1995; Surette *et al.*, 2003)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*		Black paper, cotton, grapevine, winged pea, **lettuce**, maize, potato, rice, spinach, wheat	Leaf, nodule, root, stem, seed, tuber	AR, AT, AR, CA, IT, IN, AU	CD	(Aravind *et al.*, 2009; Bell *et al.*, 1995; Bulgari *et al.*, 2009; Jackson et al., 2013; Mano and Morisaki, 2008; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Muresu *et al.*, 2008; Rascovan *et al.*, 2016; Reiter *et al.*, 2002; Sturz *et al.*, 1998; West *et al.*, 2010)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium albidum*	Maize	Shoot	PT	CD	(Pereira and Castro, 2014)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium citrenum*	Potato, red clover	Nodule, root, stem, taproot, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium flaccumfaciens*	**Carrot**, citrus plant, maize, grapevine	Brache, cane, leaf, root, shoot, trunk, xylem sap	AT, CA, PT, ES, USA	CD	(Araújo *et al.*, 2002; Bell *et al.*, 1995; Pereira and Castro, 2014; Rosenblueth and Martinez-Romero, 2006; Surette *et al.*, 2003; West *et al.*, 2010)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium herbarum*	Maize	Shoot	PT	CD	(Pereira and Castro, 2014)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium luteum*	Grapevine, potato, red clover	Cane, leaf, nodule, root, stem, tuber, taproot, trunk	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998; West *et al.*, 2010)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium oceanosedimentum*	Maize	Shoot	PT	CD	(Pereira and Castro, 2014)
Actinobacteria	*Microbacteriaceae*	*Curtobacterium*	*Curtobacterium pusillum*	Grapevine	Xylem sap	CA	CD	(Bell *et al.*, 1995)
Actinobacteria	*Microbacteriaceae*	*Frigoribacterium*		**Lettuce**	Leaf	USA	CD	(Jackson et al., 2013)
Actinobacteria	*Microbacteriaceae*	*Frigoribacterium*	*Frigoribacterium faeni*	Common bean, maize	Leaf, shoot	BR, PT	CD	(de Oliveira Costa *et al.*, 2012; Pereira and Castro, 2014)
Actinobacteria	*Microbacteriaceae*	*Herbiconiux*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Microbacteriaceae*	*Leifsonia*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Microbacteriaceae*	*Leifsonia*	*Leifsonia aquatica*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Actinobacteria	*Microbacteriaceae*	*Leifsonia*	*Leifsonia poae*	**Lettuce**	Leaf	USA	CD	(Jackson et al., 2013)

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Actinobacteria	*Microbacteriaceae*	*Microbacterium*		Bell pepper, winged pea, **lettuce**, maize, **radish**, spinach	Leaf, nodule, root, shoot, steem	AT, IT, KO, USA	CD	(Jackson et al., 2013; McInroy and Kloepper, 1995; Muresu *et al.*, 2008; Rasche *et al.*, 2006; Seo *et al.*, 2010)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium arborescens*	**Cucumber**, onion	Crude extract	USA	CD	(Nithya and Babu, 2017)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium esteraromaticum*	**Carrot**, marigold	Root	CA	CD	(Rosenblueth and Martinez-Romero, 2006; Sturz and Kimpinski, 2004; Surette *et al.*, 2003)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium foliorum*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium hydrocarbonoxydans*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium imperiale*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium oleivorans*	Bell paper, **carrot**, common bean, maize, **tomato**, watermelon	Crude extract, root, shoot	CO, USA	CD	(Lopez-Lopez *et al.*, 2010; Nithya and Babu, 2017; Xia *et al.*, 2015)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium phyllosphaerae*	Betula, common bean, ginseng	Leaf, root, stem	BR, KO, ES	CD	(Cho *et al.*, 2007; de Oliveira Costa *et al.*, 2012; Mesa *et al.*, 2017; Vendan *et al.*, 2010)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium saperdae*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium schleiferi*	**Cucumber**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium testaceum*	Maize, common bean, **tomato**	Crude extract, leaf, root, shoot, stem	BR, IN, NE, PT, USA	CD	(de Oliveira Costa *et al.*, 2012; Nithya and Babu, 2017; Pereira and Castro, 2014; Rai *et al.*, 2007; Rosenblueth and Martinez-Romero, 2006; Zinniel *et al.*, 2002)
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	*Microbacterium trichothecenolyticum*	Alfalfa	Root	SR	CD	(Miliute *et al.*, 2015; Stajković *et al.*, 2009)
Actinobacteria	*Microbacteriaceae*	*Pseudoclavibacter*	*Pseudoclavibacter helvolus*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Actinobacteria	*Microbacteriaceae*	*Salinibacterium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*		Black pepper, **cucumber**, coton, ginseng, winged pea, maize	Nodule, root, stem, shoot	AT, IT, IN, KO, USA	CD	(Aravind *et al.*, 2009; Cho *et al.*, 2007; Hallmann *et al.*, 1997; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Muresu *et al.*, 2008; Rasche *et al.*, 2006)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter agilis*	Common bean	Seed	CO	CD	(Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter globiformis*	Maize	Root, stem	IN, USA	CD	(Miliute *et al.*, 2015; Rai *et al.*, 2007; Zinniel *et al.*, 2002)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthobacter ilicis*	Red clover	Nodule, root, taproot	CA	CD	(Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter mysorens*	**Cucumber**, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter nicotianae*	**Carrot**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter nitroguajacolicus*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter protophormiae*	**Cucumber**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Actinobacteria	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter ureafaciens*	Potato	Stem, tuber	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Actinobacteria	*Micrococcaceae*	*Kocuria*	*Kocuria carniphila*	Ginseng	Root	KO	CD	(Cho *et al.*, 2007)
Actinobacteria	*Micrococcaceae*	*Kocuria*	*Kocuria kristinae*	Banana, bell pepper, **carrot**, maize, **tomato**, watermelon	Root, shoot	CA, IN, USA	CD	(Surette *et al.*, 2003; Thomas and Soly, 2009; Xia *et al.*, 2015)
Actinobacteria	*Micrococcaceae*	*Kocuria*	*Kocuria palustris*	Common bean	Leaf, root	BR, CO	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Micrococcaceae*	*Kocuria*	*Kocuria varians*	**Carrot**, marigold, grapine	Leaf, cane, root, trunk	AT, CA	CD	(Rosenblueth and Martinez-Romero, 2006; Sturz and Kimpinski, 2004; Surette *et al.*, 2003; West *et al.*, 2010)
Actinobacteria	*Micrococcaceae*	*Micrococcus*		Black pepper, **cucumber**, cotton, maize, potato, **tomato**, watermelon, wild rice	Fruit, root, shoot, stem, tuber	AT, CA, JP, NP, KE, USA	CD	(Aravind *et al.*, 2009; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Hallmann *et al.*, 1997; Mbai *et al.*, 2013; McInroy and Kloepper, 1995; Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998; Xia *et al.*, 2015)
Actinobacteria	*Micrococcaceae*	*Micrococcus*	*Micrococcus aquilus*	Banana	Shoot	IN	CD	(Thomas and Soly, 2009)
Actinobacteria	*Micrococcaceae*	*Micrococcus*	*Micrococcus luteus*	Banana, bell pepper, **carrot**, common bean, ginseng, rice	Leaf, root, seed, stem, shoot	AT, BR, CO, CA, IN, KO	CD	(de Oliveira Costa *et al.*, 2012; Lopez-Lopez *et al.*, 2010; Mano and Morisaki, 2008; Rasche *et al.*, 2006; Surette *et al.*, 2003; Thomas and Soly, 2009; Vendan *et al.*, 2010)
Actinobacteria	*Micrococcaceae*	*Micrococcus*	*Micrococcus varians*	Red clover	Nodule, root, taproot	CA	CD	(Miliute *et al.*, 2015; Sturz *et al.*, 1998)
Actinobacteria	*Micrococcaceae*	*Paenarthrobacter*	*Paenarthrobacter aurescens*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Actinobacteria	*Micrococcineae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micrococcineae*	*Cellulomonas*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micrococcineae*	*Cellulosimicrobium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micrococcineae*	*Leifsonia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micrococcineae*	*Promicromonospora*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micrococcineae*	*Sanguibacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micromonosporaceae*	*Dactylosporangium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micromonosporaceae*	*Rugosimonospora*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Micromonosporaceae*	*Salinispora*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*		Wheat, scots pine	Buds, root	AU	CD	(Conn and Franco, 2004; Miliute *et al.*, 2015; Pirttila *et al.*, 2004; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Zinniel *et al.*, 2002)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*	*Mycobacterium petroleophilum*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Actinobacteria	*Nocardiaceae*	*Nocardia*		Citrus plant	Branche	BR, ES	CD	(Araújo *et al.*, 2002; Rosenblueth and Martinez-Romero, 2006)
Actinobacteria	*Nocardiaceae*	*Rhodococcus*		Bell pepper, grapevine	Stem, shoot	AT	CD	(Hallmann *et al.*, 1997; Rasche *et al.*, 2006)
Actinobacteria	*Nocardiaceae*	*Rhodococcus*	*Rhodococcus erythropolis*	Bell pepper, betule, common bean	Leaf, root, shoot	AT, BR, ES	CD	(de Oliveira Costa *et al.*, 2012; Mesa *et al.*, 2017; Rasche *et al.*, 2006)
Actinobacteria	*Nocardiaceae*	*Rhodococcus*	*Rhodococcus fascians*	Bell pepper	Shoot	AT	CD	(Rasche *et al.*, 2006)
Actinobacteria	*Nocardiaceae*	*Rhodococcus*	*Rhodococcus luteus*	Grapevine	Stem, xylem sap	CA, AU	CD	(Bell *et al.*, 1995; Miliute *et al.*, 2015; West *et al.*, 2010)
Actinobacteria	*Nocardioidaceae*	*Aeromicrobium*		**Lettuce**	Leaf	USA	CD	(Jackson et al., 2013)
Actinobacteria	*Nocardioidaceae*	*Nocardioides*		Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Nocardioidaceae*	*Nocardioides*	*Nocardioides alkalitolerans*	Maize	Shoot	PT	CD	3
Actinobacteria	*Promicromonosporaceae*	*Cellulosimicrobium*	*Cellulosimicrobium cellulans*	**Cucumber**, **tomato**	Crude extract	USA	CD	(Nithya and Babu, 2017)
Actinobacteria	*Pseudonocardiaceae*	*Amycolatopsis*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Pseudonocardiaceae*	*Pseudonocardia*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Streptomycetaceae*	*Streptacidiphilus*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Streptomycetaceae*	*Streptomyces*		Betula, bell pepper, rice, wheat	Leaf, root, shoot	AT, AR, AU, ES	CD, CI	(Coombs and Franco, 2003; Mano and Morisaki, 2008; Mesa *et al.*, 2017; Rasche *et al.*, 2006; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006)
Actinobacteria	*Streptomycetaceae*	*Streptomyces*	*Streptomyces halstedii* subsp. *scabies*	Grapevine	Leaf, cane, trunk	AT	CD	(West *et al.*, 2010)
Actinobacteria	*Streptomycetaceae*	*Streptomyces*	*Streptomyces kunmingensis*	Common bean	Root	CO	CD	(Lopez-Lopez *et al.*, 2010)
Actinobacteria	*Streptomycetaceae*	*Streptomyces*	*Streptomyces rochei* subsp. *rochei*	Grapevine	Leaf, cane, trunk	AT	CD	(West *et al.*, 2010)
Actinobacteria	*Streptosporangiaceae*	*Microbispora*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Streptosporangiaceae*	*Nonomuraea*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Streptosporangiaceae*	*Streptosporangium*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Thermomonosporaceae*	*Actinoallomurus*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Actinobacteria	*Thermomonosporaceae*	*Actinomadura*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes				Greapwine, soybean, wheat	Root, xylem sap	AR, CA	CI, CD	(Bell *et al.*, 1995; Rascovan *et al.*, 2016)
Bacteroidetes	*Amoebophilaceae*	*Cytophagales*		Rice	Stem	n.i.	CD	(Mano and Morisaki, 2008)
Bacteroidetes	*Bacteroidaceae*	*Bacteroides*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*			Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Chitinophaga*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Filimonas*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Flavisolibacter*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Flavitalea*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Niabella*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Sediminibacterium*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Segetibacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Chitinophagaceae*	*Terrimonas*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Cytophagaceae*	*Adhaeribacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Cytophagaceae*	*Dyadobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Cytophagaceae*	*Rufibacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Flavobacteriaceae*	*Capnocytophaga*		Potato	Tuber, stem	AT, CA	CD	(Miliute *et al.*, 2015; Reiter *et al.*, 2002; Sturz *et al.*, 1998)
Bacteroidetes	*Flavobacteriaceae*	*Chryseobacterium*		Bell pepper, **lettuce**, maize, soybean, spinach, **tomato**, watermelon, wheat	Leaf, root, shoot	AR, USA	CD, CI	(Jackson et al., 2013; Rascovan *et al.*, 2016; Xia *et al.*, 2015)
Bacteroidetes	*Flavobacteriaceae*	*Chryseobacterium*	*Chryseobacterium indologenes*	**Carrot**	Root	CA	CD	(Surette *et al.*, 2003)
Bacteroidetes	*Flavobacteriaceae*	*Chryseobacterium*	*Chryseobacterium soldanellicola*	Maize	Root	PT	CD	(Pereira and Castro, 2014)
Bacteroidetes	*Flavobacteriaceae*	*Chryseobacterium*	*Chryseobacterium taichungense*	Rice	Root	n.i.	CD	(Mano and Morisaki, 2008)
Bacteroidetes	*Flavobacteriaceae*	*Elizabethkingia*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Flavobacteriaceae*	*Empedobacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*		Bell pepper, betula, cereals, cotton, **lettuce**, maize, potato, soybean, **tomato**, vegetable, watermelon, wheat, woody plants	Leaf, root, seed, shoot, stem, tuber	AR, ES, USA	CD, CI	(Hallmann *et al.*, 1997; Jackson et al., 2013; McInroy and Kloepper, 1995; Mesa *et al.*, 2017; Rascovan *et al.*, 2016; Xia *et al.*, 2015)
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium frigoris*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium gleum*	Rice	Stem	n.i.	CD	(Mano and Morisaki, 2008)
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium psychrophilum*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Bacteroidetes	*Flavobacteriaceae*	*Myroides*		**Radish**	Leaf, root	KO	CD	(Seo *et al.*, 2010)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Bacteroidetes	*Flavobacteriaceae*	*Weeksella*		**Cucumber**	Root	USA	CD	(Mahaffee and Kloepper, 1997)
Bacteroidetes	*Sphingobacteriaceae*	*Mucilaginibacter*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Bacteroidetes	*Sphingobacteriaceae*	*Pedobacter*		**Lettuce**, soybean, wheat	Leaf, root	AR, USA	CD, CI	(Jackson et al., 2013; Rascovan *et al.*, 2016)
Bacteroidetes	*Sphingobacteriaceae*	*Pedobacter*	*Pedobacter terrae*	Maize	Shoot	PT	CD	(Pereira and Castro, 2014)
Bacteroidetes	*Sphingobacteriaceae*	*Sphingobacterium*		**Lettuce**, **radish**, rice, soybean, wheat	Leaf, root	AR, KO, USA	CD, CI	(Jackson et al., 2013; Mano and Morisaki, 2008; Rascovan *et al.*, 2016; Rosenblueth and Martinez-Romero, 2006; Seo *et al.*, 2010)
Bacteroidetes	*Sphingobacteriaceae*	*Sphingobacterium*	*Sphingobacterium multivorum*	Common bean	Leaf	BR	CD	(de Oliveira Costa *et al.*, 2012)
Planctomycetes				Populus, soybean, **tomato**, wheat	Leaf, root	AR, USA	CI	(Gottel *et al.*, 2011; Rascovan *et al.*, 2016; Romero *et al.*, 2014)
Planctomycetes	*Isosphaeraceae*	*Aquisphaera*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Phycisphaeraceae*			Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Blastopirellula*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Bythopirellula*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Gemmata*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Pirellula*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Schlesneria*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Singulisphaera*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Planctomycetes	*Planctomycetaceae*	*Zavarzinella*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia				Populus, soybean, **tomato**, wheat	Leaf, root	AR, USA	CI	(Gottel *et al.*, 2011; Rascovan *et al.*, 2016; Romero *et al.*, 2014)
Verrucomicrobia	*Chthoniobacteraceae*	*Chthoniobacter*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Opitutaceae*	*Alterococcus*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Opitutaceae*	*Opitutus*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Verrucomicrobiaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Verrucomicrobiaceae*	*Luteolibacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Verrucomicrobiaceae*	*Prosthecobacter*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Verrucomicrobiaceae*	*Roseimicrobium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Verrucomicrobia	*Verrucomicrobiaceae*	*Verrucomicrobium*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Chloroflexi				Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Chloroflexi	*Caldilineaceae*	*Caldilinea*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Chloroflexi	*Chloroflexaceae*	*Chloroflexus*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Chloroflexi	*Ktedonobacteraceae*	*Ktedonobacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Chloroflexi	*Oscillochloridaceae*	*Oscillochloris*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Chloroflexi	*Sphaerobacteraceae*	*Sphaerobacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Acidobacteria				Populus, soybean, **tomato**, wheat	Leaf, root	AR, USA	CI	(Gottel *et al.*, 2011; Rascovan *et al.*, 2016; Romero *et al.*, 2014)
Acidobacteria	*Acidobacteriaceae*	*Candidatus Koribacter*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Acidobacteria	*Acidobacteriaceae*	*Edaphobacter*		Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Acidobacteria	*Blastocatellaceae*	*Blastocatella*		Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Acidobacteria	*Holophagaceae*	*Holophaga*	*Holophaga foetida*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)
Aquificae				Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Armatimonadetes	*Armatimonadaceae*	*Armatimonas*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016) (Rascovan *et al.*, 2016)
Armatimonadetes	*Chthonomonadaceae*			Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Cyanobacteria				Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Deinococcus-Thermus	*Deinococcaceae*	*Deinococcus*		Bell pepper, maize, soybean, **tomato**, watermelon	Root, shoot	AR, USA	CD, CI	(Rascovan *et al.*, 2016; Xia *et al.*, 2015)
Deinococcus-Thermus	*Deinococcaceae*	*Deinococcus*	*Deinococcus indicus*	Rice	Root	n.i.	CI	(Mano and Morisaki, 2008)

Phylum/ Class	Family	Genus	Species	Plant types	Plant parts	Country	Approach	References
Dictyoglomi				Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Gemmatimonadetes				Populus	Root	USA	CI	(Gottel *et al.*, 2011)
Gemmatimonadetes	*Gemmatimonadaceae*	*Gemmatimonas*		Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Spirochaetes				Soybean	Root	AR	CI	(Rascovan *et al.*, 2016)
Synergistetes				Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Synergistetes	*Synergistaceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Tenericutes	*Acholeplasmataceae*			Wheat	Root	AR	CI	(Rascovan *et al.*, 2016)
Tenericutes	*Acholeplasmataceae*	*Candidatus Phytoplasma*	*Candidatus Phytoplasma vitis*	Grapevine	Leaf	IT	CD	(Bulgari *et al.*, 2009)
Thermotogae				Soybean, wheat	Root	AR	CI	(Rascovan *et al.*, 2016)

The table comprises studies published between 1995-2017 with the main objective of analyzing the endophytic bacterial diversity.

Countries: AT Austria; AR, Argentina; AU, Australia; BR, Brazil; CO, Colombia; CN, China; CA, Canada; GN, Guinea; HK, Hong Kong; IN, India; IT, Italy; JP, Japan; KE, Kenya; KO, Korea; MX, Mexico; NL, The Netherlands; NP, Nepal; PH, Philippines; PK, Pakistan; PT, Portugal; SN, Senegal; ES, Spain; SR, Serbia; UG, Ugand.

n.i., not identified. CD and CI, culture-dependent and culture-independent methods, respectively.

Table S3.2. Most representative endophytic bacterial family and genera (cited in ≥ 5 publications) reported in raw-eaten vegetables (indicated in bold the ones with described increased capacity for contaminants uptake), their occurrence in wastewater and human microbiomes and their association with antibiotic resistance and pathogenicity.

Family	Genera	Raw-eaten vegetables	Vegetables parts	Wastewater Microbiome	Human microbiome[1]	Likely pathogenicity based on bacterial infectious diseases	Classes of antibiotics	ARGs[2]	References Wastewater, microbiome and pathogenicity
Enterobacteriaceae	*Enterobacter*	Black pepper, **carrot**, **cucumber**, grapwine, **lettuce**, onion, **radish** black pepper, lemon, spinach, strawberry	Crude extract, leaf, nodule, root, stem, tuber, xylem sap	+	GIT, oral, UGT	Bacteremia, pneumonia, urinary tract infections, endocarditis	AG	*aac*(3')-IIa, *aac*(3')-VI, *aac*(6')-Ib, *aac*(6')-If, *aph*(3')-Ia, *aph*(6')-Id, *ksg*A	(Amador *et al.*, 2015; Davin-Regli and Pagès, 2015; Pati *et al.*, 2018)
							β-L	bla_{GES}, bla_{VEB}, bla_{TEM}, bla_{CTX-M}, bla_{PER}, bla_{SHV-2}, bla_{BRO}, bla_{PSE-1}, bla_{OXA-2}, bla_{OXA-10}, bla_{IMP}, bla_{SIM}, bla_{VIM}, *Omp*36	
							M	*ere*A, *mac*A, *mac*B	
							S	*dfr*A1, *dfr*A3, *dfr*A5, *dfr*A15, *dfr*A17, *dfr*A19, *sul*1	
							T	*tet*C, *tet*D	
							O	*bac*A, *cat*A2, *cat*B3, *cml*E1, *fos*A, *mdt*G, *mdt*H, *acr*R, *rob*A, *acr*A, *acr*B, *tol*C	
Yersiniaceae	***Serratia***	Bell pepper, black pepper, ginseng, lemon, **lettuce**, spinach	Leaf, nodule, root, shoot, stem	+	Airways	Meningitis, pneumonia, endocarditis, bacteremia	AG	*aac*(3')-Ia, *aac*(3)-Ib, *aac*(6')-Ib, *aac*(6')Ic, *aph*(3')-Ia, *ksg*A	(Gupta and Thakur, 2015; Khanna *et al.*, 2013; Mahlen, 2011)
							β-L	bla_{SME-1}, bla_{KPC}, bla_{TEM-1}, bla_{CTX-M}, bla_{OXA-1}, bla_{IMP}, bla_{VIM}	
							Q	*qnr*B	
							S	*dfr*A1, *dfr*A12	
							T	*tet*41, *tet*B	

							O	*bac*A, *acr*B, *cat*A1, *cat*B3, *cml*E1, *fos*A *mdt*G *ros*A, *ros*B *omp*1	
Erwiniaceae	***Erwinia***	**Carrot**, **lettuce**, ginseng, sugarbeet, spinach	Root, leaf, stem, tuber	+	n.i	n.i	AG O	*aph*(33)-Ib, *aph*(6')-Id *acr*B, *bac*A	(Li *et al.*, 2010)
Moraxellaceae	***Acinetobacter***	Banana, bell pepper, **carrot**	Root, shoot, leaf	+	GIT, UGT, skin	Pneumonia, septicemia, skin infections, bacteremia, meningitis	AG β-L Q S T O	*aac*(3')-Ia, *aac*(3')-Iia, *aac*(6')-Ia, *aac*(6')-Iia, *aac*(6')-Ib, *aac*(6')-Ig, *aph*(3')-Ia, *aph*(3')-VIa, *aph*(6')-Id bla_{GES}, bla_{VEB}, bla_{TEM}, bla_{CTX-M}, bla_{PER}, bla_{SHV-2}, bla_{BRO}, bla_{PSE-1}, bla_{OXA-2}, bla_{OXA-10}, bla_{IMP}, bla_{SIM}, bla_{VIM}, *Opr*D, *amp*C *gyr*A, *par*C *dfr*A1, *dfr*A7, *dfr*A10, *dfr*A12, *dfr*A17, *sul*1 *tet*39, *tet*A, *tet*C *ade*A, *ade*B, *ade*C *cat*A1, *cat*B2, *cat*B3, *cml*E1, *cml*E3, *lpx*	(Asif *et al.*, 2018; Da Silva *et al.*, 2007)
Pseudomonadaceae	***Pseudomonas***	Banana, bell pepper, black pepper, **carrot**, **cucumber**, ginseng, grapevine, lemon, **lettuce**, onion, **radish**, strawberry, **tomato**, watermelon	Crude extract, flower, leaf, nodule, root, seed, shoot, stem, tuber	+	Airways, blood, GTI, skin, UGT	Malignant external otitis, endocarditis, meningitis, pneumonia, septicemia	AG β-L	*aac*(3')-Ia, *aac*(3')-IIa, *aac*(3')-IIi, *aac*(6')-Ib, *aac*(6')-IIa, *aac*(6')-Iib, *ant*(2")-Ia, *ant*(2")-Ib, *ant*(4)-IIa, *aph*(3')-Ib, *aph*(3')-Via, *aph*(6')-Ic, *aph*(6')-Id, *emr*E bla_{PAO}, bla_{PSE-1}, bla_{PSE-3}, bla_{GES}, bla_{KPC}, bla_{VEB}, bla_{TEM-1}, bla_{TEM-2}, bla_{PER}, bla_{VIM}, bla_{SHV-2}, bla_{ICR-1}, bla_{OXA-1}, bla_{OXA-2}, bla_{OXA}, bla_{OXA-9}, bla_{OXA-10}, bla_{GIM}, bla_{IMP}	(Azam and Khan, 2019; Gellatly and Hancock, 2013; Luczkiewicz *et al.*, 2015)

							M Q S T O	*ere*A *par*C *dfr*A1, *dfr*A2, *dfr*A12, *dfr*A15, *dfr*A17, *sul*1 *tet*A, *tet*B, *tet*G, *tet*X *bac*A, *cat*B2, *cat*B3, *cat*B4, *cml*E1, *cml*E8, *fos*C, *mex*A, *mex*B, *mex*D, *mex*E, *mex*F, *mex*X, *mex*Y, *msr*A, *opr*J, *opr*M, *opr*N, *cpx*R	
Xanthomonadaceae	***Stenotrophomonas***	Bell pepper, **carrot**, **cucumber**, ginseng, **lettuce**, onion, **radish,** spinach, tomato, watermelon	Leaf, seed, crude extract, root, shoot, stem	+	GIT	Bacteremia, urinary tract infections, endocarditis, meningitis	β-L M Q T	bla_{TEM-2}, bla_{L-1} *mph*C *sme*A, *sme*B, *sme*C, *sme*D, *sme*E, *sme*F *tet*G	(Brooke, 2012; Kalidasan *et al.*, 2018)
Xanthomonadaceae	***Xanthomonas***	**Carrot**, **cucumber**, **lettuce**, onion	Crude extract, leaf, root	n.i.	n.i	n.i.	AG	*aph*(3')-Ib, *aph*(6')-Id	n.i.
Burkholderiaceae	***Burkholderia***	Banana, bell pepper, **cucumber**, onion, pineapple, **tomato**, watermelon	Leaf, nodule, root, seed, shoot, stem	+	Airways	Melioidosis, Pneumonia	AG β-L O	*aph*(3')-Ia bla_{OXA-2} *bac*A, *ceo*A, *ceo*B, *ocp*M, *omp*38, *amr*A, *amr*B, *opr*A	(Liu *et al.*, 2014; Peng *et al.*, 2018)
Oxalobacteraceae	-	Banana, bell pepper, **lettuce**	Leaf, nodule, root, seed, shoot, stem	n.i.	Oral, GIT	Pneumonia, meningoencephalitis	n.i.	n.i.	(Baldani *et al.*, 2014)
Rhizobiaceae	***Agrobacterium***	Banana, **carrot**, **cucumber**, ginseng, **lettuce**, **radish**	Root, nodule, stem, shoot, tuber	+	n.i.	Bacteraemia	T O	*tet*30 *cat, cat*B1	(Christakis *et al.*, 2006; Yan *et al.*, 2017)

Bacillaceae	***Bacillus***	Banana, bell pepper, black pepper, **carrot**, **cucumber**, ginseng, grapevine, lemon, **lettuce**, onion, **radish**, **tomato**, watermelon	Crude extract, leaf, root, seed, shoot, stem	+	Bone, skin, UGT, GIT	Bacteremia, meningitis, endophthalmitis, pneumonia	AG	*aad*D, *aph*(3')-Ia, *aph*(3')-IVa	(Bottone, 2010; Vaz-Moreira *et al.*, 2014)
							β-L	*bla*A	
							GP	*ble*, *van*A, *van*HA, *van*RA, *van*RB, *van*SA, *van*XA, *van*YA, *van*Z	
							LP	*pgs*A	
							M	*mph*A, *mph*B, *mph*F	
							T	*tet*L, *tet*M	
							O	*bac*A, *bcr*A, *bcr*C, *fos*B *tmr*B *blt*, *bmr*, *cata*10, *cata*6, i*mr*B, *erm*B, *erm*C, *erm*D	
Staphylococcaceae	***Staphylococcus***	Banana, bell pepper, **carrot**, **cucumber**, ginseng, grapevine, onion, **tomato**, watermelon	Crude extract, leaf, root, seed, shoot, stem, xylem sap	+	Airways, blood, bone, ear, eye, GIT, nose, skin, UGT	Skin infections, pneumonia, endocarditis, osteomyelitis	AG	*aac*(6')-Ie, *aad*(9')-Ib, *aad*D, *aph*(3')-IIIa	(Feng *et al.*, 2008; Gómez *et al.*, 2016; Lakhundi and Zhang, 2018)
							β-L	bla_{LEN}, bla_{PC}, *mec*A, *mec*R1	
							GP	*ble*, *van*A, *van*HA, *van*RA, *van*SA, *van*XA, *van*YA, *van*Z	
							LP	*agr*A, *cls*, *pgs*A, *rpo*C, *wal*K, *mpr*F	
							M	*mef*A, *mph*C	
							Q	*gyr*A, *gyr*B, *par*C	
							SG	*vat*A, *vat*B, *vat*C, *vga*A, *vga*B, *vgb*A, *vgb*B	
							S	*dfr*A12, *dfr*A17	

							T O	*tet*38, *tet*K, *tet*L, *tet*M, *str* *bac*A, *bcr*A, *cat*A7, *cat*A8, *cat*A9, *cml*E1, *cml*E4 *Glp*T, *Uhp*T, *fus*A, *fus*B, *fus*E, *mur*A *ile*S, *mup*A, *mup*B *men*A *erm*A, *erm*A, *erm*C, *erm*Y, *msr*A, *inu*A, *rop*B	
Paenibacillaceae	***Paenibacillus***	Banana, bell pepper, **carrot**, ginseng, **radish, tomato**, watermelon	Crude extract, leaf, root, seed, shoot, stem	+	GIT, oral	Urinary tract infection	GP T O	*van*SA, *van*RA, *van*RB, *van*HA, *van*A, *van*XA *tet*L *bac*A	(Grady *et al.*, 2016; Mohammed *et al.*, 2017)
Microbacteriaceae	***Microbacterium***	Bell pepper**, carrot**, **cucumber**, ginseng, **lettuce,** onion, **radish**, spinach, **tomato**, watermelon	Crude extract, leaf, root, shoot, stem	+	Oral, skin	n.i.	n.i.	n.i.	(Kim *et al.*, 2011)
Micrococcaceae	***Kocuria***	Banana, bell pepper, **carrot**, ginseng, **tomato**, watermelon	Crude extract, root, shoot	+	GIT, skin	Endocarditis, peritonitis	n.i.	n.i.	(Benmalek and Fardeau, 2016; Dotis *et al.*, 2015)
Micrococcaceae	***Arthrobacter***	bell pepper, black pepper, **carrot**, **cucumber**, ginseng, **tomato**	Crude extract, root, shoot, stem	+	UGT	Endocarditis	O	*erm*R	(Agunbiade *et al.*, 2017; Bernasconi *et al.*, 2004)
Micrococcaceae	***Micrococcus***	Banana, bell pepper, black pepper, **carrot**, **cucumber**, ginseng, **tomato**, watermelon	Leaf, root, seed, shoot, stem, tuber	+	GIT, UGT, skin	Endocarditis, meningitis	n.i.	n.i.	(Liu *et al.*, 2007; Miltiadous and Elisaf, 2011)
Flavobacteriaceae	***Chryseobacterium***	Bell pepper, **carrot**, **lettuce**, spinach, **tomato**, watermelon	Leaf, root, shoot	+	UGT	Pneumonia, meningitis	β-L	*bla*B	(Chen *et al.*, 2013; Kämpfer *et al.*, 2003)

[1]Endophytic bacterial genera were screened for their occurrence in the human microbiome using database of Human Microbiome Project (https://www.hmpdacc.org/catalog/). Legend: UGT, urogenital tract; GIT, gastrointestinal tract; n.i., no info was found.

[2]Endophytic bacterial genera were screened for the presence of antibiotic resistance genes (ARGs) using database such as ARDB and CARD

(https://ardb.cbcb.umd.edu/index.html; https://card.mcmaster.ca/aro/list).

Legend for the classes of antibiotics: Aminoglycosides (AG), Beta-lactams (β-L), Glycopeptide (GP), Lipopeptides (LP), Macrolides (M), Oxazolidinones (OL), Quinolone (Q), Streptogramins (SG), Sulfonamides (S), Tetracyclines (T), Others (O) which include the following antibiotic classes: Acriflavin, Aminocoumarins, Aminonucleoside, Amphenicols, Anthracycline, Fosfomycins, Fosmidomycin, Lincosamides, Lysocin, Mupirocin, Nucleoside, Polypeptide. The main antibiotics listed in the table (AG, β-L, GP, LP, M, OL, Q, SG, S and T) represent the main antibiotics used in humans and plants (Fair and Tor, 2014; Stockwell and Duffy, 2012). n.i., no info was found; +, bacterial genera found in wastewater. In blue, bacteria phylogenetically closely related to human pathogens.

4. Limited occurrence of antibiotic resistance in culturable bacteria thriving on the surface of edible fruit and vegetable

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Authors: Nazareno Scaccia, Catarina Ferreira, Joana Abreu-Silva, Ivone Vaz-Moreira, Célia M. Manaia

Authors contributions:

	Planning	**Experimental work**				**Data analysis**	**Writing**
		Sample collection and processing	Phenotypic and genotypic isolate characterization	16Sr RNA gene sequence analysis	Horizontal gene transfer		
Nazareno Scaccia	**x**	**x**	**x**	**x**		**x**	**x**
Dr. Catarina Ferreira	**x**	**x**	**x**		**x**		
Joana Abreu-Silva				**x**	**x**		
Dr. Ivone Vaz-Moreira	**x**	**x**	**x**	**x**		**x**	**x**
Prof. Célia Manaia	**x**					**x**	**x**

4.1. Abstract

Vegetables and fruits are beneficial diet components, which regular intake contribute to prevent chronic disease. However, these products may transmit foodborne pathogens, especially if consumed without being processed. Because of the widespread distribution of antibiotic resistant bacteria (ARB) among different One-Health compartments, their occurrence in raw fresh produce and consequent transmission to humans is an issue that deserves investigation. This study aimed to explore whether unprocessed vegetables and processed fruits could host ARB, associated antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). The possibility that vegetable- and fruit-bacterial isolates could acquire ARGs by transformation assay was also assessed.

The taxonomic identification and antibiotic resistance phenotypes of culturable bacteria thriving on the surface of ready-to-eat watercress or associated with strawberry were studied.

The results showed that ready-to-eat watercress presented counts of total heterotrophs ranging from 5.7 to 7.8 log (CFU/mL) in watercress washing solution and 6.0 log (CFU/g dry weight) in unprocessed strawberry. Watercress isolates (> 40%) displayed resistance to cephalothin, amoxicillin, ticarcillin, sulfamethoxazole and sulfamethoxazole/trimethoprim, presumably intrinsic in these bacteria. A slightly distinct pattern was observed in strawberry isolates, among which was observed mostly (60%) resistance to sulfamethoxazole.

Among the Gram-negative isolates, were members of the genera *Pseudomonas, Stenotrophomonas, Erwinia, Rahnella*, *Methylobacterium*, and *Chryseobacterium*. These genera comprise previously reported endophytes as well as opportunistic pathogens capable of acquiring ARGs (e.g. *Pseudomonas* spp. and *Stenotrophomonas* spp.). However, the screening of ARGs, *intI1* and plasmid replicon types, suggested that acquired resistance among the examined plant-associated bacterial isolates was scarce. Nevertheless, the identification of ubiquitous bacteria raised the question about the capability of these bacteria to acquire ARGs. This was tested by induced transformation, which led to the emergence of tetracycline and meropenem resistance phenotype in isolates identified as *Rahnella inusitata* FCA-5D, *Stenotrophomonas rhizophila* VA.FA9 and *Pseudomonas fragi* VA.F11.

As a conclusion, and in spite of the small sample size, bacteria associated to these products were not observed to represent major vectors of antibiotic resistance. In general, these results suggest that this is an area that deserves future research.

4.2. Introduction

Human health benefits associated with the consumption of fruits and vegetables are well established (Pem and Jeewon, 2015; Slavin and Lloyd, 2012). Health agencies such as the World Health Organization (WHO) incentivize the consumption of plant-based food, as an equilibrated diet that can contribute to reduce the risks associated to noncommunicable diseases (WHO, 2019). Nonetheless, vegetables may be associated with foodborne diseases (via infection or toxicoinfection) (Bhunia, 2008), mainly when are consumed raw or unprocessed (Alegbeleye *et al.*, 2018; Soderqvist *et al.*, 2017). For example, vegetable- and fruit-borne bacterial outbreaks, such as *Escherichia coli* gastroenteritis, salmonellosis and campylobacteriosis have been reported (Callejon *et al.*, 2015; Carstens *et al.*, 2019; de Oliveira Elias *et al.*, 2018; Fröhling *et al.*, 2018; Mohammadpour *et al.*, 2018). The risks associated with plant-based food, particularly raw-eaten vegetables, as vehicles of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been also recognized (Blau *et al.*, 2018; Chen *et al.*, 2019; Hölzel *et al.*, 2018).

The plant microbiome has been suggested as a major route by which humans can come in contact with environmental bacteria, including ARB (Chen *et al.*, 2019). Although plants can internalize these bacteria from the surrounding ecosystem, bacteria with intrinsic or acquired ARGs can contaminate the surface of plants at the pre- (e.g. through manure application or irrigation practices) or post-harvest procedure (Alegbeleye *et al.*, 2018).

ARB are increasingly regarded as human and animal health threats (WHO, 2018b). The use of antibiotics for bacterial human, animal and plant disease has contributed to shaping the antibiotic resistome, which refers to the set of genes conferring antibiotic resistance in pathogenic and non-pathogenic bacteria (Chen *et al.*, 2019; Sundin and Wang, 2018; Wright, 2010). If genetic resistance determinants are present within mobile genetic elements (MGEs) (e.g. plasmids) they can be transferred among bacteria by horizontal gene transfer (HGT) processes (Chen *et al.*, 2019; Sundin and Wang, 2018). The plant phyllosphere and the rhizosphere have been indicated as sites where HGT may occur and, therefore, where ARGs dissemination may eventually occur (Blau *et al.*, 2018; Sundin and Wang, 2018). Although the knowledge about vegetables and fruits microbiome resistome are still sparse (Chen *et al.*, 2019; Sundin and Wang, 2018), the fact that plant associated ARB might impact the human and/or animal health cannot be overlooked.

Fresh produce is mostly commercialized unprocessed or minimally processed, in the case of ready-to-eat products (Mohammadpour *et al.*, 2018). Washing, cutting and packaging are the processing stages before reaching the retail market (Fröhling *et al.*, 2018;

Mohammadpour *et al.*, 2018). However, it has been argued that if these ready-to-eat products are contaminated with helminths, pathogens or ARB they will be important paths of transmission of these microbiological agents to humans (Bekele and Shumbej, 2019; Maldonade *et al.*, 2019; Mohammadpour *et al.*, 2018; Zhou *et al.*, 2020). Moreover, although when food products are processed and the biological contaminants load is reduced, it is possible that a part will persist (Verraes *et al.*, 2013). This consequence will be particularly relevant when light processing is used as is the case of fresh produce.

This study was designed as a set of preliminary monitoring assays to assess the occurrence of ARB and eventual ARGs and MGEs on the surface of ready-to-eat watercress and cultivable epiphytic and endophytic bacteria from processed strawberry. It was also aimed to assess whether these isolates were able to acquire ARGs by induced transformation.

4.3. Materials and methods

4.3.1. Watercress sampling and bacterial enumeration

Two samples of ready-to-eat watercress were purchased from two different local supermarkets (A and B) in Porto (Portugal) in November 2017 and March 2018, respectively. Each of the packaged watercress samples purchased were kept at refrigerator temperature before analysis. Approximately 50 g of watercress leaves of each sample were washed in 500 mL of sterile saline solution (0.85% w/v NaCl) vigorously for 30 seconds to detach bacteria from the watercress surface, and then ten-fold diluted. Decimal dilutions of the washing water were filtered using cellulose nitrate membranes (0.45µm porosity) and placed onto Plate Count Agar (PCA) medium (Liofilchem®, Italy), Membrane Fecal Coliform (mFC) Agar medium (BD Difco™), or onto these culture media supplemented with amoxicillin (AML) (32 mg/L). The medium mFC was used to investigate the presence of entereobacteria and AML supplementation as an attempt to detect ARB, both likely contaminants in watercress coming from water irrigation. Cultures were incubated at 30 °C for 4 days, and the number of Colony Forming Units (CFUs) was determined. Colonies with distinct morphologies on each of those culture media were isolated, purified and further characterized as described below.

4.3.2. Sampling of strawberries along industrial processing

Strawberries samples were collected along the line of production and processing in an industrial plant producing yogurts or other food products additives (**Figure 4.1**). Strawberry samples were collected in April 2017 from 4 points along the process – production field,

transport to the industrial unit, washing and freezing (**Figure 4.1**). Samples were named as S1 - harvested in the production field, S2 – S1 batch after transportation to the industrial unit and peduncle removal, S3 – S2 after washing with a solution of sodium hypochlorite 200 ppm (v/v) and a re-wash with tap water to remove the excess of sodium hypochlorite, and S4 - after fruit cutting and individual quick freezing (IQF) (**Figure 4.1**). Strawberries samples were collected into sterile plastic bags in triplicate and stored at 4 °C until further analyses. Based on the hypothesis that the strawberries washing water although disinfected with sodium hypochlorite could promote the recirculation of bacteria among distinct batches, washing water was also sampled and analysed (sample W, **Figure 4.1**). A volume of 5 L of washing water (sample W) was collected using a sterilized plastic bottle and sodium hypochlorite was neutralized with sodium thiosulfate [0.01% (w/v)].

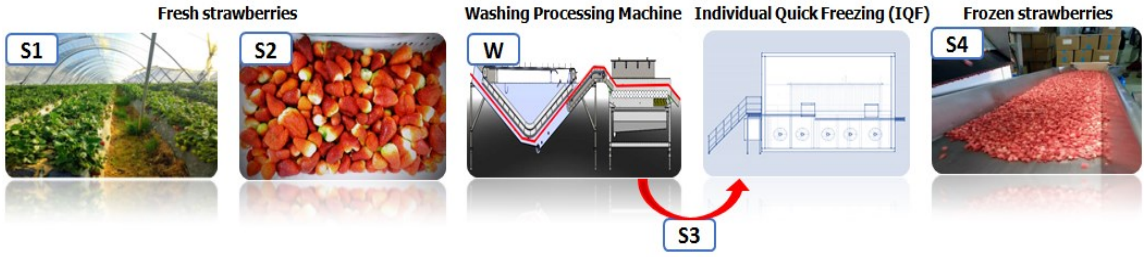

Figure 4.1. Representative scheme of the industrial strawberry processing. Samples collected are indicated by letters S and W. Legend: S1, strawberries harvested directly from the field; S2, strawberries before disinfection process (peduncle has already been removed); S3, strawberries after disinfection process; S4, frozen strawberries; W, washing water used to disinfect the strawberries

Approximately 100 g of each fruit sample was macerated in a Stomacher at high speed for 5 minutes. Ten-fold serial dilutions for each sample were made, and the enumeration of cultivable bacteria using the filtration method was performed. Cellulose nitrate membranes (0.45 µm porosity) after filtration were placed onto PCA and incubated at 30 °C for 7 days. The strawberry dry weight was determined for 1 mL of strawberry slurry, collected in triplicate, by incubation at 60 ºC, until a constant weight was achieved.

Expecting low bacterial loads, samples S3, S4 and W were enriched in Luria-Bertani broth medium (LB, Life Technologies™, USA), by adding 1 mL of strawberry slurry or water to the LB (9 mL) and incubated for 7 days (samples E). After the incubation time, a volume of 100 µL of each enrichment culture was plated on PCA and incubated for 24-48 h at 30 ºC. Colonies with distinct morphologies on PCA were isolated, purified and further

characterized. The purified cultures were preserved in LB medium supplemented with 15% (v/v) of glycerol and stored at – 80 °C.

4.3.3. Characterization of bacterial isolates

All the bacterial isolates were successively streaked onto PCA and further characterized for colony and cellular morphology, catalase, cytochrome c oxidase, and Gram-staining (Smibert and Krieg, 1994). In addition, all the isolates were tested for susceptibility to 12 antibiotics based on the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015). Specifically to, amoxicillin (AML, 25 μg) and ticarcillin (TIC, 75 μg) for penicillin antibiotic class, ceftazidime (CEF, 30 μg) and cephalothin (CP, 30 μg) for cephalosporin antibiotic class, meropenem (MER, 10 μg) for carbapenem antibiotic class, tetracycline (TET, 30μg) for tetracycline antibiotic class, sulfamethoxazole (SUL, 25 μg) and sulfamethoxazole/trimethoprim (SXT, 25 μg) for sulphonamide antibiotic class, gentamicin (GEN, 10 μg) and streptomycin (STR, 10 μg) for aminoglycoside antibiotic class, ciprofloxacin (CIP, 5 μg) for quinolone antibiotic class, and colistin sulphate (CT, 50 μg) for polypeptides antibiotic class. The antibiotic susceptibility testing was performed on Mueller-Hinton agar (OXOID) incubated at 30 °C for 24 h. The strain *E. coli* ATCC 25922 was used as control, and the bacterial phenotypes were classified as resistant (R), susceptible (S) or intermediate (I) according to the CLSI guidelines (CLSI, 2015). Gram-negative isolates with resistance phenotypes to antibiotics belonging to at least three distinct classes were selected for further analysis.

This selection, which excluded identical bacterial isolates recovered on the same isolation event, corresponded to 6 strawberry and 22 watercress isolates (Table 1). These isolates were identified based on the 16S rRNA gene sequence analysis as described previously (Ferreira da Silva *et al.*, 2007), using the EzBioCloud 16S rRNA database (Yoon *et al.*, 2017). The same DNA extracts were used to survey the presence of the target genes class 1 integrase gene *intl1* and the ARGs *sul1*, *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA-A}}$ and *bla*$_{\text{SHV}}$ or the plasmid replicon types FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA. These analyses were based on conventional Polymerase Chain Reaction (PCR) as described before (Carattoli *et al.*, 2005; Narciso-da-Rocha *et al.*, 2018).

4.3.4. Transformation assay

The limited occurrence of antibiotic resistance in the examined strawberry and watercress bacterial isolates, motivated the assessment if some of these isolates might be

able to uptake exogenous DNA by transformation and acquire resistance to antibiotics. This possibility was assessed in isolates identified as *Pseudomonas paralactis* (strain CAA-6H, from watercress sample A), *P. fragi* (strain VA.F11, from watercress sample B), *S. rhizophila* (strain VA.FA9, from watercress sample B), *Rahnella inusitata* (strain FCA-5D, from watercress sample A), *R. aquatilis* (strain EFS4.3, from the enrichment of the strawberry sample S4), and *Erwinia persicina* (strain EFS3.2, from the enrichment of the strawberry sample S3). These strains were selected as receptors because besides their possible ubiquity suggested by the taxonomic identification and because two of them harboured the genes *intI1* and HI1, whose presence might hint some capabilities in HGT processes. Receptor cells cultivated on PCA at 30 °C for 24 hours were made competent by calcium chloride exposure [$CaCl_2$ solution: 60 mM $CaCl_2$ in 15% glycerol and 10 mM PIPES (piperazine-N,N′-bis(2-hydroxypropanesulfonic acid)), pH 7] (Chang *et al.*, 2017). The donor DNA consisted on plasmid DNA extracted from two strains, the tetracycline- and quinolone-resistant *E. coli* strain H1FC54, harbouring the ARGs $bla_{\text{OXA-1}}$, bla_{TEM}, $bla_{\text{SHV-12}}$ and *aac(6')-Ib-cr* (Ferreira *et al.*, 2019) and the carbapenem-resistant *Klebsiella pneumoniae* strain KP-349, harbouring the ARG bla_{KPC} (Ferreira et al. submitted for publication). DNA extracts were obtained using the QIAGEN Plasmid Midi Kit (QIAGEN, Germany), according to manufacturer's instructions. The competent cell receptors (50 µL) were defrosted on ice and added to 5 µL of plasmid DNA extract (*E. coli* H1FC54 or *K. pneumoniae* KP-349) at 25 ng/µL. The mixture of competent cell receptors and free-DNA was gently mixed, placed first on ice for 20 minutes and then at a temperature 42 °C for 75 seconds. A volume of 950 µL of pre-warmed (30 °C) SOC medium was then added to each mixture and left at 30 °C for 3 hours at 62 rpm. In order to obtain possible transformants, 150 µL of each mixture were plate in duplicate on LA medium supplemented with the respective antibiotic (TET, 8 mg/L or MER, 0.25 mg/L) and incubated overnight at 30 °C.

The six competent cell receptors were tested with the DNA extract of the *E. coli* H1FC54, and four were tested with the DNA extract of the *K. pneumoniae* KP-349, since the strains CAA-6H and FCA-5D presented resistance and intermediate phenotype to meropenem, used as selective factor.

Putative transformants were cultured on LA medium supplemented with the antibiotics used as selective factors in transformation assays, cryopreserved and further analysed for phenotypic and genotypic acquired resistance. The authenticity of the recipients was confirmed based on Random Amplified Polymorphic DNA (RAPD), the acquisition of the antibiotic resistance phenotypes tested by disk diffusion method as described above or by

determination of the minimum inhibitory concentration with the MICE (OXOID, United Kingdom) for meropenem (32–0.002 μg/mL). The possibly acquired genes were screened by PCR (*bla*$_{OXA-1}$, *bla*$_{TEM}$, *bla*$_{SHV-12}$ and *aac(6')-Ib-cr* or *bla*$_{KPC}$) as described before (Ferreira *et al.*, 2019). The amplicons with the expected molecular weight were further analysed by Sanger nucleotide sequencing.

4.3.4. Statistical analyses

CFUs counts were expressed per mL of watercress washing solution or per strawberry dry weight. The determination of statistically significant differences ($p < 0.05$) of bacterial counts were performed by one-way analysis of variance (ANOVA) test (SPSS Statistics for Windows v.24.0; IBM Corp., Armonk, NY, USA) analysis.

4.4. Results

4.4.1. Watercress bacterial enumeration and characterization of the bacterial isolates

The bacterial enumeration of watercress samples purchased in two different supermarkets is summarized in **Figure 4.2**. The counts on mFC and on PCA were not statistically different, probably of the limited selective character of mFC incubated at 30 ºC in this type of sample. The watercress samples from supermarket A had significantly higher bacterial counts ($7.0 \times 10^7 \pm 1.0 \times 10^7$ and $6.4 \times 10^7 \pm 1.4 \times 10^7$ CFU/mL of watercress washing solution on PCA and mFC media, respectively) compared to supermarket B ($5.4 \times 10^5 \pm 6.2 \times 10^4$ and $5.1 \times 10^5 \pm 8.4 \times 10^4$ CFU/mL of watercress washing solution on PCA and mFC media, respectively). Bacterial counts on culture media with AML were of $7.7 \times 10^6 \pm 7.5 \times 10^5$ CFU/mL on PCA and $5.1 \times 10^6 \pm 6.4 \times 10^5$ CFU/mL on mFC, corresponding to > 8% of resistant bacteria for supermarket A and of $3.1 \times 10^5 \pm 6.1 \times 10^4$ CFU/mL on PCA and $3.8 \times 10^5 \pm 5.8 \times 10^4$ CFU/mL on mFC for supermarket B, corresponding to > 57% of resistant bacteria (**Figure 4.2**).

A total of 68 bacterial isolates were recovered from watercress washing solution, being 37 isolates from samples A and 31 isolates from samples B. Most of the bacterial isolates stained Gram-negative, with only 4 and 7 strains isolated from the watercress samples A and B staining Gram-positive, respectively.

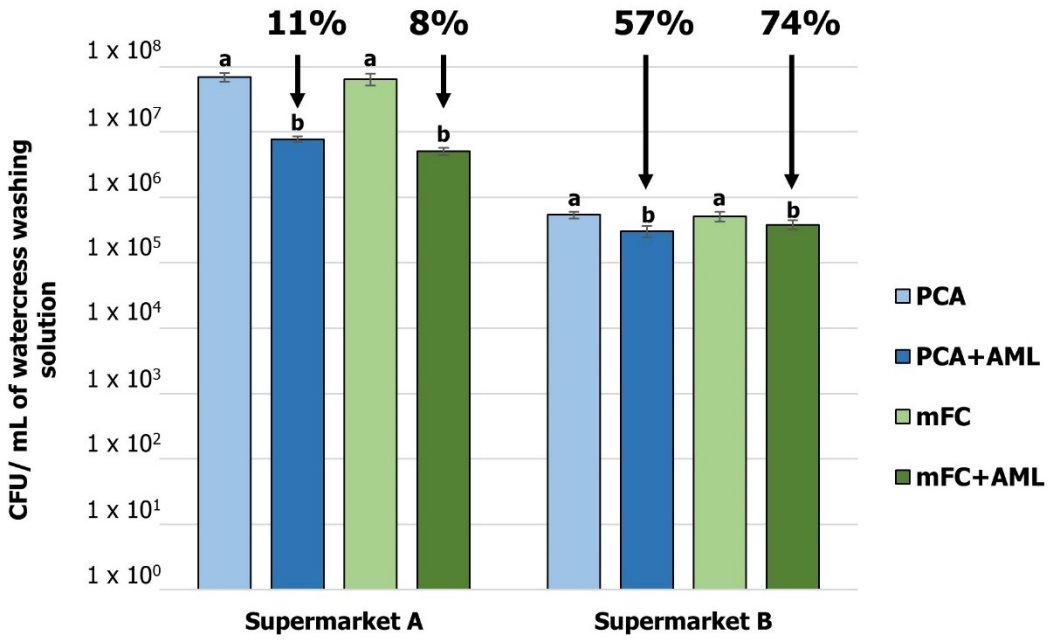

Figure 4.2. Bacterial enumeration on the culture media PCA (blue bars) and mFC (green bars) for watercress samples from two different supermarkets (A and B). Letters a and b indicate statistically significant differences ($p < 0.05$) between bacterial counts. The percentage value on the top of the bars refers to ratio of counts on amoxicillin (AML) (value of bacteria counts in medium supplemented with AML divided by value of bacteria counts in medium without AML).

4.4.2. Strawberries microbial load variations along its industrial processing and bacterial isolates characterization

Fruits sampled directly from the field (S1) have shown bacterial counts of 9.1 x 10^5 ± 1.8 x 10^5 CFU/g of strawberry dry weight (**Figure 4.3**, S1). Fruits sampled directly from the field (S1) have shown bacterial counts of 9.1 x 10^5 ± 1.8 x 10^5 CFU/g of strawberry dry weight (**Figure 4.3**, S1). After the first processing step (S2), peduncle removal, the bacterial load significantly decreased to 5.2 x 10^5 ± 6.8 x 10^4 CFU/g of strawberry dry weight. Strawberry disinfection, fruits re-washing with tap water to remove the excess of disinfectant, cut and quick freezing, lowered the bacterial counts to 7.6 x 10^4 ± 1.1 x 10^4 CFU/g of strawberry dry weight (**Figure 4.3**, S3). After the freezing process (S4) the bacterial counts significantly increased to 3.3 x10^5 ± 9.0 x 10^4 CFU/g of strawberry dry weight values close to the pre-disinfection step. Contrary to the hypothesis that bacteria on the surface of the fruits would be washed out for the water bath promoting a high concentration of bacteria in this water, a low microbial load was observed in the washing water (sample W, data not shown). The measured chlorine concentration in the washing solution was of 400 ppm. From the strawberry samples (n=30), disinfection water (n=8) and enrichment culture (n=14), a total of 52 isolates were obtained. Most of the isolates

recovered from the strawberries before processing (S1) were Gram-negative (83%) (**Figure 4.4**). After processing (samples S2 and S3; **Figure 4.4**), Gram-negative and Gram-positive isolates were recovered in the same proportion (**Figure 4.4**). After freezing and in disinfection water, the Gram-positive bacteria predominated (83 and 100%, respectively) (sample S4 and W, **Figure 4.5**).

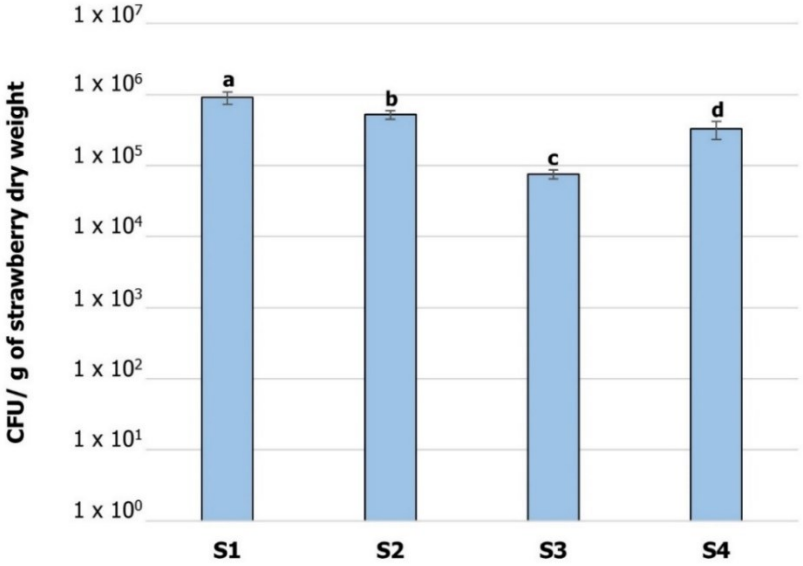

Figure 4.3. Bacterial load determination in strawberries samples. Legend: S1, strawberries harvested directly from the field; S2, strawberries before disinfection process (peduncle has already been removed); S3, disinfected strawberries; S4, frozen strawberries. Letters a-d indicate statistically significant variation ($p < 0.05$) between samples.

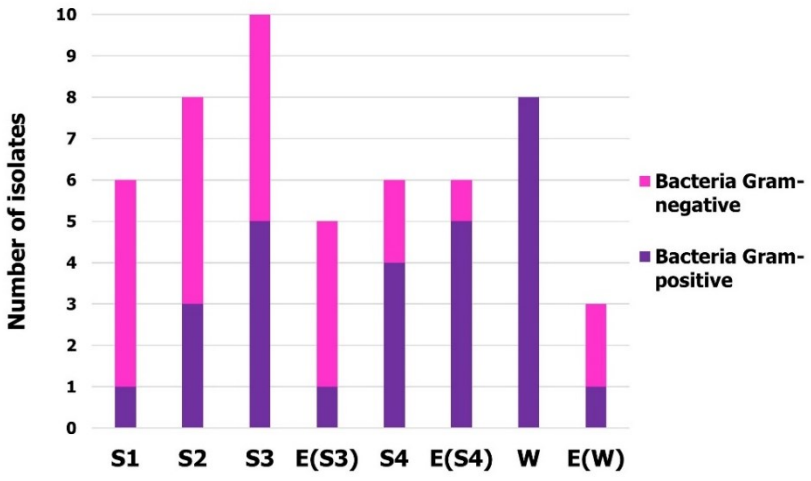

Figure 4.4. Number of Gram-negative and Gram-positive bacterial isolates recovered from (n values are indicated on the y-axis): S1, strawberries harvested directly from the field; S2, strawberries peduncle free; S3, disinfected strawberries; S4, frozen strawberries; W, disinfection water; E, enrichment culture of S3, S4 and W samples.

4.4.3. Antibiotic resistance phenotyping

The isolates obtained (68 isolates from watercress and 52 isolates from strawberries) were characterized for their antibiotic resistance phenotype. Most bacterial watercress and strawberry isolates (90 - 95%) were susceptible to tetracycline (TET), ciprofloxacin (CIP), and gentamicin (GEN) (Figure 5). Watercress isolates were predominantly resistant to cephalothin (CP, 91%), amoxicillin (AML, 70%), ticarcillin (TIC, 64.5%), sulfamethoxazole/trimethoprim (SXT, 44%) and sulfamethoxazole (SUL, 56%) (Figure 5 A). The cultivable bacterial fraction from strawberry samples were mainly resistant to sulfamethoxazole (SUL, 61.5%), ceftazidime (CEF, 36.5%) and cephalothin (CP, 34.5%) (**Figure 4.5** B).

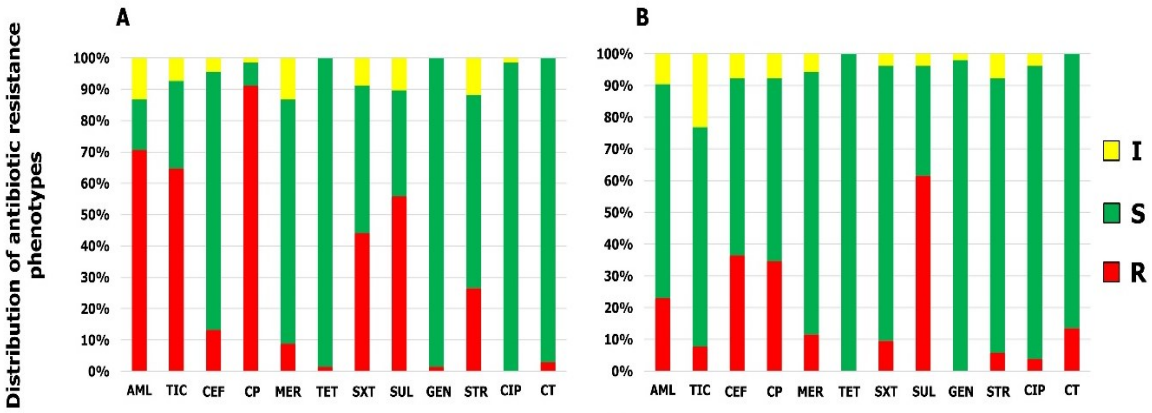

Figure 4.5. Antibiotic resistance phenotypes of watercress (A) (n=68) and strawberry isolates (B) (n=52). Antibiotic legend: AML, amoxicillin; TIC, ticarcillin; CEF, ceftazidime; CP, cephalothin; MER, meropenem; TET, tetracycline; SXT, sulfamethoxazole/trimethoprim; SUL, sulfamethoxazole; GEN, gentamicin; STR streptomycin; CIP, ciprofloxacin; CT, colistin sulphate. Bacterial phenotype legend: I, intermediate; S, susceptible; R, resistant.

Twenty-six out of thirty-one watercress isolates from samples purchased at the supermarket B showed resistance to different classes of antibiotics such as penicillins, sulphonamides, cephalosporins and aminoglycosides. One of the isolates, *P. paralactis* (strain VA.FA5) (**Table 4.1**), was resistant to most of the antibiotics tested, being susceptible only to tetracycline, gentamicin, and ciprofloxacin. Seventeen out of thirty-seven isolates recovered from watercress samples from supermarket A showed also resistance to different antibiotic classes, with one isolate, *P. paralactis* (strain CAA-6H) (**Table 4.1**), exibiting resistance to amoxicillin, ticarcillin, ceftazidime, cephalothin, meropenem and streptomycin.

In the group of isolates recovered from strawberries only 6 out of 52 were resistant to at least three classes of antibiotics, being the resistance to penicillins, cephalosporins and sulphonamides the core resistance phenotype observed (**Table 4.1**).

4.4.4. Identification and characterization of antibiotic resistant bacteria

From a total of 120 bacterial isolates (52 isolates from strawberries and 68 isolates from watercress), a group of 28 non-repetitive Gram-negative isolates (22 from watercress samples, and 6 from strawberry samples) have presented resistance phenotypes to antibiotics belonging to at least three distinct classes. These 28 bacterial isolates were identified as belonging to 22 bacterial species (**Table 4.1**). Most of these species belong to the *Proteobacteria* (*Alpha-* and *Gamma-proteobacteria* classes) and *Bacteroidetes* (*Flavobacteriia* class) phyla. Specifically, the *Gammaproteobacteria* were identified as members of the genera *Pseudomonas* (samples S1, WA and WB), *Rahnella* (samples E(S4) and WA), *Erwinia* (sample E(S3)) and *Stenotrophomonas* (samples E(W1) and WB) and in the class *Alphaproteobacteria* were members of the genus *Methylobacterium* (samples S1 and S2; **Table 4.1**). The *Flavobacteriia* class was represented only by members of the genus *Chryseobacterium* (sample WA). The genus *Pseudomonas* was identified in all the samples.

Among the *Pseudomonas* species identified were *P. extremorientalis* in sample S1, *P. reidholzensis*, *P. putida* and *P. parafulva* in sample WA, *P. paralactis* in sample WA and WB, *P. laurylsulfatiphila*, *P. koreensis*, *P. fragi*, *P. orientalis* and *P. poae* in sample WB (**Table 4.1**). Other species identified were within the order *Enterobacterales, R. inusitata* (WA) and *R. aquatilis* (E(S4)), *E. persicina* (E(S3)), in the order *Rhizobiales, Methylobacterium adhaesivum* and *M. gossipiicola* (S1 and S2, respectively) and in the order *Flavobacteriales, Chryseobacterium indoltheticum* (WA). *S. rhizophila* was the only species identified in both strawberries and watercress samples.

Selected genetic determinants - *sul1*, bla_{TEM}, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$ and bla_{SHV}, class 1 integron integrase gene (*intI1*), and plasmid replicon types (FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA), were screened in these isolates. Curiously, only two out of the 28 isolates, both from watercress A (CAA-6H, FCA-5D) (**Table 4.1**) presented the one of the searched genetic elements. The presence of the gene *intI1* was detected in *Pseudomonas paralactis* (strain CAA-6H) and the plasmid replicon type HI1 was detected in *R. inusitata* (strain FCA-5D), isolated from watercress A on PCA and mFC medium supplemented with amoxicillin, respectively.

Table 4.1. Watercress and strawberry ARB isolates. Identification (based on 16S rRNA gene sequence analysis), source, antibiogram and target genes and Inc groups found within the ARB identified, is listed. The presence of the genes is marked with "+" whilst, the absence of the gene is marked with "-".

				Antibiogram												Target genes and Inc groups	
	Isolates	Source (culture medium with or without antibiotic)	Species identification (% similarity)	AML	TIC	CEF	CP	MER	TET	SXT	SUL	GEN	STR	CIP	CT	*intI1*	HI1
Watercress	CA-5E	WA (PCA)	*Pseudomonas reidholzensis* (100%)	I	I	I	R	R	S	R	S	S	S	S	S	-	-
	CAA-5A	WA (PCA+AML)	*Chryseobacterium indoltheticum* (98%)	R	R	S	S	S	R	S	R	S	I	S	S	-	-
	CAA-6H	WA (PCA+AML)	*Pseudomonas paralactis* (99%)	R	R	R	R	R	S	S	S	S	R	S	S	+	-
	FC-5F	WA (mFC)	*Pseudomonas putida* (99%)	R	R	S	R	S	S	R	R	S	I	S	S	-	-
	FC-6D	WA (mFC)	*Pseudomonas parafulva* (99%)	S	R	S	R	S	S	S	R	S	S	S	S	-	-
	FCA-5D	WA (mFC+AML)	*Rahnella inusitata* (99%)	S	R	S	R	I	S	S	R	S	R	S	S	-	+
	FCA-5E	WA (mFC+AML)	*Pseudomonas putida* (99%)	R	R	I	R	S	S	R	R	S	S	S	S	-	-
	VA.P1	WB (PCA)	*Pseudomonas extremaustralis* (99%)	R	S	S	R	S	S	R	R	S	S	S	S	-	-
	VA.P6	WB (PCA)	*Pseudomonas viridiflava* (99%)	R	R	S	R	S	S	R	R	S	R	S	S	-	-
	VA.P8	WB (PCA)	*Pseudomonas paralactis* (99%)	R	R	S	R	S	S	R	R	S	R	S	S	-	-
	VA.PA2	WB (PCA+AML)	*Pseudomonas paralactis* (99%)	R	R	S	R	S	S	R	I	S	R	S	S	-	-
	VA.PA3	WB (PCA+AML)	*Pseudomonas poae* (99%)	R	R	S	R	S	S	R	R	S	S	S	S	-	-
	VA.PA11	WB (PCA+AML)	*Pseudomonas orientalis* (99%)	R	R	S	R	S	S	R	I	S	S	S	S	-	-
	VA.PA12	WB (PCA+AML)	*Pseudomonas paralactis* (100%)	R	R	S	R	S	S	R	R	S	S	S	S	-	-
	VA.F11	WB (mFC)	*Pseudomonas fragi* (99%)	I	R	S	R	S	S	R	R	S	S	S	S	-	-
	VA.F12	WB (mFC)	*Pseudomonas koreensis* (99%)	R	R	S	R	S	S	R	R	S	S	S	S	-	-
	VA.FA2	WB (mFC+AML)	*Pseudomonas paralactis* (100%)	R	R	S	R	S	S	R	R	S	R	S	S	-	-
	VA.FA5	WB (mFC+AML)	*Pseudomonas paralactis* (99%)	R	R	R	R	R	S	R	R	S	R	S	R	-	-
	VA.FA6	WB (mFC+AML)	*Pseudomonas laurylsulfatiphila* (99%)	R	R	R	R	S	S	R	R	S	R	S	S	-	-
	VA.FA7	WB (mFC+AML)	*Pseudomonas paralactis* (99%)	R	R	S	R	S	S	R	R	S	I	S	S	-	-
	VA.FA9	WB (mFC+AML)	Stenotrophomonas rhizophila (99%)	R	S	S	R	S	S	S	R	S	S	S	S	-	-
	VA.FA12	WB (mFC+AML)	*Pseudomonas koreensis* (99%)	R	R	S	R	S	S	R	S	S	S	S	S	-	-
Strawberry	FS1.17	S1 (PCA)	*Pseudomonas extremorientalis* (99%)	R	R	R	R	S	S	R	R	S	S	S	S	-	-
	FS1.16	S1 (PCA)	*Methylobacterium gossipiicola* (99%)	R	R	S	R	S	S	R	R	S	R	S	S	-	-
	FS2.9	S2 (PCA)	*Methylobacterium adhaesivum* (99%)	R	S	I	S	R	S	I	R	I	S	S	R	-	-
	EFS3.2	ES3 (PCA)	*Erwinia persicina* (99%)	R	S	S	R	S	S	S	R	S	S	S	S	-	-
	EFS4.3	ES3 (PCA)	*Rahnella aquatilis* (99%)	R	R	S	R	S	S	S	R	S	S	S	S	-	-
	EFW1.1	EW1 (PCA)	*Stenotrophomonas rhizophila* (99%)	R	S	R	S	R	S	S	R	S	S	S	R	-	-

Classes of antibiotics: AML, amoxicillin; TIC, ticarcillin; CEF, ceftazidime; CP, cephalothin; MER, meropenem; TET, tetracycline; SUL, sulfamethoxazole; SXT, sulfamethoxazole/trimethoprim; GEN, gentamicin; STR, streptomycin; CIP, ciprofloxacin and CT, colistin sulphate. In red, resistant bacteria; in green, susceptible bacteria; in yellow, intermediate bacteria. Legend of the source: WA, watercress sample from supermarket A; WB, watercress sample from supermarket B; S1, strawberries harvested directly from the field; S2, strawberries without peduncle; ES3, enrichment culture of S3 (disinfected strawberries); ES4, enrichment culture of S4 (frozen strawberries); EW1, enrichment culture of W (disinfection water). Culture media: PCA, Plate Count Agar medium; PCA+AML, PCA supplemented with amoxicillin (32 mg/L); mFC, membrane fecal coliform agar; mFC+AML, mFC supplemented with amoxicillin (32 mg/L).

4.4.5. Transformation assay

Preliminary results of the transformation assay led to the isolation of putative transformants from LA media supplemented with tetracycline or meropenem. The recipient strain *R. inusitata* FCA-5D acquired the capacity to grow in the presence of tetracycline, and the strains *S. rhizophila* VA.FA9 and *Pseudomonas fragi* VA.F11 acquired the capacity to grow in the presence of meropenem. These traits were stable after five successive transfers, and the authenticity of the isolates could be confirmed by RAPD. Strain *S. rhizophila* VA.FA9 acquired resistance to meropenem, with an increase of MIC value from 0.25 µg/mL to 4-12 µg/mL and also to sulfamethoxazole/trimethoprim. In the transformants of the *Pseudomonas fragi* VA.F11 was observed an increase in the meropenem MIC value from 0.06 µg/mL to 0.25-2 µg/mL. In spite of these MIC increase values, it was not possible to confirm the presence of any of the screened ARGs present in the plasmid DNA used for transformation assays.

4.5. Discussion and conclusion

The bacterial isolates recovered from watercress and strawberries presented a limited antibiotic resistance profile. Among the ARB recovered from watercress, *Pseudomonas* spp., along with *Chryseobacterium indoltheticum*, *R. inusitata* and *S. rhizophila* species were detected (Table 1). The genus *Pseudomonas* is well-known for its high metabolic versatility which makes it a ubiquitous bacterium largely distributed in different environments (Moradali *et al.*, 2017). Bacterial species under the genus *Pseudomonas* may be closely related to human pathogens and can host a wide range of ARGs (Moradali *et al.*, 2017) (Chapter 3). The majority of the watercress bacterial isolates belonged to the genus *Pseudomonas* and the species *P. paralactis* was the most commonly identified. Members of the genus *Pseudomonas* have already been found as the most abundant bacterial group in ready-to-eat leafy salad, such as rocket and spinach (Tatsika *et al.*, 2019). Members of the genus *Chryseobacterium* have been reported in several habitats (e.g. humans, animals, soil and water) as well as associated with raw-eaten vegetables and harbouring ARGs (Chapter 3). *Rahnella* spp. have been found in water, soil, and more seldom in food and clinical specimens of immunocompromised patients (Koczura *et al.*, 2016; Mohamaden *et al.*, 2019). Additionally, *S. rhizophila* was isolated from watercress samples. The genus *Stenotrophomonas* has already been observed as endophyte in carrot, cucumber, lettuce, radish, and tomato, harbouring several ARGs (Chapter 3). It is also a member of the same

genus as the emerging multidrug resistant opportunistic pathogen *S. maltophilia* (Berg and Martinez, 2015).

Among the watercress samples was identified the replicon type HI1 in *R. inusitata* (strain FCA-5D) (Table 1). The plasmids IncHI1 are usually associated with ARB of human, animal and environmental origin which are involved in the dissemination of antibiotic resistance among bacteria (Cain and Hall, 2013; Mutai *et al.*, 2019; Popowska and Krawczyk-Balska, 2013). In *P. paralactis* (strain CAA-6H), from watercress sample, was also detected the gene *intl1* (Table 1), considered an indicator of class one integrons which can be involved in the dissemination of antibiotic resistance (Gillings *et al.*, 2015; Narciso-da-Rocha *et al.*, 2014).

From strawberry, ARB from S1 and S2 samples such as *P. extremorientalis*, *M. adhaesivum* and *M. gossipiicola* were observed. Members of the genus *Pseudomonas* have been reported as endophytes in strawberry (Chapter 3**)**. From enrichment cultures of disinfected strawberry, *E. persicina* and *R. aquatilis* were identified (Table 1). *Erwinia* spp. have been found as endophytic bacteria associated with lettuce and carrot having ARGs (Chapter 3). Furthermore, associated with enrichment cultures of disinfection water*, S. rhizophila* was detected.

The antibiotic resistance profile observed in watercress and strawberry bacteria might be due to intrinsic properties of these bacteria. Indeed, the genus *Pseudomonas* for instance, it is well-known to have an extensive intrinsic resistome due to different factors (Fajardo *et al.*, 2008; Moradali *et al.*, 2017). It is also worth mentioning that plant-associated bacteria might also have a broad spectrum of efflux pumps for bacteria/plant interactions which might confer a wide resistance phenotype (Chapter 3). However, in the presence of selective pressure (e.g. antibiotics), bacteria intrinsically resistant might possess a better fitness, and genetic determinants conferring resistance may be acquired from other bacteria or free DNA by HGT processes, becoming stable genetic elements. Therefore, acquired antibiotic resistance may be favoured by intrinsic resistance.

Although none of the identified bacteria isolated either from strawberry or watercress samples are recognized foodborne pathogens (CDC, 2020), these plant-based products were observed to contain some ubiquitous bacterial groups. At least three isolates have developed resistance phenotypes through transformation, although it was not possible to determine which genes or genetic elements were associated with the acquisition of the resistance phenotypes. These results suggest that these types of food products may harbour environmental bacteria which characteristics may depend on the hygiene and

microbiological quality of the products. The occurrence of bacteria with acquired resistance or able to acquire it cannot be discarded.

5. Persistence of wastewater antibiotic resistant bacteria and their genes in human fecal material

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Authors: Nazareno Scaccia, Ivone Vaz-Moreira and Célia M. Manaia

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5.1. Abstract

Domestic wastewater is a recognized source of antibiotic resistant bacteria and antibiotic resistance genes (ARB&ARGs), whose risk of transmission to humans cannot be ignored.

The fitness of wastewater ARB in the complex fecal microbiota of a healthy human was investigated in feces-based microcosm assays (FMAs). FMAs were inoculated with two wastewater isolates, *Escherichia coli* strain A2FCC14 (MLST ST131) and *Enterococcus faecium* strain H1EV10 (MLST ST78), harbouring the ARGs bla_{TEM}, bla_{CTX}, bla_{OXA-A} and *van*A, respectively. The FMAs, incubated in the presence or absence of oxygen or in the presence or absence of the antibiotic cefotaxime or vancomycin, were monitored based on cultivation, ARGs quantification and bacterial community analysis. The fecal bacterial community was dominated by members of the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. The ARGs harboured by the wastewater isolates could be quantified after one week, in FMAs incubated under both aerobic and anaerobic conditions. These observations were not significantly different in FMAs incubated anaerobically, supplemented with sub-inhibitory concentrations of cefotaxime or vancomycin. The observation that ARGs of wastewater ARB persisted in presence of the human fecal microbiota for at least one week supports the hypothesis of a potential transmission to humans, a topic that deserves further investigation.

Keywords: human fecal microbiota, microcosm assays, microcosm effect, antibiotic resistance transmission, antibiotic resistant bacteria, antibiotic resistance genes.

5.2. Introduction

Antibiotic resistance, defined as the capability of bacteria to survive and proliferate in the presence of antibiotics, is a natural bacterial property (Davies and Davies, 2010).

Bacteria resistant to antibiotics, against which were once susceptible, owe that capability to the acquisition of antibiotic resistance genes (ARGs), most of the times through horizontal gene transfer (Bengtsson-Palme *et al.*, 2018; Summers, 2006). Acquired antibiotic resistance emergence and proliferation have been attributed to factors such as the presence of antibiotics and other antimicrobials, metals or conditions still unknown, which through their stressor effects exert what has been designated as selective pressures (Martínez, 2008; Rosenblatt-Farrell, 2009). Ubiquitous bacteria, harbouring acquired antibiotic resistance genes, can thrive in the environment, in particular in wastewater, water, soil and wildlife (Berendonk *et al.*, 2015; Huddleston, 2014). The paths of transmission of these bacteria back to humans are not fully understood and the probability of such occurrence hardly can be estimated based on the current knowledge (Manaia, 2017). A still unanswered question refers to the capability of antibiotic resistant bacteria (ARB) from environmental origin, as well as their genes, to survive or persist in the human body (Bengtsson-Palme *et al.*, 2018; Larsson *et al.*, 2018; Manaia, 2017). Specifically, if it is assumed that the digestive tract is the entry portal, one of the questions would be if these bacteria would be able to survive the competition of complex intestinal microbiome (Manaia, 2017; Vaz-Moreira *et al.*, 2014).

The role of the digestive tract as a relevant entry portal assumes a particular likeliness in situations of ingestion of raw vegetable-based food products, therefore acting as potential sources of ARB to humans (Hölzel *et al.*, 2018; Valerio *et al.*, 2006; Zhang *et al.*, 2019).

The risks of ARB occurrence in vegetables can be enhanced in scenarios of manure soil amendment or water reuse for irrigation (Becerra-Castro *et al.*, 2015; Heuer *et al.*, 2011; Marti *et al.*, 2013). Indeed, several recent studies have shown the presence of ARB and ARGs in raw-eaten products (e.g. lettuce), raising concerns for consumers, particularly for immunocompromised people (Araújo *et al.*, 2017; Blau *et al.*, 2018; O'Flaherty *et al.*, 2019; Zhang *et al.*, 2017; Zhu *et al.*, 2017). However, even after ingestion, the success of allochthonous bacteria in the intestinal tract is supposedly antagonized by the gut microbiota, a complex and dynamic community of microorganisms colonizing the gastrointestinal tract of humans since birth (Donaldson *et al.*, 2016; Gibson *et al.*, 2014). While indigenous (or autochthonous) microorganisms may have the intestine as a long term or almost permanent niche, allochthonous microorganisms may colonize transiently the human gut, although some, the most fitted, will be able to persist for long time periods (Milani *et al.*, 2017;

Ventura *et al.*, 2009). Among the most fitted bacterial groups, it can be hypothesized that bacteria of enteric origin, like *Escherichia coli* or *Enterococcus* spp., are good candidates to survive the competition of the fecal microbiome of a healthy individual. These can certainly be part of the 10^6 to 10^9 bacterial cells that, depending on the different dietary intake, can be ingested daily (Derrien and van Hylckama Vlieg, 2015; Lang *et al.*, 2014). If part of these bacteria carry acquired ARGs, such fact can hypothetically increase their fitness in the presence of antibiotics and/or facilitate the interchange of those genes with the native community (Salyers *et al.*, 2004). Therefore, the contamination of the human food chain with environmental ARB might represent a risk for the subsequent transmission to humans.

Among the multiple bottlenecks that may hamper the successful human-gut colonization by allochthonous bacteria, are the capacity to survive the complex native microbiome and/or the stability of the respective ARGs. These questions boosted this study that used the fecal material of a healthy infant as a model of human gut microbiota to assess the persistence of enteric ARB isolated from wastewater, *Escherichia coli* strain A2FCC14 and *Enterococcus faecium* strain H1EV10, harbouring the ARGs bla_{TEM}, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$ and *van*A, respectively. Specifically, the objectives of this work were to assess: 1) if those wastewater ARB and the respective ARGs were outcompeted in the presence of the human fecal microbiota, 2) if the cell-free ARGs could persist in that environment, and 3) the influence of the presence of oxygen or of antibiotics on the survival and persistence of the ARB and ARGs measured in 1) and 2). Assuming successful colonization, for which it is necessary that the exogenous enteric bacteria can thrive in the presence of fecal material, an additional question is if the acquired ARGs will be lost because represent a fitness cost.

5.3. Material and methods

The survival of wastewater antibiotic resistant isolates and persistence of the respective ARGs was assessed in the presence of the complex human fecal microbial community. The experiments were conducted in FMAs composed of human stool specimens spiked with two wastewater isolates: *E. coli* strain A2FCC14 (harbouring the ARGs bla_{TEM}, bla_{CTX} and $bla_{\text{OXA-A}}$) and *Ent. faecium* strain H1EV10 (harbouring the ARG *van*A), or with the respective DNA extracts. The environmental variables tested in the FMAs were aerobic *vs*. anaerobic conditions, and the effect of single or multiple doses of sub-inhibitory concentrations of cefotaxime or vancomycin, under anaerobic conditions. The FMAs were incubated at 37 ºC, and samples were collected at 0, 1, 3 and 7 days. Monitoring was based on the enumeration of culturable bacteria, quantitative PCR (qPCR) analysis of antibiotic

resistance and 16S rRNA genes and bacterial community analyses based on 16S rRNA gene amplicon sequencing.

5.3.1. Bacterial strains

The strains *E. coli* A2FCC14 (isolated from raw municipal wastewater) and *Ent. faecium* H1EV10 (isolated from untreated hospital effluent) (Varela *et al.*, 2013) were used to inoculate the fecal microcosm assays (FMAs). The Whole Genome Shotgun projects of strains A2FCC14 and H1EV10 have been deposited at DDBJ/ENA/GenBank under the accession numbers WSZB00000000 and WSZC00000000, respectively. These strains have an Average Nucleotide Identity (ANI) of orthologous gene pairs shared between two microbial genomes with the type strains of the species of 98.5% and 99.5%, respectively. ARGs conferring resistance to beta-lactam (bla_{TEM}, $bla_{\text{CTX-M}}$, and $bla_{\text{OXA-A}}$), harboured by the *E. coli* strain and to vancomycin (*van*A) harboured by the *Ent. faecium* strain were monitored in FMAs. For inoculum preparation, both strains were handled as follows: *E. coli* A2FCC14 was cultivated on mFC agar medium (Difco BD) supplemented with cefotaxime 4 mg L^{-1} and incubated overnight at 37 °C and *Ent. faecium* H1EV10 was cultivated on m-Enterococcus agar medium (Difco BD) supplemented with vancomycin 16 mg L^{-1} and incubated for 48 hours at 37 °C. The biomass collected from each of those bacterial cultures was used to prepare a bacterial suspension in saline solution (0.85% (w/v) NaCl) with a cell density of approximately 10^8 and 10^7 Colony Forming Units (CFU) mL^{-1} for *E. coli* A2FCC14 and *Ent. faecium* H1EV10, respectively. This cell density was in accordance with the average density of bacteria of these groups in the human microbiota and although higher than can be expected from a possible ingestion and digestion, it overcomes the experimental risk of reaching the values below the limits of quantification. After an initial calibration of CFU versus turbidity at 610 nm, the cell suspensions used to inoculate each FMA were measured by spectrophotometry.

5.3.2. Feces-based microcosm assays (FMAs)

The FMAs prepared with fecal material of a healthy child were used as a model to assess the fate of ARB and ARGs. All assays used fecal material supplied by a single healthy donor, aged 40 to 58 months during this study, and who was never submitted to antibiotherapy. The option for a donor with these characteristics was because although the gut microbiota was certainly affected by diet and lifestyle, it was not rearranged due to previous antibiotic

exposure, a control considered relevant for the present experimental design. In total, were collected twelve stool samples, each used to run an independent FMA (**Table 5.1**). For each FMA experiment, were collected ≥ 80 g of fecal material that was stored at 4 °C for no more than 3 days. Each FMA comprised inoculated assays (M-assays) and the respective non-inoculated controls (C-assays). FMAs were tested under aerobic and anaerobic conditions or under anaerobic conditions spiked with antibiotics. The evaluation of the effect of oxygen was considered of interest given the fact that *E. coli* are facultative anaerobes and enterococci are aerotolerant, supporting some inference about the influence of the fitness of these exogenous bacteria versus the effect of competition by the fecal microbiota. In total, four FMAs were incubated under aerobic conditions [40 (C and M); 44 (C and M); 48 (C and M); 50 (C and M)], three under anaerobic conditions [50 (C and M); 54 (C and M); 58 (C and M)] and four under anaerobic conditions, spiked with subinhibitory concentrations of antibiotics. Antibiotic spiking was done in a single-dose [54-C+cefotaxime (54-C.C), 54-C+vancomycin (54-C.V), 54-M+cefotaxime (54-M.C) and 54-M+vancomycin (54-M.V)] or in multiple-doses [58-C+cefotaxime (58-C.C), 58-C+vancomycin (58-C.V), 58-M+cefotaxime (58-M.C) and 58-M+vancomycin (58-M.V)] (**Table 5.1**). Anaerobic FMAs were handled and incubated in an anaerobic chamber (Whitley Workstation A35, containing a gas mixture of 85% carbon dioxide, 10% nitrogen, 5% hydrogen). Each experimental set (FMA) comprised 24 vial assays, corresponding to triplicates of spiked and non-spiked assays to be sacrificed for analyses after 0, 1, 3 and 7 days of incubation (3 replicates x spiked/non-spiked x 4 incubation periods). Although the transit time of bacteria in the large intestine is estimated to be ~2.5 days, ingested bacteria can be detected in the intestine for one week (Berg, 1996; Derrien and van Hylckama Vlieg, 2015). Based on this note and preliminary assays, an incubation period of 7 days (extended to 30 days for occasional analysis) was selected.

To prepare these experimental sets, stool samples were diluted five times with sterile saline solution (0.85% (w/v) NaCl) and divided in 24 aliquots of 15 mL each. Half of these 24 aliquots were inoculated with 2 mL of a bacterial cocktail, described in the previous section, composed by the mixture of both strains, *E. coli* A2FCC14 and *Ent. faecium* H1EV10 (M-assays). The other half of the aliquots, corresponding to non-inoculated controls (C-assays), was spiked with 2 mL of sterile saline solution. FMAs 40, 44 and 48 were incubated aerobically. FMA50 comprised two parallel FMAs (24 vials incubated aerobically and 24 anaerobically).

Table 5.1. Composition and conditions of the different microcosm assays. Each FMA comprised 24 vials (12 non-inoculated - C and 12 inoculated - M).

Experiment	Inoculum				Conditions used for each assay				16S rRNA based microbiome analysis
	ARB (cell density OD_{610})		Free-DNA		Oxygen availability		Antibiotic presence		
FMA	*E. coli* A2FCC14 cell density (approx. CFU mL^{-1})	*Ent. faecium* H1EV10 cell density (approx. CFU mL^{-1})	*E. coli* A2FCC14 (µg)	*Ent. faecium* H1EV10 (µg)	Aerobic	Anaerobic	Cefotaxime (4 mg L^{-1})	Vancomycin (16 mg L^{-1})	
40-C	-	-	-	-	+	-	-	-	T0; T7
40-M	10^7	10^6	-	-	+	-	-	-	T0; T7
44-C	-	-	-	-	+	-	-	-	T0; T7
44-M	-	-	2.0	1.5	+	-	-	-	T0; T7
48-C	-	-	-	-	+	-	-	-	
48-M	10^7	10^6	-	-	+	-	-	-	
48-M	-	-	2.0	1.5	+	-	-	-	
50-C	-	-	-	-	+	-	-	-	T0; T7
50-M	10^7	10^6	-	-	+	-	-	-	T0; T7
50-C	-	-	-	-	-	+	-	-	T7
50-M	10^7	10^6	-	-	-	+	-	-	T0; T7
54-C	-	-	-	-	-	+	-	-	T0; T7
54-M	10^7	10^6	-	-	-	+	-	-	T0; T7
54-C.C	-	-	-	-	-	+	+ (1 dose)	-	T7
54-M.C	10^7	10^6	-	-	-	+	+ (1 dose)	-	T0; T7
54-C.V	-	-	-	-	-	+	-	+ (1 dose)	T7
54-M.V	10^7	10^6	-	-	-	+	-	+ (1 dose)	T0; T7
58-C	-	-	-	-	-	+	-	-	T0; T7
58-M	10^7	10^6	-	-	-	+	-	-	T0; T7
58-C.C	-	-	-	-	-	+	+ (3 doses)	-	T7
58-M.C	10^7	10^6	-	-	-	+	+ (3 doses)	-	T0; T7
58-C.V	-	-	-	-	-	+	-	+ (3 doses)	T7
58-M.V	10^7	10^6	-	-	-	+	-	+ (3 doses)	T0; T7

C, control - non-inoculated assay; C.C, control spiked with cefotaxime; C.V, control spiked with vancomycin. M, ARB-inoculated assay; M.C, ARB-inoculated assay spiked with cefotaxime; M.V, ARB-inoculated assay spiked with vancomycin.
T0, beginning; T7, seven days of incubation.

FMA54 and FMA58 were incubated anaerobically and were spiked with antibiotics. Briefly, FMA54 comprised 24 vials without antibiotic, 24 spiked with one dose of cefotaxime (4 mg L^{-1} for each microcosm) and 24 spiked with one dose of vancomycin (16

mg L^{-1} for each microcosm). FMA58 differed from FMA54 on the method of antibiotic spiking which in FMA58 was supplied at three antibiotic moments (3 x 4 mg L^{-1} for cefotaxime or 3 x 16 mg L^{-1} for vancomycin) at time 0, 1 day and 3 days, before each sample collection. Cell-free DNA supplemented FMAs, FMA44 and FMA48, were spiked with DNA extracts from *E. coli* A2FCC14 and *Ent. faecium* H1EV10. The DNA fragment length of the DNA extract of *E. coli* A2FCC14 was assessed in an agarose gel (1 %) electrophoresis, originating a single band with a molecular weight 10-50 000 bp, with no smearing effect, which would suggest that DNA was degraded. The quantity of DNA used was 2.0 and 1.5 µg of DNA extracted from *E. coli* A2FCC14 and *Ent. faecium* H1EV10, corresponding to the cell density used in the ARB inoculated FMAs (**Table 5.1**). FMA44 was designed aiming at assessing the persistence of the spiked free DNA and FMA48 aimed at assessing the potential occurrence of natural transformants, able to uptake the free DNA.

Therefore FMA48 was composed of 3 separated assays (6 vial assays corresponding to triplicates of non-spiked assays, sampled at T0 and T7, 6 vial assays corresponding to triplicates of bacteria-spiked assays, sampled at T0 and T7 and, 6 vial assays corresponding to triplicates of assays spiked with cell-free DNA extracted from the test bacteria, sampled at T0 and T7). Natural transformants were tentatively isolated on the mFC or m-Enterococcus culture media supplemented with antibiotic (cefotaxime or vancomycin, respectively). Cultivable bacteria counts were processed immediately, and aliquots for dry weight determination and total DNA extraction were stored at -20 and -80 ºC, respectively, until used. All determinations were done in triplicate. The stool dry weight was determined in 1 mL fecal slurry samples by incubation at 60 ºC, until a constant weight was reached, which corresponded to approx. 5 days. This study was approved by the Ethics Committee of the Universidade Católica Portuguesa in Porto.

5.3.3. Enumeration of cultivable bacteria in FMAs

Luria-Bertani Agar (LA) (Invitrogen), mFC agar (Difco BD) and m-Enterococcus agar (Difco BD) were used for enumeration of total heterotrophic bacteria (HB), enterobacteria and enterococci, respectively. When necessary, these culture media were supplemented with cefotaxime (4 mg L^{-1}; Sigma-Aldrich, St Louis, USA) or vancomycin (16 mg L^{-1}; Sigma-Aldrich, St Louis, USA), at concentrations corresponding to minimum inhibitory concentration (MIC) for *E. coli* or enterococci (CLSI, 2014). For bacterial enumeration, volumes of 1 mL were collected from each FMA, serially diluted in sterile saline solution

(0.85% (w/v) NaCl) and plated on the adequate culture medium using the Miles and Misra method (Miles *et al.*, 1938). Cultures were incubated at 37 ºC for 24 h on LA and mFC, or 48 h on m-Enterococcus agar. All bacterial counts were performed in triplicate.

5.3.4. DNA extraction

Total DNA was extracted from 1 mL of fecal slurry (corresponding to approximately 240 mg of wet sedimented stool) using the NZY Tissue gDNA Isolation kit (Nzytech, Portugal) according to the manufacturer's instructions. The DNA concentration in the extracts was quantified using Qubit fluorometer (Thermo Fisher Scientific, USA). Extracts were preserved at -20 ºC until qPCR or microbial community analyses were performed.

5.3.5. Quantitative PCR

The abundance of the genes *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{OXA-A}$ and *van*A, harboured by the inoculated bacteria, was monitored based on real-time quantitative PCR (qPCR). The total bacterial abundance was assessed based on the housekeeping gene 16S rRNA. The qPCR was conducted in a StepOne™ Real-Time PCR System (Life Technologies, Carlsbad, CA) following the conditions previously described (Narciso-da-Rocha *et al.*, 2018) for the ARGs *bla*$_{TEM}$, *bla*$_{CTX}$ and *bla*$_{OXA-A}$ and the 16S rRNA gene. The qPCR conditions for the detection of the gene *van*A were set up in 25 µL volume using the Power SYBR Green mastermix (Thermo Fisher Scientific, Austin, USA) containing 10 µM of each primer: VnF 5'-ATCGGCAAGACAATATGACAGC-3' and VnR 5'-AGCCTGATTTGGTCCACCTC-3' (Lata *et al.*, 2009). The PCR program was initiated by a period of 5 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec. The standard curve for *van*A gene was prepared using a clone of the *van*A gene of *Ent. faecium* H1EV10 with an efficiency between 96 and 105%.

5.3.6. Bacterial community analysis

The bacterial community composition of FMA40, 44, 50, 54 and 58 was analysed at time zero (T0) and after 7 days of incubation (T7), based on the hypervariable region V3/V4 of the 16S rRNA gene, using paired-end Illumina MiSeq® Sequencing (Genoinseq, Portugal) as previously described by Narciso-da-Rocha *et al.* (2018). The primers used were forward primer Bakt_341F 5'- CCTACGGGNGGCWGCAG -3' and reverse primer Bakt_805R 5'-GACTACHVGGGTATCTAATCC -3' according to the manufacturer's instructions

(Illumina, San Diego, CA, USA). Demultiplexed raw reads were extracted from Illumina MiSeq® System in fastq format and the reads were processed and analysed using Quantitative Insights Into Microbial Ecology (QIIME2) pipeline (version 2017.10; http://qiime2.org/) (Bolyen *et al.*, 2018). Sequences shorter than 200 bp and with average quality scores lower than 25 were eliminated. Sequences with average quality lower than 25 in a window of 5 bases were trimmed using the software PRINSEQ (Schmieder and Edwards, 2011). Sequences were filtered, merged and, chimeric reads were removed by the DADA2 software package enclosed in QIIME2 (Callahan *et al.*, 2016). Taxonomy was assigned to the amplicon sequence variants (ASVs), sequences with 100% identity, using the ARB SILVA database release 132 (Yilmaz *et al.*, 2014). A total of 4 750 244 reads (ranging from 22 176 to 112 134 reads per sample) and 1714 ASVs (ranging from 171 to 263 per sample) were obtained from the 105 datasets, corresponding to triplicates of 35 samples of C and M FMAs.

5.3.7. Statistical analyses

Cultivable bacteria counts were expressed as log values of colony-forming units (CFUs) per g of stool dry weight. Gene abundance was expressed as gene copy number per g of dry weight (abundance) or per 16S rRNA gene copy number (relative abundance or prevalence).

One-way analysis of variance (ANOVA), Tukey's and Bonferroni post-hoc tests and *t*-test (SPSS Statistics for Windows v.24.0; IBM Corp., Armonk, NY, USA) were used for determination of statistically significant differences ($p < 0.01$) of cultivable bacteria counts, abundance and prevalence of measured genes when comparing different incubation times or conditions. The bacterial community composition was expressed as the relative abundance of reads number of a specific bacterial group per total reads number. Correlations between the relative abundance of bacterial groups at phylum and family level were analysed using the statistical analysis of taxonomic and functional profiles using the software STAMP v2.1.3 and Canoco 5.01 (Parks *et al.*, 2014; Šmilauer and Lepš, 2014). Statistically significant differences between bacterial phyla or family relative abundances were determined using a two-tailed *t*-test ($p < 0.01$) and the p values were corrected for multiple testing using the Benjamini-Hochberg FDR (Benjamini and Hochberg, 1995).

5.4. Results and discussion

5.4.1. Microbial community composition of the fecal material

Since the fecal microbiota composition and the respective temporal variations could somehow influence the fate of exogenous ARB and ARGs, the analysis of the fecal bacterial community was necessary in this study. This part of the work had two aims, assess the phylogenetic diversity in the fecal material and assess the variations that were due to the microcosm effect. Over the study period, the infant (40-58 months) fecal microbiota was dominated by members of the bacterial phyla *Firmicutes* (41.6-47.1%) and *Bacteroidetes* (23.5-36.2%), followed by *Actinobacteria* (7.2-22.5%), *Proteobacteria* (1.5-7.4%) and *Verrucomicrobia* (1.2-7.4%) (**Table S5.1**). These results are in line with similar studies involving healthy individuals in the same age range (Monira *et al.*, 2011). Statistically significant variations were observed over the study period for all the above-mentioned phyla, although none with a clear trend of increase or decrease. These variations might be due to diet and/or the natural dynamic processes observed in infants gut microbiota (Milani *et al.*, 2017; Zmora *et al.*, 2019). Although these variations were smooth and with little expected impacts on the survival of the exogenous bacteria, whenever adequate they will be used to discuss the results.

5.4.2. Effect of incubation condition on the microbial community composition

The microcosm effect, meaning the bacterial community variations that occurred during the incubation period (7 days), which could allegedly influence the survival or persistence of exogenous bacteria or their ARGs, was assessed in non-inoculated FMAs, under both aerobic and anaerobic conditions. Besides the variation of phyla composition, it was also assessed the variation in the relative abundance of members of the families that include the wastewater ARB surrogates used in the inoculated FMAs, *Enterobacteriaceae* and *Enterococcaceae* (**Table 5.2**). The relative abundance of *Proteobacteria* significantly ($p<0.01$) increased (ratio $T7/T0 > 1$) during the incubation under aerobic, but not under anaerobic conditions (**Table 5.2**). It was also observed that the relative abundance of *Enterobacteriaceae* in the non-inoculated assays was always higher than in inoculated microcosms (**Table 5.2**), which might be due to a steady-state-like for *Enterobacteriaceae*

Table 5.2. Microcosm effect in fecal microcosm assays (FMAs) based on a single healthy donor aged 40, 44, 50, 54 and 58 months. Control, non-inoculated (C), and test, ARB-inoculated (M), FMAs incubated under aerobic (40-C, 40-M; 44-C, 44-M; 50-C, 50-M) or anaerobic (50-C, 50-M; 54-C, 54-M; 58-C, 58-M) conditions. Phyla with relative abundance equal or below 2% are designated as other phyla. The values correspond to the ratio of relative abundance (number of reads/total number of reads) of each replicate at T7 of C or M per the average values of the relative abundance at the respective T0 of C or M. The values are expressed as the average of triplicates ± the standard deviation.

FMAs	**Condition**	**Phylum relative abundance at T7/ phylum relative abundance at T0**						**Family relative abundance at T7/ family relative abundance at T0**	
		Bacteroidetes	*Actinobacteria*	*Verrucomicrobia*	*Firmicutes*	*Proteobacteria*	Other phyla (≤ 2%)	*Enterobacteriaceae*	*Enterococcaceae*
40-C	Aerobic	0.85±0.01*	1.02±0.02	0.80±0.08	0.78±0.01*	7.53±0.44*	24.94±0.71	51.90±9.43*	n.d.
40-M		0.90±0.02*	1.10±0.06	1.47±0.26	0.82±0.04*	4.81±0.59*	2.81±0.69	20.08±4.26*	0.69±0.14
44-C		1.06±0.03	1.77 ±0.05*	1.43±0.08*	0.65±0.01*	1.62±0.10*	3.12±0.74	3.05±0.14*	n.d.
44-M		1.12±0.05*	1.54±0.19*	1.41±0.07*	0.62±0.03*	1.62±0.04*	3.69±0.29	2.86±0.19*	0.26±0.45
50-C		0.79±0.05	1.13±0.06	0.64±0.07	0.99±0.09	4.83±1.02*	1.70±0.29	27.59±6.48*	n.d.
50-M		0.86±0.03	1.18±0.03	0.62±0.13	0.90±0.01	4.95±0.16*	2.18±0.46	20.72±0.38*	4.75±0.17*
50-C	Anaerobic	0.94±0.02	1.39±0.07	0.43±0.09	0.98±0.00	0.39±0.01*	2.16±0.08	0.36±0.08*	n.d.
50-M		0.93±0.09	1.44±0.19	0.47±0.13	0.96±0.01	0.33±0.05*	1.00±0.19	0.31±0.02	6.31±0.18*
54-C		0.91±0.03	1.59±0.14*	0.65±0.08*	0.83±0.04*	2.18±0.25*	1.40±0.44	28.80±3.51*	n.d.
54-M		0.98±0.03	1.32±0.13*	0.83±0.18	0.89±0.03*	0.70±0.10*	2.86±0.71	1.34±0.23	1.14±0.15
58-C		1.09±0.02	1.24±0.02*	1.28±0.18	0.81±0.01*	0.96±0.21	1.53±0.18	48.37±17.22*	n.d.
58-M		1.06±0.06	1.31±0.13	1.09±0.12	0.74±0.04*	2.46±0.43*	1.78±0.38	6.56±1.33*	3.91±0.45*

n.d., not detected both at T0 and T7.

*; statistically significant variation ($p<0.01$) between T0 and T7 for each FMA.

in the fecal microbial community, in which these bacteria, in equilibrium with the remaining community, are kept at a certain level and eventually exogenous bacteria, as were the wastewater isolates in this case, may have a limited proliferation capacity.

The relative abundance of the bacterial phylum *Firmicutes* was fairly stable over the 7 days period, under both aerobic and anaerobic conditions, with the ratio T7/T0 varying between 0.65 and 0.99 in non-inoculated FMAs (**Table S5.3**). Curiously, members of family *Enterococcaceae*, of the phylum *Firmicutes*, were not detected in non-inoculated FMAs.

The observation that anaerobic conditions had a lower impact on the microcosm effect than aerobic conditions, mainly in the groups that include the surrogates used, recommended that the effect of antibiotics would be better examined under anaerobiosis. In the FMA54 and FMA58, the addition of cefotaxime or vancomycin only affected significantly ($p<0.01$) the relative abundance of *Proteobacteria* (**Table S5.3**). Curiously, this effect was different when a single- (FMA54) or multiple-antibiotic-dose (FMA58) was used (**Table S5.3**). A single cefotaxime dose (54-C.C and 54-M.C) significantly ($p<0.01$) reduced the relative abundance of *Proteobacteria* in both inoculated and non-inoculated FMAs (ratio antibiotic/no antibiotic < 1). In contrast, multiple cefotaxime doses led to a significant ($p<0.01$) reduction on the relative abundance of *Proteobacteria* only in inoculated FMAs (58-M.C). The use of a single vancomycin dose significantly ($p<0.01$) reduced the relative abundance of Proteobacteria in the non-inoculated samples (54-C.V; **Table S5.3**). In contrast, three doses of vancomycin led to a significant ($p<0.01$) increase of the Proteobacteria relative abundance, only in non-inoculated FMA (58-C.V). As noted above for oxygen, also the antibiotic effect on members of the Enterobacteriaceae followed the same pattern as for the Proteobacteria phylum (**Table S5.3**). In contrast, no noticeable effects of antibiotics were observed on Enterococcaceae in inoculated FMAs.

These different behaviors of the bacterial community in presence of a single or multiple doses of antibiotics may suggest the rearrangement of the community or the charity among community members that able to degrade the antibiotics. The Principal Component Analysis (PCA) of the non-inoculated assays suggest that irrespective of the antibiotic and spiking method, members of the families Burkholderiaceae (phylum Proteobacteria) and Lachnospiraceae (phylum Firmicutes) were those whose relative abundance decreased during the incubation period. The contribution of these groups to the community rearrangement may have been exacerbated in the presence of antibiotics (**Table S5.1** A, B, C, and D). In general, the results suggest that antibiotics might have important effects on the fecal bacterial community composition and structure, with effects being noticed in the

relative abundance of members of the phyla Firmicutes, Actinobacteria and Bacteroidetes (**Table S5.3**). The relative abundance of Actinobacteria was significantly lower in the fecal material used in the FMA where a single antibiotic dose was tested (FMA 54) than in that where three successive doses were assayed (FMA 58). The opposite was observed for the relative abundance of Bacteroidetes (**Table S5.1**). Since both phyla members were observed to have strong correlation with antibiotic spiking, the distinct behaviour observed in response to one or multiple antibiotic doses may be also a result of distinct microbiota composition.

The importance of these bacterial groups as potential barriers or facilitators for antibiotic-induced bacterial community rearrangements may deserve further investigation. In general, the PCA results (**Table S5.1**) are in agreement with the literature that suggests that the oral or intravenous antibiotic administration can promote the disturbance of the human gut microbiome (Bhalodi et al., 2019; Francino, 2016). The PCA analysis (**Table S5.1**) suggests that the significant variations observed in Proteobacteria relative abundance (**Table S5.3**), might be due to complex fecal bacterial community rearrangements rather than to the increase or decrease of a specific bacterial lineage. Indeed, it is this type of indirect effect that was demonstrated for vancomycin, a glycopeptide antibiotic used against Gram-positive bacterial infection. In that case, it was associated with the decrease of Firmicutes that led to the increase of Proteobacteria in the fecal microbial community (Isaac et al., 2017; Vrieze et al., 2014). Indeed, although there is no evidence specifically for vancomycin and cefotaxime, the ability of sub-inhibitory concentrations of antibiotics to modify the competition between bacterial species within a microbial community has been discussed (Hall and Corno, 2014; Martinez, 2009).

5.4.3. Monitoring of culturable bacteria and antibiotic resistance genes

The native and exogenous culturable populations of enterobacteria, enterococci and/or total heterotrophic bacteria were monitored over time and under distinct conditions.

Heterotrophic and enterobacteria counts presented similar variation patterns in inoculated and non-inoculated FMAs and, in both cases, it was possible to infer about the beneficial oxygen effect on the first day of incubation, characterized by a slight CFU increase. Therefore, it is suggested that the fitness of enteric bacteria cannot be explained based only on the fecal microbiota competition, it is also explained by the survival capacity, in this case, higher in the presence of oxygen (**Figure 5.1**). In contrast, the behaviour of the aerotolerant enterococci was identical in the presence or absence of oxygen.

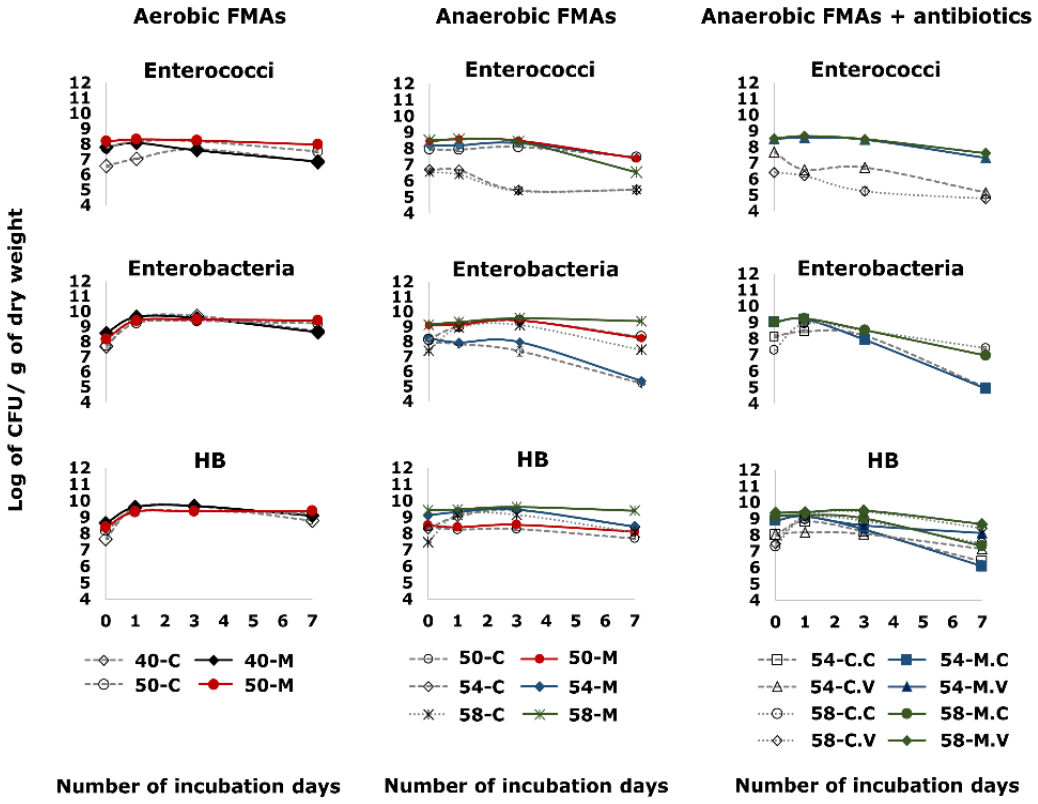

Figure 5.1. Colony forming units (CFUs) enumeration per gram of dry weight of stool samples, non-inoculated (C, dashed grey lines with empty symbols) or ARB-inoculated (M, solid colourful lines with full symbols) for FMAs incubated under aerobic (column on the left hand side) and anaerobic (column in the middle) conditions, and in the presence of antibiotics (column on the right hand side). FMA54 were performed in the presence of a single-dose of cefotaxime (54-M.C, blue squares) or vancomycin (54-M.V, blue triangles), while FMA58 were conducted in the presence of multiple-doses of cefotaxime (58-M.C, green circles) or vancomycin (58-M.V, green rhombus). CFUs were enumerated on m-Enterococcus, mFC and LA medium for enterococci, enterobacteria and heterotrophic bacteria (HB) counts, respectively. The CFU values are the average of triplicates with the standard deviation. Note: At T0 of FMA-54 and FMA-58, the log CFUs on antibiotic supplemented culture media per gram of feces dry weight of the ARB-inoculated FMAs was 9.03 ± 0.11 and 9.13 ± 0.14 (54 and 58) on mFC agar with cefotaxime and, 8.55 ± 0.06 and 8.47 ± 0.55 (54 and 58) on m-Enterococcus agar with vancomycin, being below the detection limit in non-inoculated controls. At time 7 days of FMA-54 and FMA-58, the log CFUs on antibiotic supplemented culture media per gram of feces dry weight of the ARB-inoculated FMAs was 6.38 ± 0.07 and 8.06 ± 0.12 (54 and 58) on mFC agar with cefotaxime and, 7.40 ± 0.07 and 6.58 ± 0.08 (54 and 58) on m-Enterococcus agar with vancomycin, being below the detection limit in non-inoculated controls.

Also, enterococci counts presented a similar variation pattern in inoculated and non-inoculated assays (**Figure 5.1**). In general, under anaerobic conditions, enterococci, enterobacteria and heterotrophic bacteria counts presented in average reductions of 1.1, 1.0 and 0.1 log units over the 7 incubation days, respectively (**Figure 5.1**). However, the

stochastic nature of these variations is suggested, when different FMAs are compared (**Figure 5.1**). The exogenous bacteria were wastewater isolates, *E. coli* A2FCC14 and *Ent. faecium* H1EV10, belonging to the multilocus sequence types ST131 and ST78, respectively, therefore genetically related with widespread pathogens (Khan *et al.*, 2010; Nicolas-Chanoine *et al.*, 2014). These exogenous bacteria were traced based on their ARGs bla_{TEM}, bla_{CTX-M}, bla_{OXA-A} and *van*A (**Figure 5.2**). Under aerobic conditions, the 16S rRNA gene abundance per gram of feces dry weight, decreased during the incubation period on average 0.26 log-units in inoculated FMAs (40-M and 50-M) and 0.32 log-units for non-inoculated FMAs (40-C and 50-C). These values were comparatively higher under anaerobic conditions, of 0.48 log-units in inoculated FMAs (50-M, 54-M, and 58-M) and of 0.42 log-units in non-inoculated FMAs (50-C, 54-C and 58-C).

Among the analysed ARGs, only bla_{TEM} was detected in non-inoculated microcosms (**Figure 5.2**). The bla_{TEM} gene may have been ingested by the donor (e.g. fresh produce) (Blau et al., 2018), and its occurrence in the gut microbiota of healthy individuals, even if never exposed to antibiotics, has been reported (Fouhy et al., 2014; Sommer et al., 2009). In general, the vanA gene abundance (per gram of dry weight) did not vary significantly from T0 to T7, with average measurements at T0 and T7 of 5.87 ± 0.11 and 5.79 ± 0.15 log-units under aerobic conditions, and 7.34 ± 1.17 and 7.17 ± 1.12 log-units under anaerobic conditions (**Figure 5.2**). In contrast, occasionally, although under both aerobic and anaerobic conditions (40-M and 58-M, **Figure 5.2**) the prevalence of *van*A (per 16S rRNA gene) increased significantly ($p<0.01$), which may be due to the decrease of the overall bacterial population, herein measured in 16S rRNA gene abundance. Aerobically, on average (FMAs 40-C, 50-C, 40-M and 50-M), the abundance of bla_{TEM} gene had a significant ($p<0.01$) increase from 7.13 ± 0.55 log-units at T0 to 8.23 ± 0.44 log-units at T7 (**Figure 5.2**).

Anaerobically, bla_{TEM} was not detected in one of the non-inoculated assays (58-C) and it decreased significantly ($p<0.01$) in the FMA50 (50-C, 50-M; **Figure 5.2**). In the FMA54, bla_{TEM} gene increased and decreased significantly ($p<0.01$) in the non-inoculated and inoculated assays, respectively (54-C, 54-M; **Figure 5.2**). On the average of the FMAs (50-C, 54-C, 50-M, 54-M, and 58-M), its abundance varied from 7.61 ± 1.12 log-units at T0 to 8.18 ± 0.74 log-units at T7 (**Figure 5.2**). The prevalence of the bla_{TEM} gene (per 16S rRNA gene) followed the same pattern of variation of the bla_{TEM} gene abundance along time, which suggests that the variations of bla_{TEM} gene are mainly due to total bacteria variations (**Figure 5.2**; **Figure S5.2**).

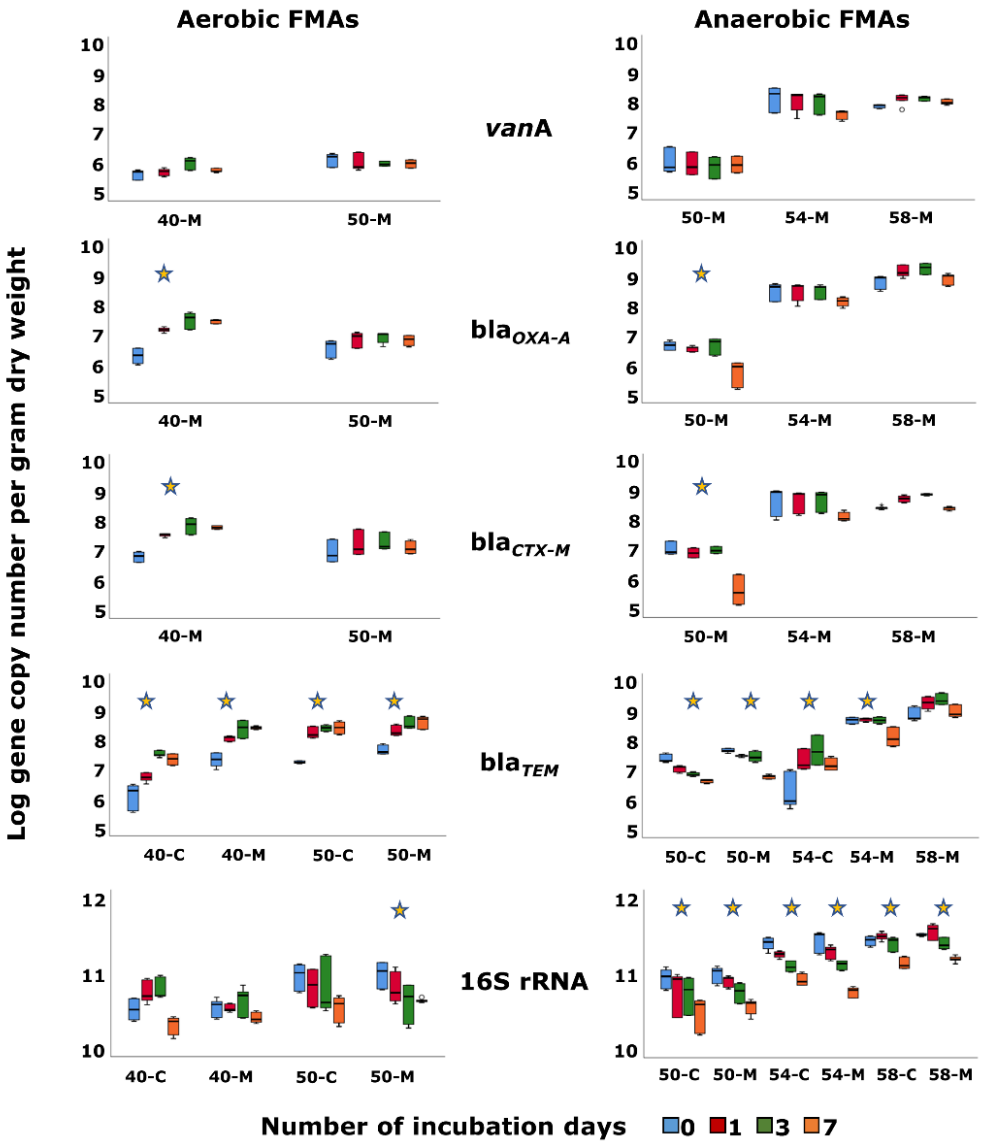

Figure 5.2. Variation of 16S rRNA and antibiotic resistance genes over time. The abundance of the genes (vanA, bla_{OXA-A}, bla_{CTX-M}, bla_{TEM}, and 16S rRNA) per g of dry weight of stool, of non-inoculated (C) and ARB-inoculated (M) FMAs under aerobic (column on the left side) and anaerobic (column on the right) conditions is shown. The variation of each gene along time among each FMA is indicated by coloured boxplot graphs (blue, red, green and orange corresponding to 0, 1, 3 and 7 days, respectively). The FMAs 40-C, 40-M, 50-C and 50-M were performed aerobically. The FMAs 50-C, 50-M, 54-C, 54-M, 58-C and 58-M were performed in anaerobic condition. With exception of the gene bla_{TEM}, the ARGs bla_{CTX}, bla_{OXA-A} and vanA were not detected in the non-inoculated microcosms. However, the gene bla_{TEM} was not detected in the non-inoculated microcosms 58-C. Genes abundance are the average values of all FMAs replicates with the standard deviation. Stars indicate statistically significant variation ($p<0.01$) of the genes abundance between T0 and T7 for each FMA. In cell-free DNA supplemented FMAs, FMA44 and FMA48, the spiked genes (in log copy number per gram of stool dry weight) were 5.92 ± 0.09 for bla_{CTX-M}, 5.99 ± 0.43 for bla_{OXA-A}, and 4.02 ± 0.13 for vanA and 9.24 ± 0.30 for bla_{TEM}, a value that might be overestimated, given the natural occurrence of this gene in the non-inoculated microcosms. After one day of incubation the bla_{CTX-M}, bla_{OXA-A}, and vanA genes were below the limit of quantification.

The abundance (per gram of dry weight) of the *bla*$_{CTX-M}$ gene increased significantly ($p<0.01$) under aerobic incubation (40-M; **Figure 5.2**) ranging from 6.83 ± 0.15 to 7.80 ± 0.05 log-units and, the same gene decreased significantly ($p<0.01$) under anaerobic incubation (50-M; **Figure 5.2**) varying from 7.04 ± 0.21 to 5.63 ± 0.46 log-units. Similar results were observed for *bla*$_{OXA-A}$ gene, which abundance (per gram of dry weight) varied significantly ($p<0.01$) from 6.37 ± 0.22 to 7.49 ± 0.06 log-units under aerobic conditions (40-M; **Figure 5.2**) and from 6.68 ± 0.14 to 5.75 ± 0.42 log-units under anaerobic conditions (50-M; **Figure 5.2**).

In general, it was observed that the abundance of the beta-lactamase genes increased under aerobic conditions and decrease under anaerobic conditions (**Figure 5.2**). This result that is aligned with the enumeration of culturable bacteria, suggests that the fitness of the exogenous bacteria, and not only the competition by the native microbiota, may dictate the fate of enterobacteria in the FMAs. The prevalence (expressed per 16S rRNA gene copy number) of both *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ increased under aerobic (40-M, 50-M) and anaerobic conditions (54-M, 58-M, except 50-M) (**Figure S5.2**). Comparing the FMA-40 and FMA-50 the abundance and prevalence of the *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ follows the same pattern suggesting that the variations of the beta-lactamase genes are mainly due to bacterial host variations (**Figure 5.2**; **Figure S5.2**).

To test the hypothesis that the fate of the exogenous ARGs is mainly dictated by the survival and integrity of the host cell, cell-free DNA extracts were used for spiking the FMAs (FMA44). The ARGs *bla*$_{CTX-M}$, *bla*$_{OXA-A}$, and *van*A, detected after inoculation at doses of 4-5 log units were below the detection limit after 1 day of incubation (**Figure 5.2**), suggesting the rapid DNA degradation by the native fecal microbiota (e.g. extracellular enzymes or bacteria feeding in naked DNA). This result supported the hypothesis that viable or at least integer bacterial hosts are necessary to ensure the ARGs persistence. Moreover, any attempts to isolate transformants from free-DNA spiked FMA48 were unfruitful, therefore failing the evidence of antibiotic resistance acquisition by transformation.

5.4.3.1. Antibiotics effect on exogenous antibiotic resistant bacteria

Culturable enterococci showed a different pattern of variation in inoculated and non-inoculated assays spiked with vancomycin, although in both was observed a significant ($p<0.01$) decrease along time (**Figure 5.1**). In the presence of a single-dose of cefotaxime (FMA54; **Figure 5.1**), culturable enterobacteria in inoculated and non-inoculated FMAs

undergone a significant ($p<0.01$) decrease of approximately 2 log-units after 7 days. A similar, although less intense effect, was observed after administration of a multiple-dose of cefotaxime, with enterobacteria reductions of approximately 1 log-unit in inoculated and non-inoculated microcosms after a week (**Figure 5.1**). Generally, in the presence of low concentrations of cefotaxime or vancomycin, heterotrophic bacteria counts presented a similar trend along the time in both inoculated and non-inoculated microcosms (**Figure 5.1**).

Using a single-dose of cefotaxime (FMA 54-M.C, 54-C.C; **Figure 5.1**) or vancomycin (FMA 54-M.V, 54-C.V; **Figure 5.1**), heterotrophic bacteria counts of inoculated and non-inoculated samples decreased significantly ($p<0.01$) of approximately 3 or 1 log-units, respectively, after 7 days. A similar, albeit less intense effect, was observed after administration of a multiple-dose of cefotaxime (FMA 58-M.C, 58-C.C; **Figure 5.1**) where heterotrophic bacteria counts of inoculated and non-inoculated assays decreased significantly ($p<0.01$) of approximately 2 log-units after 7 days.

The abundance of the 16S rRNA gene per gram of feces dry weight, a measure of bacterial abundance, was not significantly different in inoculated and non-inoculated assays, and decreased significantly ($p<0.01$) over the incubation period, in inoculated (from 11.45 ± 0.19 to 10.88 ± 0.28 log-units; FMA 54-M.C, 54-M.V; FMA 58-M.C and 58-M.V) and in non-inoculated microcosms (from 11.38 ± 0.11 to 10.89 ± 0.11 log-units; FMA 54-C.C, 54-C.V; FMA 58-C.C and 58-C.V), irrespective of the use of one or three doses of antibiotic (**Figure 5.3**). In the presence of one or three doses of cefotaxime or vancomycin, the abundance of *vanA* gene did not change significantly (FMA 54-M.C, 54-M.V; FMA 58-M.C and 58-M.V; **Figure 5.3**). However, in the presence of one or three doses of cefotaxime the *van*A per 16S rRNA gene copy number (prevalence) increased significantly ($p<0.01$) on average from -3.66 ± 0.04 to -3.27 ± 0.08 log-units in inoculated microcosms (FMA 54-M.C and FMA 58-M.C; **Figure S5.3**). Similarly, in the presence of three doses of vancomycin, the prevalence (per 16S rRNA gene) of *van*A increased significantly ($p<0.01$) from -3.51 ± 0.06 to -2.95 ± 0.01. Since culturable enterococci decreased over this period, the observed increase is probably due to the sharp decrease of bacteria (16S rRNA gene abundance) observed during incubation. In the presence of one-dose of cefotaxime, the abundance (per dry weight) of the ARGs bla_{TEM}, $bla_{\text{CTX-M}}$ and $bla_{\text{OXA-A}}$ decreased significantly ($p<0.01$) (FMA 54-M.C; **Figure 5.3**) while in the presence of three-doses of cefotaxime, the abundance of these genes did not vary significantly (FMA 58-M.C; **Figure 5.3**).

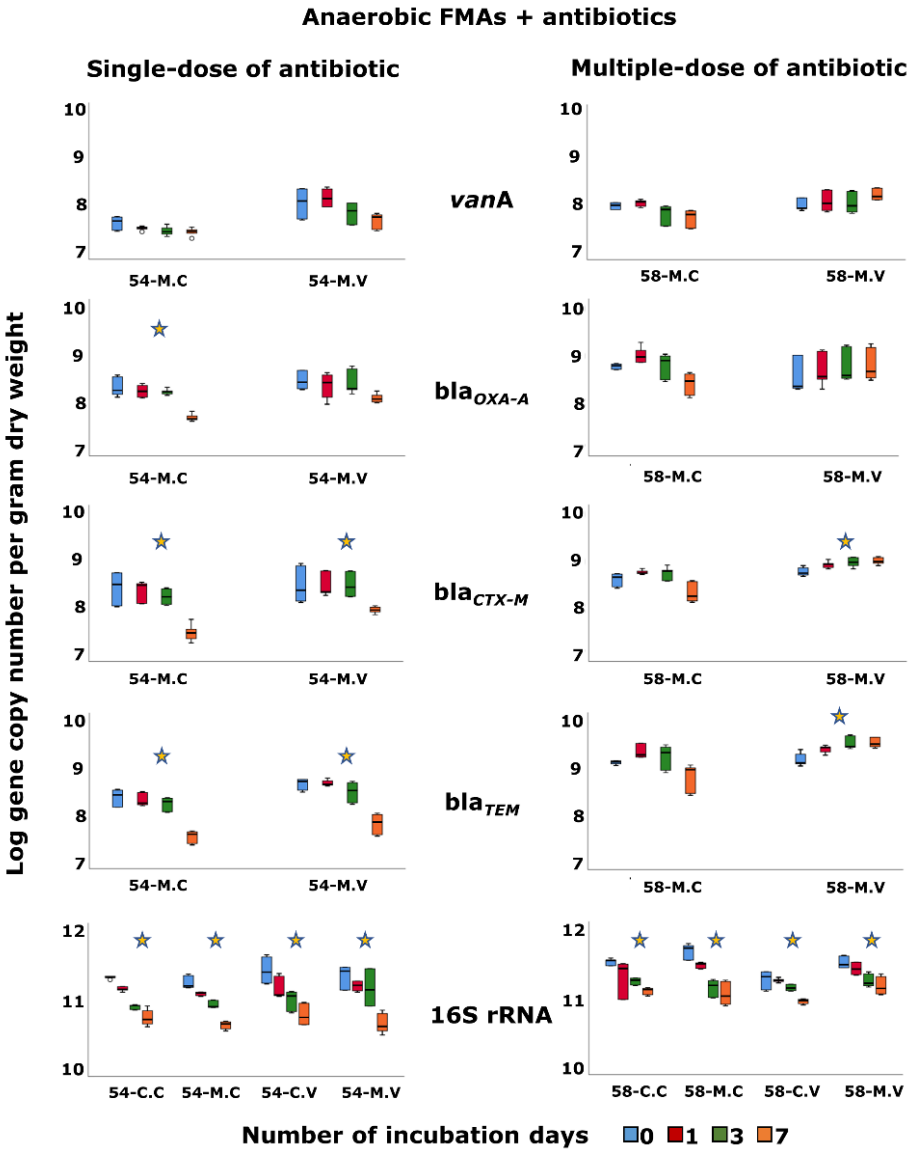

Figure 5.3. Effect of antibiotic on the variation of 16S rRNA and antibiotic resistance genes along the time. The abundance of the genes (vanA, bla_{OXA-A}, bla_{CTX-M}, bla_{TEM} and 16S rRNA) per g of dry weight of stool, of non-inoculated (C) and ARB-inoculated (M) FMAs under anaerobic conditions in the presence of antibiotics is shown. The variation of each gene along time for each FMA is indicated by colored boxplot graphs (blue, red, green and orange corresponding to 0, 1, 3 and 7 days, respectively). FMAs were performed in the presence of a single-dose of cefotaxime (54-C.C, 54-M.) or vancomycin (54-C.V, 54-M.V) or, in the presence of multiple-doses of cefotaxime (58-C.C, 58-M.C) or vancomycin (58-C.V, 58-M.V). The ARGs bla_{TEM}, bla_{CTX}, bla_{OXA-A} and vanA were not detected in the non-inoculated microcosms. Genes abundance are the average values of all FMAs replicates with the standard deviation. Stars indicated statistically significant variation ($p<0.01$) of genes abundance between T0 and T7 for each FMA.

The prevalence (per 16S rRNA gene) of the ARGs *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ increased significantly ($p<0.01$), but only when three doses of cefotaxime were supplied (FMA 58-M.C; **Figure S5.3**).

In the presence of one or three doses of vancomycin, the abundance (per dry weight) of the ARGs *bla*$_{TEM}$ significantly ($p<0.01$) decreased and of *bla*$_{CTX-M}$ significantly ($p<0.01$) increased, while the gene *bla*$_{OXA-A}$ no significant variations were observed (FMA 54-M.V and FMA 58-M.V; **Figure 5.3**). The prevalence of *bla*$_{TEM,}$ *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ increased significantly ($p<0.01$) only in the presence of three doses of vancomycin (FMA 58-M.V; **Figure S5.3**). The abundance and prevalence of the genes *bla*$_{TEM}$ and *bla*$_{CTX-M}$ followed the same pattern of variation when vancomycin was supplemented at three doses suggesting that the variations of these two genes are mainly due to bacterial host variations. Nevertheless, the prevalence increment of the gene *bla*$_{OXA-A}$ in the presence of three-doses of vancomycin suggests that this antibiotic might favour the survival or the persistence of bacteria harbouring the *bla*$_{OXA-A}$ gene along the period of incubation.

5.4.4. Relationship between bacterial community and antibiotic resistance genes

To unravel the possible relationship between ARGs persistence and the variation of the fecal bacterial community in the presence of a single- or multiple-dose of cefotaxime or vancomycin, a Canonical Correspondence Analysis (CCA) was performed. This analysis confirmed the microbiota rearrangements due to the microcosm effect (**Table 5.2**) by the separation between T0 and T7 samples and highlighted the effects of antibiotics (**Table S5.3**, **Table S5.1**) (T7 vs T7.C or T7.V, **Figure 5.4**). The use of a single- or multiple- antibiotic doses highlighted distinct patterns of correlation between the fecal bacterial community composition and the quantified genes. While with a single-dose, the ARGs variation was co-linear, with all genes showing the same pattern of variation (**Figure 5.4** A and B), for multiple-doses were observed distinct patterns of variation (**Figure 5.4** C and D). With a single cefotaxime or vancomycin dose, the quantified genes were correlated with groups observed to decrease over the incubation period such as *Ruminococcaceae* and *Burkholderiaceae* (for cefotaxime), and *Lachnospiraceae* (for both antibiotics), as could be observed over axis 1, which explains >85% of the variation. The use of multiple doses of cefotaxime or of vancomycin produced distinct correlation patterns. For three cefotaxime doses, the genes 16S rRNA, *bla*$_{CTX-M}$ and *bla*$_{TEM}$ had a co-linear variation and were correlated with the decrease during incubation of populations such as *Lachnospiraceae,* as can be seen over axis 1 that explains >70% of the variation. The variation of the genes *bla*$_{OXA-A}$ and *van*A was co-linear and negatively correlated with *Firmicutes* of the families *Christensenellaceae* and *Enterococcaceae* (**Figure 5.4** C).

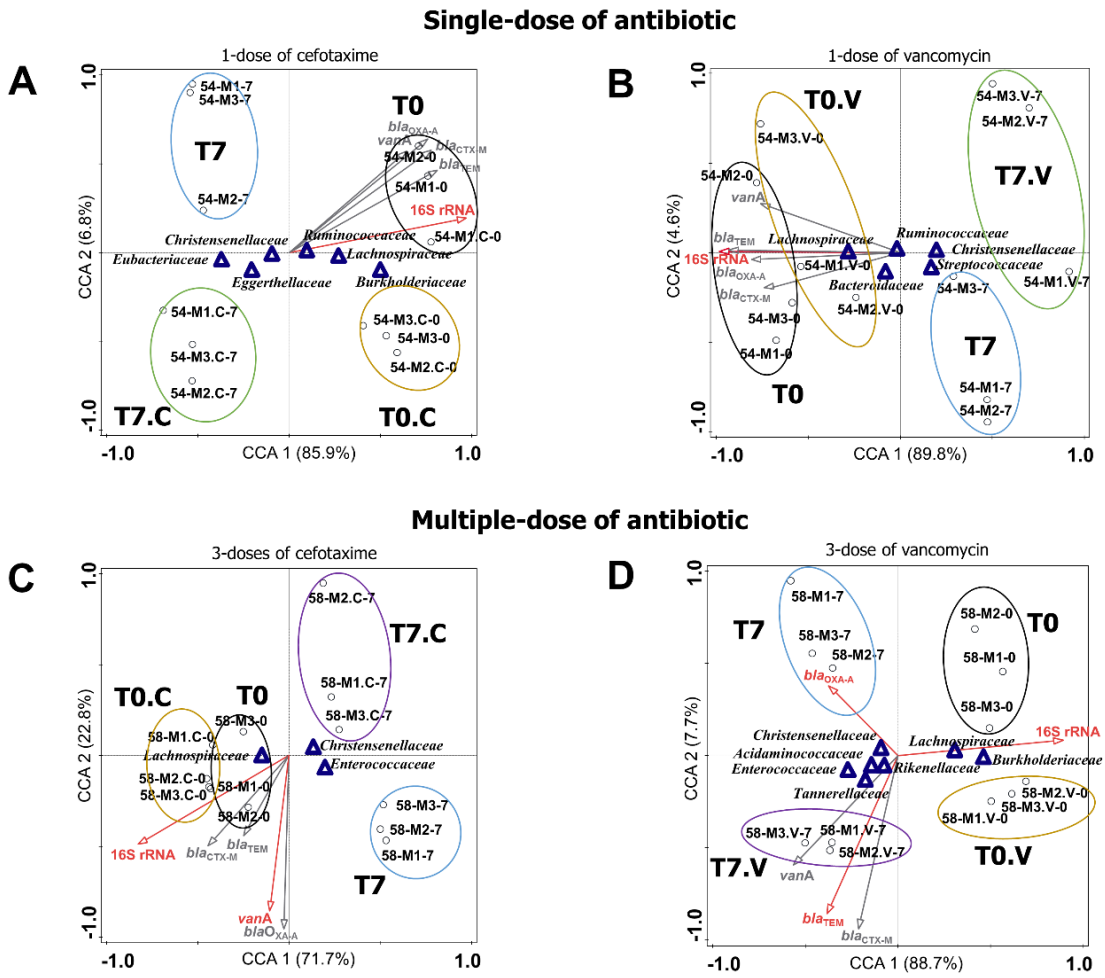

Figure 5.4. Canonical Correspondence Analysis (CCA) of bacterial families (with relative abundance > 1%, and with the highest fit values, > 0.90) of the ARB-inoculated assays in the presence of a single-dose of cefotaxime (**A**) or vancomycin (**B**) or, in the presence of a multiple-dose of cefotaxime (**C**) or vancomycin (**D**). All the CCA represent microcosms at the beginning (T0) and after seven days of incubation (T7) without antibiotic (black circle and light blue circle, respectively), T0 and T7 in the presence of a single-dose of cefotaxime (T0.C, yellow circle; T7.C, green circle) or vancomycin (T0.V, yellow circle; T7.V, green circle) and, T0 and T7 in the presence of a multiple-dose of cefotaxime (T0.C, yellow circle; T7.C, purple circle) or vancomycin (T0.V, yellow circle; T7.V, purple circle). The red arrows show the significant explanatory variables ($p<0.05$) while grey arrows represent the explanatory variables with no significant correlation.

For three vancomycin doses, the 16S rRNA gene was positively correlated with the *Lachnospiraceae* and *Burkholderiaceae*, as can be observed over axis 1 explaining >88% of the variation. With opposite distributions over axis 2 that explains < 8% of variation were the genes *bla*$_{\text{OXA-A}}$ and *van*A, *bla*$_{\text{CTX-M}}$ and bla$_{\text{TEM}}$ with a co-linear variation. The three latter were positively correlated with *Tannerellaceae* (phylum *Bacteroidetes*) and the *Enterococcaceae*, *Acidaminococcaceae* and *Christensenellaceae* (phylum *Firmicutes*) (**Figure 5.4** D).

This study aimed at assessing if ARGs harboured by exogenous wastewater ARB in fecal material and if aerobiosis or antibiotics could influence their survival. The rationale was that variations of exogenous bacteria could be due to their fitness or due to the influence of the fecal microbiota rearrangements, or both. Previous studies have shown that enterobacteria spiked in animal feces can maintain viability for up to three months in ambient air (Scott *et al.*, 2006; Segura *et al.*, 2018; Sinton *et al.*, 2007; Walters and Field, 2009). In the present study, it was used a period of 7 days, considering this would be an acceptable time period for transient intestinal colonization with wastewater ARB. The PrCA and CCA results suggest that exogenous bacteria survival or proliferation is unpaired by the autochthonous fecal microbiota, mainly *Firmicutes* and *Bacteroidetes* families. However, the assayed exogenous bacteria could not be eliminated until 7 days and their genes could be detected in FMAs incubated for 30 days (data not shown).

Overall these results suggest that: i) the fate of ARGs is mainly determined by the fitness of the host bacteria; ii) in spite of the ARGs host decay, the overall decrease of the fecal bacterial population (e.g. due to adverse conditions or antibiotherapy) may lead to apparent increases of ARGs prevalence, and iii) even if ARGs host cells lose viability they may protect the ARGs, as long as cells integrity is maintained. The role of the competitor native fecal microbiota is unquestionable, and it may be influenced by both diversity and abundance. Although it is difficult to estimate the likelihood of transmission of wastewater-ARB to humans, the fact that these may be able to survive the competition by native microbiota is a topic that should not be neglected.

Supplementary Information

Chapter 5. Persistence of wastewater antibiotic resistant bacteria and their genes in human fecal material

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Authors: Nazareno Scaccia, Ivone Vaz-Moreira and Célia M. Manaia

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Table S5.1. Bacterial community composition of non-inoculated (C) FMAs, expressed as phylum relative abundance. Legend: 40-C0, 44-C0, 50-C0, 54-C0 and 58-C0 are non-inoculated FMAs at time zero. The relative abundance of each phylum was estimated as the ratio between the number of reads of a given phylum and the total number of reads obtained for the FMA. Phyla with relative abundance equal or below 2% are presented as other phyla. The values are the average of triplicates ± the standard deviation.

Phylum	Phylum relative abundance (%) in each FMA				
	40-C0	44-C0	50-C0	54-C0	58-C0
Firmicutes	46.19 ± 0.49^{a}	43.82 ± 0.40^{b}	41.57 ± 0.20^{c}	46.57 ± 1.14^{a}	47.07 ± 0.48^{a}
Bacteroidetes	31.35 ± 0.74^{a}	36.15 ± 0.62^{b}	30.10 ± 0.65^{a}	29.77 ± 1.44^{a}	23.53 ± 1.13^{c}
Actinobacteria	18.87 ± 0.66^{ab}	7.24 ± 1.27^{c}	18.81 ± 1.65^{ab}	16.84 ± 0.77^{a}	22.45 ± 1.92^{b}
Proteobacteria	2.15 ± 0.12^{a}	7.35 ± 0.18^{b}	1.66 ± 0.06^{c}	1.54 ± 0.03^{c}	1.69 ± 0.17^{c}
Verrucomicrobia	1.20 ± 0.14^{a}	4.93 ± 0.27^{b}	7.38 ± 1.23^{b}	4.55 ± 0.46^{b}	4.54 ± 0.56^{b}
Other phyla (≤ 2 %)	0.04 ± 0.01^{a}	0.17 ± 0.03^{b}	0.06 ± 0.01^{a}	0.19 ± 0.01^{b}	0.15 ± 0.02^{b}

Legend: a-d; statistically significant difference ($p<0.01$) between FMAs.

Table S5.2. Oxygen effect on bacterial community in non-inoculated (50-C) and ARB-inoculated (50-M) FMAs. The presented values are the ratios of the relative abundance of each replicate at T7, for C or M, with oxygen and the average values of the relative abundance at T7, for C or M, without oxygen. The values are expressed as average of triplicates ± the standard deviation.

FMAs	**Phylum relative abundance with oxygen/ phylum relative abundance without oxygen**						**Family relative abundance with oxygen/ family relative abundance without oxygen**	
	Bacteroidetes	*Actinobacteria*	*Verrucomicrobia*	*Firmicutes*	*Proteobacteria*	Other phyla ≤ 2%	*Enterobacteriaceae*	*Enterococcaceae*
50-C	0.85±0.05	0.81±0.04	1.50±0.16	1.01±0.09	12.31±2.61*	0.79±0.13	76.34±17.94*	n.d.
50-M	0.97±0.04	0.79±0.02	1.47±0.31	0.89±0.01	14.96±0.49*	1.66±0.35	1.00±0.02	1.00±0.04

*, statistically significant variation ($p<0.01$) between incubation under aerobic and anaerobic conditions for the non-inoculated (50-C) and ARB-inoculated (50-M) microcosms.
n.d., not detected at T7.

Table S5.3. Antibiotic effect on non-inoculated (C) and ARB-inoculated (M) FMAs exposed to the addition of a single- (54-C.C, 54-M.C, 54-C.V, and 54-M.V) or a multiple-dose (58-C.C, 58-M.C, 58-C.V, and 58-M.V) of antibiotic. Legend: 54-C or 54-M and 58-C or 58-M, non-inoculated (C) or ARB-inoculated (M) FMA without addition of antibiotics; 54-C.C or 54-M.C and 58-C.C or 58-M.C, FMAs with addition of a single- or a multiple-dose, respectively, of cefotaxime (4 mg L^{-1}); 54-C.V or 54-M.V and 58-C.V or 58-M.V, FMA with addition of a single- or a multiple-dose, respectively, of vancomycin (16 mg L^{-1}). Phyla with relative abundance equal or below 2% were designated as other phyla. The values correspond to the ratios of the relative abundance of each replicate at T7 of C or M in the presence of antibiotic and, the average values of the relative abundance at T7 of C or M without the addition of antibiotics. The values are expressed as the average of triplicates ± the standard deviation.

FMAs	**Conditions**	**Phylum relative abundance with antibiotic/ phylum relative abundance without antibiotic**						**Family relative abundance with antibiotic/ family relative abundance without antibiotic**	
		Bacteroidetes	*Actinobacteria*	*Verrucomicrobia*	*Firmicutes*	*Proteobacteria*	Other phyla (≤ 2%)	*Enterobacteriaceae*	*Enterococcaceae*
54-C	Single-dose of antibiotic cefotaxime (C) or vancomycin (V)	1.00±0.03	1.00±0.09	1.00±0.13	1.00±0.04	1.00±0.11	1.00±0.31	1.00±0.12	n.d.
54-M		1.00±0.03	1.00±0.10	1.00±0.21	1.00±0.03	1.00±0.14	1.00±0.25	1.00±0.17	1.00±0.13
54-C.C		1.08±0.08	0.86±0.09	1.20±0.14	1.08±0.02	0.26±0.04*	1.25±0.21	0.12±0.03*	n.d.
54-M.C		1.00±0.09	0.86±0.12	1.26±0.30	1.09±0.04	0.39±0.02*	1.07±0.09	0.14±0.03*	1.04±0.15
54-C.V		1.02±0.10	0.90±0.12	1.28±0.22	1.10±0.01	0.19±0.02*	1.10±0.23	0.06±0.01*	n.d.
54-M.V		0.96±0.04	0.98±0.07	1.14±0.20	1.06±0.04	0.45±0.16	1.01±0.25	0.23±0.17	1.88±0.79
58-C	Multiple-dose of antibiotic cefotaxime (C) or vancomycin (V)	1.00±0.02	1.00±0.02	1.00±0.14	1.00±0.01	1.00±0.22	1.00±0.12	1.00±0.36	n.d.
58-M		1.00±0.06	1.00±0.10	1.00±0.11	1.00±0.05	1.00±0.17	1.00±0.22	1.00±0.20	1.00±0.12
58-C.C		0.80±0.07	1.19±0.08	0.73±0.12	1.01±0.00	1.30±0.39	1.52±0.48	1.46±0.47	n.d.
58-M.C		0.89±0.13	1.19±0.16	0.91±0.23	1.09±0.01	0.18±0.03*	1.27±0.33	0.13±0.03*	0.78±0.05
58-C.V		0.80±0.38	1.16±0.51	0.71±0.32	0.98±0.44	2.76±1.22*	1.07±0.51	3.47±0.85*	n.d.
58-M.V		1.01±0.05	1.02±0.04	0.94±0.17	1.05±0.06	0.68±0.14	0.63±0.15	0.63±0.15	1.18±0.17

n.d., not detected at T7.

*, statistically significant difference ($p<0.01$) between the FMAs with the addition of antibiotic versus no addition of antibiotic, for each FMA at time 7 day.

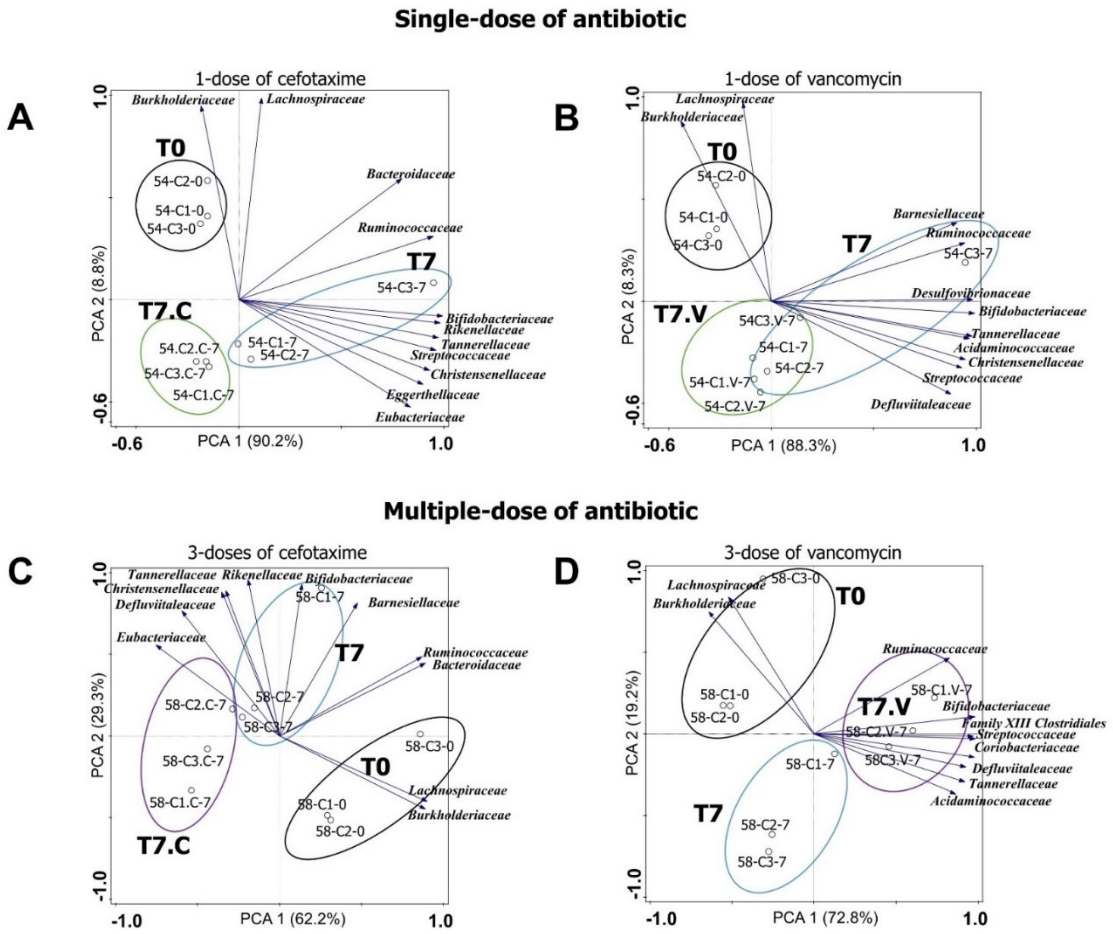

Figure S5.1. Principal component analysis (PCA) of bacterial family composition variation (relative abundance > 1%, with the highest fit values, > 0.90) in non-inoculated assays, in the presence of a single-dose of cefotaxime (**A**) or vancomycin (**B**) or, in the presence of a multiple-dose of cefotaxime (**C**) or vancomycin (**D**) or in the respective antibiotic-free controls. All the PCA represent microcosms at time 0 (T0) and after seven days (T7) without the addition

of antibiotic (black and light blue circle, respectively) and, T7 in the presence of a single-dose of cefotaxime or vancomycin (T7.C or T7.V; green circle) or in the presence of a multiple-dose of cefotaxime or vancomycin (T7.C or T7.V; purple circle).

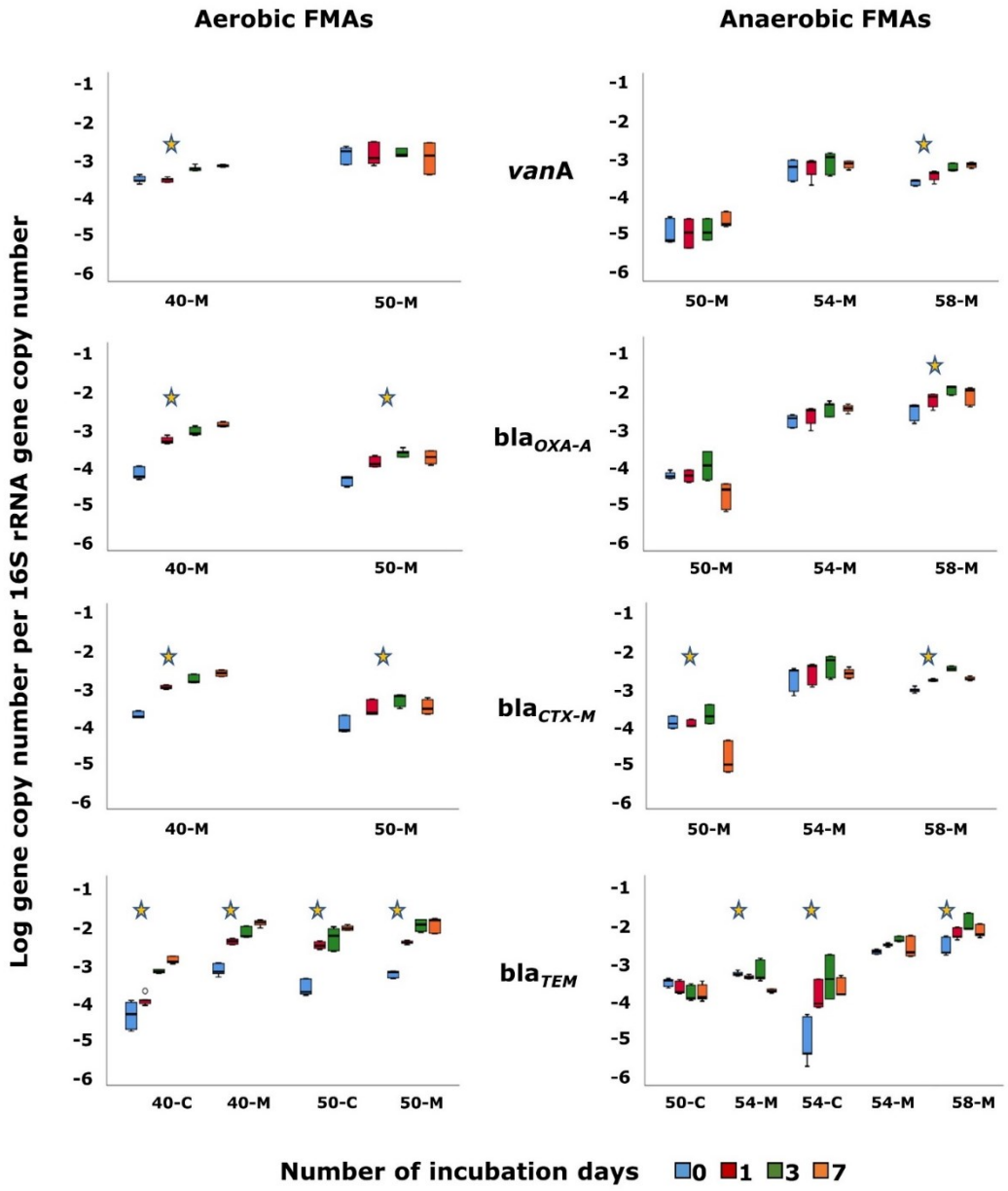

Figure S5.2. Variation of antibiotic resistance genes (bla_{TEM}, bla_{CTX}, bla_{OXA-A} and vanA) prevalence (per 16S rRNA) over time, in non-inoculated (C) and ARB-inoculated (M) FMAs under aerobic (column on the left side) or anaerobic (column on the right) conditions. The variation of each gene along time for each FMA is indicated by coloured boxplot graphs (blue, red, green and orange corresponding to 0, 1, 3 and 7 days, respectively). The FMAs 40-C, 40-M, 50-C and 50-M were incubated aerobically. The FMAs 50-C, 50-M, 54-C, 54-M, 58-C and 58-M were incubated under anaerobic condition. With exception of the gene bla_{TEM}, the ARGs bla_{CTX}, bla_{OXA-A} and vanA were not detected in the non-inoculated microcosms. However, the gene bla_{TEM} was not detected in the non-inoculated microcosms 58-C. Genes prevalence (or relative abundance) are the average values of all FMAs replicates with the standard deviation. Stars indicated statistically significant variation ($p<0.01$) between T0 and T7 of the genes for each FMA.

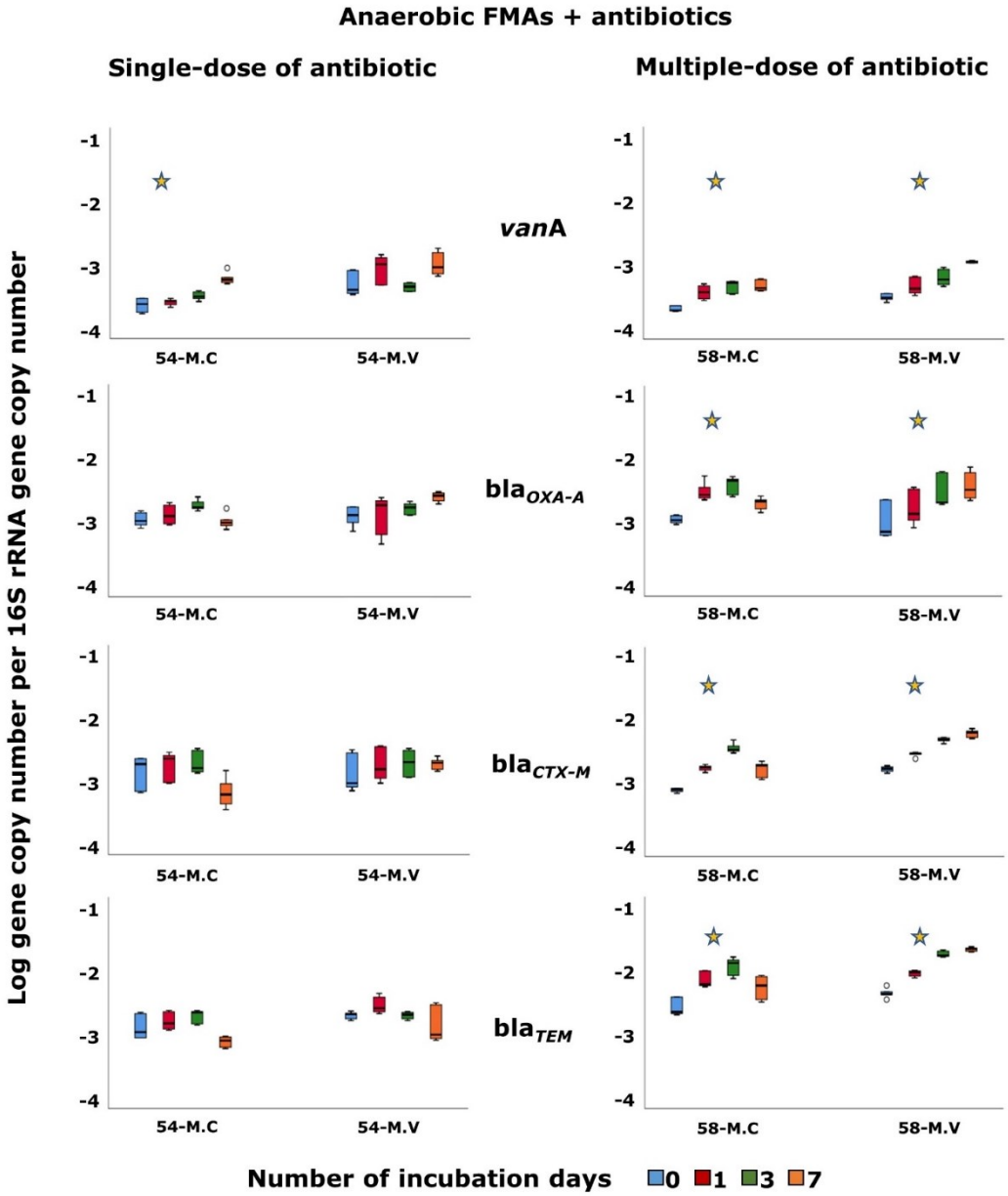

Figure S5.3. Effect of antibiotic addition on the variation of antibiotic resistance genes (bla_{TEM}, bla_{CTX}, bla_{OXA-A} and vanA) prevalence (per 16S rRNA) over time, in non-inoculated (C) and ARB-inoculated (M) FMAs under anaerobic conditions in the presence of antibiotics. The variation of each gene along time for each FMA is indicated by coloured boxplot graphs (blue, red, green and orange corresponding to 0, 1, 3 and 7 days, respectively). FMAs were performed in the presence of a single-dose of cefotaxime (54-C.C, 54-M.C) or vancomycin (54-C.V, 54-M.V) or, in the presence of multiple-doses of cefotaxime (58-C.C, 58-M.C) or vancomycin (58-C.V, 58-M.V). The ARGs bla_{TEM}, bla_{CTX}, bla_{OXA-A} and vanA were not detected in the non-inoculated microcosms. Genes abundance are the average values of all FMAs replicates with the standard deviation. Stars indicate statistically significant variation ($p<0.01$) of genes prevalence between T0 and T7, for each FMA.

6. General Discussion

The use of treated wastewater to irrigate crops may be a source of antibiotic resistant bacteria and antibiotic resistance genes capable of contaminating the human food chain, and therefore threat human health. A major question about wastewater reuse, particularly in agriculture, is if reclaimed water may act as a vehicle for the transmission of hazardous microorganisms (e.g. pathogenic, opportunistic, antibiotic resistant) that could colonize the plant, either on the surface or the plant vascular system, and therefore represent a human threat.

While the adsorption of microorganisms onto crop's surfaces has been demonstrated, the uptake as endophytes from contaminated soil has been poorly investigated (Zhang *et al.*, 2017). This might be due to the fact that it may be technically challenging since even if some microorganisms are uptake, they may be at extremely low levels that hardly can be detected. Based on this rational, and aware that this is a field requiring further research, we speculated that ARB thriving in wastewater can be uptake by plants, after exposure to irrigation practices. If wastewater-ARB have the potential to colonize the plant, these bacteria may be transmitted to humans via food-chain.

Although phyllosphere bacteria can easily be removed by washing or disinfection procedures, endophytic bacteria might be more persistent and not eliminated before consumption. These observations motivated the literature survey focused on endophytic bacteria (Chapter 3). The idea was to gain an overview of diversity of endophytic bacteria associated with crops that have the potential to live in different habitats (wastewater, human and plant) and may harbour acquired antibiotic resistance. It was observed that bacterial groups belonging to the genera *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Burkholderia*, *Serratia*, *Stenotrophomonas* and *Bacillus,* closely related to human pathogens and recognized ARGs hosts, may indeed be endophytic bacteria, and it seems possible that these bacteria might move across wastewater, plant and human. Among the aforementioned bacterial genera, *Pseudomonas* and *Stenotrophomonas* were also found in ready-to-eat watercress and strawberry (Chapter 4). However, among the *Pseudomonas* and *Stenotrophomonas* species identified in watercress and strawberry, from the genes tested only *intI*1 was identified in one strain of *Pseudomonas paralactis* (**Table 4.1**, Chapter 4). Also, the endophytic ARB herein found can be associated with important food crops to human food supply such as lettuce, carrot, radish, tomato and cucumber which are generally consumed raw. Therefore, the regular consumption of these products may contribute to

enrich the antibiotic resistome of consumers. Although the ingestion of these bacteria may be innocuous to humans, in cases of debilitated patients, human health risks can arise, especially if these bacteria host virulence factors as well as intrinsic or acquired antibiotic resistance.

To proceed with the study of potential plant-associated ARB present in crops consumed raw, we investigated the presence of ARB and ARGs within the cultivable fraction of the phyllosphere and endophytic bacterial community of ready-to-eat watercress and strawberry (Chapter 4). Additionally, another question regarded whether plant-associated bacteria may be or not susceptible to acquire ARGs and thus be involved in the dissemination of antibiotic resistance. Also, it was observed that the antibiotic resistance phenotypes associated with watercress and strawberry is probably due to intrinsic resistance properties and, albeit further studies are need, they might be able to acquire new resistance phenotypes.

These evidences suggested that during irrigation practices, wastewater-ARB could be transferred to plant crops and thus reach humans through the food-chain supply, mainly if the crops are consumed raw. If this is the case and although other barriers exist, another question would be whether ARB may or not survive and persist within humans. For that, in a second approach, the implementation of feces microcosm assays permitted to investigate the ability of wastewater-ARB to survive in human gut microbiota (Chapter 5). This work showed that ARGs harboured by wastewater bacterial isolates could persist in healthy infant´s stool-based microcosms under different conditions (e.g. including the presence of sub-inhibitory concentrations of antibiotics) and thus wastewater-ARB-ARGs may be able to persist in the gut microbiota environment. The persistence of the tested ARB was observed under both aerobic and anaerobic conditions as well as under anaerobic conditions in the presence of antibiotics. However, anaerobic conditions may have had a slight positive effect on ARB elimination. On the other hand, feces microcosm assays spiked with cell-free DNA showed that the ARGs could not be detected after one day of incubation. This suggests that the ARGs could readily be degraded by the fecal material and therefore, the persistence of the ARGs relied on the host cell integrity. Additionally, anaerobic feces microcosm experiments inoculated with wastewater-ARB in the presence of single- or multiple-dose of antibiotics, did not show significant variation on the persistence of the exogenous ARB and theirs associated ARGs.

To mitigate the risks of ARB ad ARGs dissemination in the environment and their transmission to humans, control strategies need to be developed. Since the agricultural fields

can be cultivated with vegetables for human consumption, the quality and safety of such products irrigated with treated wastewater, must be assured.

Further efforts need to be performed to understand the behaviour of ARB and ARGs in the environment and whether these contaminants can reach humans or animals, in order to develop risk-assessment guidelines to ensure the public health and promote the safe reuse of treated wastewater.

7. Conclusions

Crops can be involved in the dissemination of antibiotic resistance, and raw-eaten vegetables such as lettuce, carrot, radish, cucumber or tomato can host endophytic bacteria, belonging to bacterial groups frequently associated to antibiotic-resistance, are potential vehicles of antibiotic resistance transmission to humans.

Antibiotic-resistant endophytic bacteria, not eliminated during the washing of edible vegetables, might be vectors of transmission of antibiotic resistance to consumers. Among the most common bacteria in raw eaten crops are members of genera *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Burkholderia*, *Serratia*, *Stenotrophomonas* and *Bacillus*. Most of these bacterial genera, particularly *Enterobacter*, *Acinetobacter*, *Pseudomonas* and *Staphylococcus* have members that have been described as leading human antibiotic resistant opportunistic pathogens, found to yield a broad diversity of antibiotic and metal resistance genes.

Watercress and strawberry associated bacteria showed mainly intrinsic antibiotic resistance phenotypes. However, the ubiquitous character of these bacteria and potential to acquire antibiotic resistance genes are aspects that need further investigation.

Antibiotic resistance genes hosted by wastewater bacteria such as *Escherichia coli* (strain A2FCC14) and *Enterococcus faecium* (strain H1EV10), persisted in the presence of human fecal microbiota until a week, being detected up to at least one month. Fecal microcosms assays inoculated with free-DNA that was rapidly degraded demonstrated that the fate of antibiotic resistance genes depends on the fitness of the host, and therefore it is suggested that ARGs can persist as long as the integrity of the host cell is maintained. Sub-inhibitory concentrations of cefotaxime or vancomycin, did not exhibited any marked selective pressure effect on the exogenous ARB.

8. Suggestions of Future Work

The present study sheds some light on the likely transmission of wastewater antibiotic resistant bacteria to humans from wastewater-irrigated crops. The hypotheses about 1) plant contamination by wastewater antibiotic resistant bacteria due to irrigation practices, and 2) wastewater plant-associated bacteria transmission to humans are now stronger. Nevertheless, other works that should be addressed in future studies might regard:

- Further investigation about antibiotic resistant endophytic bacteria could explore the presence of metal resistance genes and antibiotic resistance genes to unravel whether they might be located on MGEs and thus, infer about the ability of endophytic bacteria in the spreading of antibiotic resistance.

- Conduct lab-scale experiments using a sprout germination assay, as initiated (Annex IV) in order to study the interaction between ARB and the plant. Also, a field-scale approach to simulate real conditions of raw crops irrigated with fresh water and treated wastewater could be taken into consideration.

- Explore more in deep the interaction between wastewater antibiotic resistant bacteria and gut microbiota. In particular, perform horizontal gene transfer experiments to study the likelihood of exchange of antibiotic resistance genes between the antibiotic resistant bacteria from wastewater and gut bacteria. Also, further experiments, for instance, using the simulator of the complete human intestinal tract, may allow to better understand the survival of exogenous bacteria within the human gastro-intestinal tract taking into account also other variables such as food matrix, stomach acidity or periplasmic movements. Additionally, it would be important to understand whether wastewater ARB may colonize the digestive tract.

Annexes

Annex I. Training courses, outreach/dissemination tasks, scientific publications outside of the Ph.D. thesis and conferences

ANSWER training activities:

- ANSWER Summer school and Training course on "*Mechanisms and processes involved in the crops uptake*". 13 – 23 June 2016, Spanish National Research Council (CSIC), Barcelona, Spain.

  The summer school focused on lectures and case studies related to wastewater reuse, current challenges, and opportunities. The training course "*Mechanisms and processes involved in the crops uptake*" instead, was focused on plants uptake of micro-contaminants from soil and water.
- "*Wastewater microbiota and the effects of treatment processes*". 5 – 7 July 2017, Escola Superior de Biotecnologia (ESB) of Universidade Católica Portuguesa (UCP), Porto, Portugal.

  In this course, lectures on wastewater microbiota and the effects of treatment processes as well as a one-day course on entrepreneurship "*Fostering entrepreneurship - from business models to clients*" were undertaken.
- "*Wastewater treatment by advanced technologies and risk assessment framework"*. 4 – 6 September 2017, Università degli Studi di Salerno (UNISA) Fisciano, Italy.

  The training event focused on wastewater treatment by advanced technologies and risk assessment framework. Also, a workshop "*Risk prognosis of environmental and public health aspects of A&ARB&ARG*" was followed.
- "*Microbiology in wastewater treatment; Design criteria for wastewater treatment plants; Horizontal resistance gene transfer in soil*". 23 – 27 April 2018, Technische Universität Wien (TU-Wien) Vienna, Austria.

  The training event aimed to give an overview on microbiology in wastewater treatment, design criteria for wastewater treatment plants and mechanisms of horizontal resistance gene transfer. Additionally, a workshop on 16S rRNA microbial community analysis was followed. Moderation of project meetings; Gender balance in the research and science arena and multicultural awareness as complementary/soft skills training was also performed.

- "*Environmental and human health risk assessment of antibiotics*". 18 – 21 June 2018, KWR Watercycle Research Institute (KWR) Utrecht, The Netherland.
  Lectures about environmental and human health risk assessment of antibiotics and 2nd workshop on "*Modelling and risk assessment tools towards sustainable wastewater reuse*" occurred. Working in Policy or Business and How to Secure a Good Job as complementary/soft skills training was also carried out.

Training activities outside ANSWER Network:

- BIOTechnology tools for food, environmental and health Applications course (BIOTA). 13 – 24 February 2017, ESB-UCP, Porto, Portugal.
  The course was focused to inform the Ph.D. students about the available techniques and tools at the ESB-CBQF in the area of Biotechnology in order to plan experiments using real projects undergoing at the ESB-CBQF institution. It was also intended to develop and improve skills in the delineation of experimental protocols, the chronogram of experiments and communication of biotechnology themes to a diverse audience.
- Introduction to Portuguese Culture and Language course (IPCL). 26 May – 21 May 2019, UCP, Porto, Portugal.
  The focus of this course was to establish opportunities for knowing the Portuguese culture through direct and indirect contact with some of its forms and expressions.

Additionally, monthly seminars and Ph.D. meetings (twice per year) taking place at the ESB-UCP were followed.

Outreach/Dissemination activities:

- Open week at the ESB-UCP. 4 – 8 April 2016 (and 19 – 21 April 2017), Porto, Portugal.
  This initiative was intended to allow the students from high school to have contact with different aspects of science through activities planned by researchers.
- Visit to a high school. 20 January 2017, Colégio Luso-Internacional do Porto (CLIP) Porto, Portugal.
  One-hour lecture entitled "*Antibiotic Resistance: Environmental and public health problem*" at the Oporto International school to the 11th grade students. During this lecture, together with ESR1 and ESR10, we talked about the antibiotic resistance

concept and the spreading of antibiotic resistance in the environment (e.g. wastewater) and what ANSWER is about.

- Video clip. June 2017, ESB-UCP, Porto, Portugal.
  A 3 minutes video was created. This activity was initiated by the ESB at the UCP. The video presents the ESR7's lab, his Ph.D. work and its contribution to ANSWER project.
- Cafè scientifique. 30 November 2017, E-learning Café, Porto, Portugal.
  Together with ESR10, 11, 12 and 13, we organized this evening event in a café (E-learning Café Asprela) in order to have an open discussion about the EU ANSWER project, wastewater reuse and risk of antibiotic resistance dissemination.
- Visit to a high school. 07 December 2017, CLIP school, Porto, Portugal.
  One-hour lecture entitled "*Antibiotic Resistance and its dissemination in the environment*" at the Oporto International high school to the 11th grade students. During this lecture, together with ESR11, ESR 12 and ERS13, we discussed about antibiotic resistance, the cycle of water and urban wastewater treatment plants, wastewater reuse and antibiotic resistance in the environment.
- Poster pitch and poster presentation. 20 August 2017, Julius Kühn-Institut (JKI), Braunschweig, Germany.
  The Julius Kühn-Institut Mini-Symposium on Networking and cooperation in microbial ecology was attended. During this mini-symposium, a poster pitch of 3 minutes about the poster entitled: *"Can wastewater antibiotic resistant bacteria survive a healthy human gut microbiome?"* was given.

Also, presentation of the ANSWER project along with the Ph.D. research project "*Evaluation of possible risks of antibiotic resistance transmission to humans by treated wastewater-irrigated crops*" were presented at the ESB-UCP (during Ph.D. meetings), Julius Kühn-Institut, Istituto Superiore di Sanità as well as during several ANSWER meetings.

Scientific publication outside of the Ph.D. thesis:

- Manaia, C. M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I. C., Zammit, I., Kampouris, I., Vaz-Moreira, I., & Nunes, O. C. 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. *Environ Int,* **115**: 312-324. doi.org/10.1016/j.envint.2018.03.044.

- Blau, K., Scaccia, N., Jechalke, S., Sib, E., Bierbaum, G., & Smalla, K. Detection of antibiotic resistant bacteria, human pathogens and transferable resistome of lettuce hydroponically grown in treated wastewater. In preparation by JKI.

Media article:

- Il Progetto Europeo ANSWER: il rischio dell'antibiotico resistenza nel riuso delle acque reflue. Manganelli, M., Scaccia, N., Testai, E. Notiziario dell'Istituto Superiore di Sanità, 32 (2019), pp. 3-6. http://old.iss.it/binary/publ/cont/aprile.pdf.

Main conferences and posters:

- Poster presentation: "*Wastewater antibiotic resistant bacteria survive within a healthy human gut microbiome*". Scaccia, N., Vaz-Moreira, I., Manaia, C.M. MICROBIOTEC'17 conference. 7th - 9th December 2017, Porto, Portugal.
- Poster presentation: "*Can wastewater antibiotic resistant bacteria survive a healthy human gut microbiome?*". Scaccia, N., Vaz-Moreira, I., Manaia, C.M. 17th International Symposium on Microbial Ecology (ISME17). 12th - 17th August 2018, Leipzig, Germany.
- Poster presentation: "*Plant-associated bacteria as potential carriers of antibiotic resistance from the environment to humans*". Scaccia, N., Vaz-Moreira, I., Manaia, C.M. XENOWACII conference. 10th - 12th October 2018, Limassol, Cyprus.
- Oral Presentation: "*Survival of wastewater antibiotic resistant bacterial isolates in a healthy human gut microbiome*". Scaccia, N., Vaz-Moreira, I., Manaia, C.M. 5th International Symposium on the Environmental Dimension of Antibiotic Resistance (EDAR5) conference. 9th - 14th June 2019, Hong Kong.
- Poster presentation: "*Detection of antibiotic resistant bacteria, human pathogens and transferable resistome of lettuce hydroponically grown in treated wastewater*". Blau, K., Scaccia, N., Jechalke, S., Sib, E., Bierbaum, G., & Smalla, K. EDAR5 conference. 9th - 14th June 2019, Hong Kong.

Award:

- In the contest of "Programa de mentorado comendador Armenio Miranda" induced by Frulact Academy, a research proposal of 1 year was submitted in June 2017. Our research work entitled "*May environmental bacteria contaminate vegetables and reach the human food chain?*" was selected along with other 5 finalists and a cash price of 500€ was gain. The idea behind this scientific work was the study of the bacterial internalization by plants. The sprouting germination system developed within this Ph.D. work (Annex IV) was also supported by this scientific competition.

Annex II. Secondment at the Julius Kühn-Institut (JKI)

Title of the project: *Detection of antibiotic resistant bacteria, human pathogens and transferable resistome of lettuce hydroponically grown in treated wastewater.*

JKI supervisors: Dr. Khald Blau, Prof. Kornelia Smalla.

As already mentioned in the introduction, treated wastewater (TWW) might be a source of nutrients and therefore is proposed to irrigate agriculture crops. Similarly, TWW has been suggested as a water source for hydroponics culture (Magwaza *et al.*, 2020). Nevertheless, TWW is a reservoir of ARB or ARGs, often located on MGEs, that might contaminate the human food chain and therefore threaten human health. Thus, leaf vegetables irrigated with treated wastewater may be a vehicle of antibiotic resistance transmission to humans (Chapter 3). Particularly, vegetables such as lettuce which is generally consumed raw, may be a source of antibiotic resistant bacteria and their associated resistance determinants that could be transferred to the gut bacteria (Blau *et al.*, 2018).

The major objective of this work was to unravel the effects of differently treated wastewater (by activated carbon filter, sequencing batch reactor and ozone), used as medium for hydroponically grown lettuce, on the abundance and diversity of ARB, human pathogens and the transferable resistome. Lettuce from hydroponic plant production were grown in a PVC pipe inside a greenhouse system, next to a WWTP in Wolfsburg-Hattorf (Germany) (https://www.bmbf-wave.de/en/1455.php; http://www.hypowave.de/projekt/).

Nutrient solution (Hoagland solution), as well as different types of treated wastewater, were pumped inside tubes to bath the roots of the plants (**Figure II.1**, picture on the top of the diagram). Lettuce and wastewater during three different sampling campaign (July, September and October 2018) were collected (in triplicate) and analysed, based on cultivation-dependent and -independent approaches, to i) enumerate total cultivable bacteria and ARB, ii) exogenous isolation of MGEs and iii) analysis of the ARGs and MGEs. The flow diagram of the experimental design is shown **Figure II.1**.

Lettuce and TWW samples, before microbial and molecular analyses, were processed as previously described (Blau *et al.*, 2018).

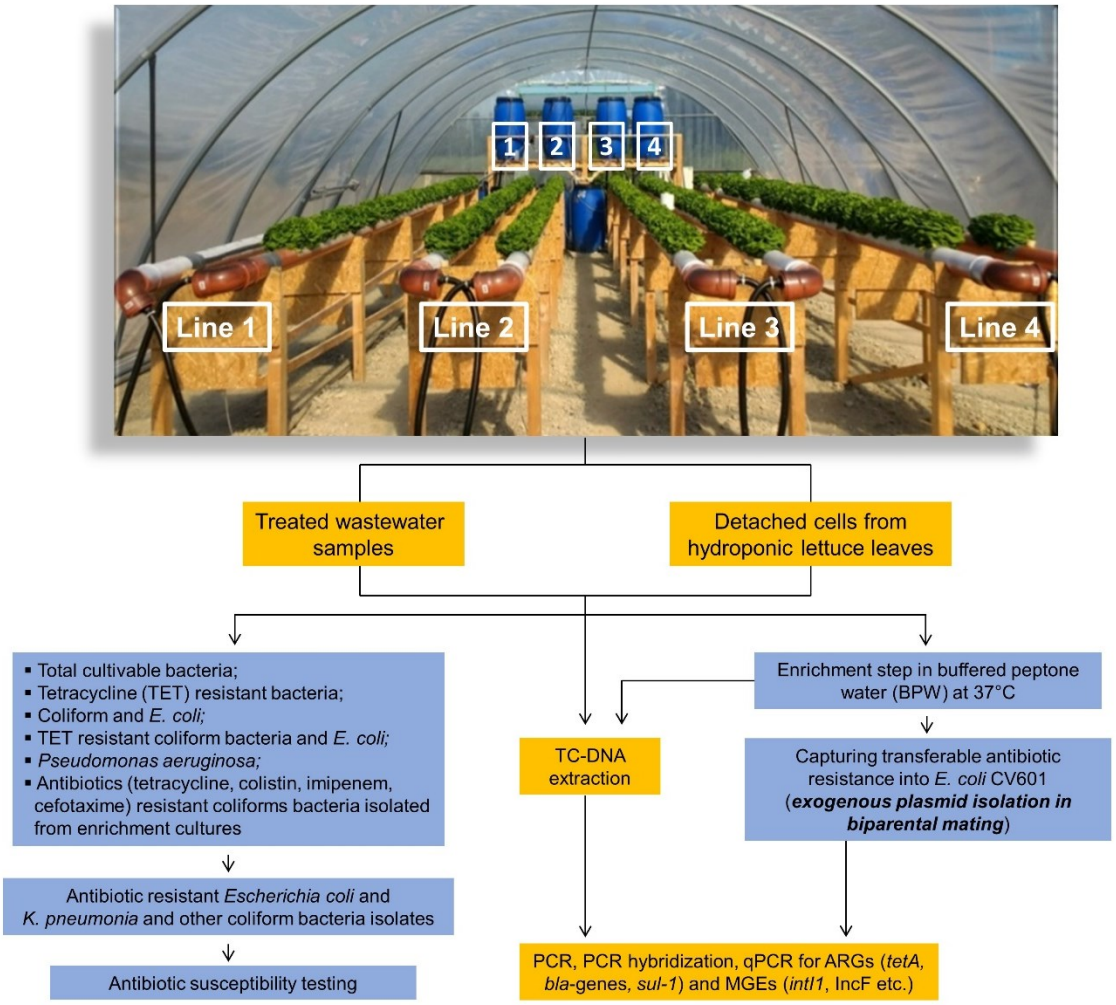

Figure II.1. Experimental workflow used to unravel antibiotic resistance in lettuce and treated wastewater through culture-dependent (blue square) and -independent (yellow square) techniques. Legend: Line 1, Hoagland solution pipeline; Line 2, effluent treated by activated carbon filter; Line 3, effluent treated by sequencing batch reactor; and Line 4, effluent treated by ozone. TC-DNA, total community DNA. This flow diagram was adapted by (Blau *et al.*, 2018).

Specifically, lettuce samples were cut into small pieces and weighted to be used for enrichment culture or, Stomaker treatment to detach bacterial cells from lettuce leaves. Similarly, TWW samples were used for enrichment culture or, to make 10-fold serial dilutions. Subsequentially, enumeration of cultivable bacteria (only directly from samples), isolation of ARB (directly from samples and from the enrichment culture), plasmids isolation (only from enrichment culture), and ARGs and MGEs analysis (directly from samples and from the enrichment culture) were performed.

Total bacteria, coliform and *E. coli* counts were performed on Reasoner's 2A agar (R2A) and Chromocult coliform agar (ChCA) respectively. Enumeration and isolation of ARB were performed using R2A or ChCA culture media supplemented with antibiotics.

Particularly, R2A was supplemented with tetracycline (10 mg/L) and ChCA was supplemented with tetracycline (10 mg/L), cefotaxime (2 mg/L) or colistin (4 mg/L). In order to capture tetracycline or cefotaxime resistance plasmids, exogenous plasmid isolation via biparental mating was performed using kanamycin- and rifampin-resistant *E. coli* CV601, which also harbour the green fluorescence protein (GFP) (Heuer *et al.*, 2002), as a recipient. The transconjugants were determined by green fluorescence, PCR analysis and confirmed by BOX-PCR (Blau *et al.*, 2018).

ARB isolates were characterized by conventional PCR analysis such as *gad*A or *ycc*T gene detection for *E. coli, opr*L gene for *P. aeruginosa* and *glt*A gene for *K. pneumoniae.* Moreover, all the isolates were screened for the presence of ARGs and MGEs by qPCR and PCR-southern blot hybridization. Total community DNA (TC-DNA) directly from samples and from enrichment culture was extracted and ARGs (i.e. *tet*A, *sul*1 and bla genes) and MGEs (i.e. *intl*1, IncF, IncP-1) by qPCR and PCR-southern blot hybridization were analyzed.

As main conclusions, first of all, antibiotic resistant coliforms bacteria (i.e. *E. coli*, *Klebsiella pneumoniae*) were isolated mainly from enrichment cultures of lettuce leaves. Also, analysis of ARGs and MGEs were conducted by PCR, qPCR and PCR hybridization. The gene *tet*(A) was detected by PCR in TC-DNA extracted from hydroponic lettuce before and after enrichment culture. However, PCR hybridization techniques, have shown higher sensitivity compared to conventional PCR. Additionally, MGEs, as well as ARGs, were detected in TC-DNA extracted from enrichment cultures suggesting that the leaf-associated transferable resistome became detectable only after enrichment culture, indicating a low abundance in the microbiota of lettuce leaves. Finally, it was also investigated the presence of transferable plasmids by exogenous plasmid capture using *E. coli* CV601 as recipient. *E. coli* CV601 transconjugants with acquired tetracycline or cefotaxime resistance from hydroponic lettuce and treated wastewater were obtained by bi-parental mating. Albeit this work is still ongoing at the JKI, these initial results indicate that lettuce hydroponically grown in TWW might be a source of transferable resistances and potential threats for human health which should deserve further assessment.

Annex III. Secondment at the Istituto Superiore di Sanità (ISS)

Title of the project: *Preliminary application of predictive food microbiology models to data on survival of antibiotic resistant bacteria and antibiotic resistance genes in the human gut microbiome.*

ISS supervisors: Dr. Maura Manganelli, Dr. Emanuela Testai.

The aim of this secondment, in collaboration with the Istituto Superiore di Sanità, was the use of predictive microbiology models to develop a possible model to predict the survival of ARB within the human gut microbiome. Moreover, this approach might help future design experiments about the adequacy of measuring the survival of ARB rather than ARGs quantification or vice versa. For that, the Predictive Microbial Modelling Lab (PMM-Lab) (https://foodrisklabs.bfr.bund.de/pmm-lab/) (Tenenhaus-Aziza and Ellouze, 2015) open-source software was selected. PMM-Lab is an open-source extension to the Konstanz Information Miner (KNIME) Analytics Platform software (https://www.knime.com/). PMM-Lab consists of three components: i) a library of KNIME nodes (called PMM-Lab), ii) a library of "standard" workflows and iii) a database to store experimental data and microbial models (https://foodrisklabs.bfr.bund.de/pmm-lab/). Users can apply PMM-Lab to proprietary or public data and create bacterial growth/ survival/ inactivation models. The framework can easily be extended to other model types, e.g. growth/ no-growth boundary models. PMM-Lab has been initiated and provided by the German Federal Institute for Risk Assessment – BfR (Berlin, Germany). Among others, PMM-Lab was chosen because of its free accessibility and versatility. Additionally, PMM-Lab software provided several modules such as database browser, growth/no growth predictor, growth fitting tool, inactivation fitting tool, growth predictor, and inactivation predictor (Tenenhaus-Aziza and Ellouze, 2015).

Predictive microbiology aims at describing microbial behaviour in different environmental conditions to prevent food-borne illnesses as well as products safety and shelf-life. Effects of specific conditions on bacterial growth, survival, and inactivation in food matrices (food and storage properties) can be predicted by quantitative mathematical models that combine the effects of environmental parameters on bacterial behaviour. A great variety of

mathematical models exists to describe the growth, survival or inactivation of microorganisms population in foods as well as in fluids. The main classes of models referred to primary and secondary models. Primary models describe the microbial evolution, exposed to a specific condition such as temperature, pH, *etc*. as a function of time. So, they can be applied to estimate kinetic parameters, for instance, growth or death in the population of microorganisms. Secondary models describe how the kinetic parameters are influenced by environmental conditions. For instance, they express how the bacterial growth of a specific bacteria population changes with the increase (or decrease) of temperature. Finally, tertiary models integrate primary and secondary models to predict bacterial parameters according to the level of independent variables in secondary models. Therefore, mathematical models might be an easy and quick way to simulate different scenarios in food safety without run those conditions in real situations - i.e. what happens to a specific microorganism in a determinate food under different storage temperatures. Following the same knowledge used in the predictive microbiology, we wanted to simulate the behaviour of wastewater ARB within the human fecal microbiome under different conditions such as presence or absence of oxygen as well as presence or absence of antibiotics.

Initially, we inserted our experimental data obtained (CFU/g or ARGs copy per g of dry weight) (Chapter 5) in the software and then we fitted our data with formulas (provided by the software) in order to determine the best-fit model for each microbial data set (bacterial growth on different culture media (LA, mFC, and m-Enterococcus) or ARGs). We observed that several primary models fitted our microbial data set and decided to use the one that significantly fitted the highest number of experimental conditions. Once the primary model is assigned, a secondary model needed to be found in order to consider the effects of our environmental parameters (oxygen and antibiotics). However, in our case we have only binary variables (presence or absence of oxygen; presence or absence of cefotaxime; presence or absence of vancomycin) and, among the secondary models available in the PMM-Lab database, none of the models take into account only three binary variables. Nevertheless, the software allows the insertion of equations to create a secondary model based on specific data. So far, we tried the equation y = a*oxygen + b*Cefotaxime, for *E. coli* A2FCC14 and its ARGs, which gave a low R-square value, meaning that a different model should be applied. However, these preliminary results show the applicability of such models to fields different from food safety and the relative simplicity and versatility of the software used. A deeper knowledge of the literature on secondary model and a short training

course to use the software in the most appropriate way will allow to design the best predictive model on our data.

Annex IV. Sprout germination assay to assess wastewater antibiotic resistant bacteria internalization in plant

To explore the ability of bacteria to enter the plant, we implemented a sprout germination assay. In this experiment, the environmental isolate *Escherichia coli* A2FCC14 (previously characterized from our lab as a multi-drug resistant strain) (Chapter 5) and the probiotic strain *Lactobacillus plantarum* NCFB 1752 isolated from pickled cabbage (Couto and Hogg, 1994) were used as model bacteria.

Alfalfa and Adzuki bean seeds purchased at the market were germinated in the presence or absence of the model bacteria. Seeds were disinfected by immersion in a bleach aqueous solution at 50% (v/v) and subsequently incubated overnight with the bacterial suspension (10^7 cell density for each strain) or only with sterilized water, as a control. Seeds were then germinated in a sterilized glass jar containing wet filter paper at room temperature in the dark until sprouts were obtained (5-7 cm). Once per week, seeds were wet with 2 mL of sterilized tap water. Alfalfa sprouts were obtained after 5 days while Adzuki bean sprouts a month later were produced. To check the presence of the test bacteria inside the plant, plant-surface disinfection of Alfalfa and Adzuki bean sprouts were performed. Particularly, Alfalfa sprouts were disinfected using 70% (v/v) ethanol followed by 25% (v/v) bleach solution, while Adzuki bean sprouts were disinfected using 36% (v/v) formaldehyde solution and 1M sodium hydroxide solution. Thereafter, in order to remove the disinfectants residues, the sprouts of both seeds were washed 3 times with sterile distilled water. To test the effectiveness of the disinfection process, seedling plants were plated directly onto Plate Count Agar medium, mFC medium and De Man, Rogosa and Sharpe agar (MRS) medium to enumerate total bacteria, *E. coli* A2FCC14, and *L. Plantarum*, respectively. All cultures were incubated at 37 °C per 72 hours. The absence of bacterial growth meant sprouts were successfully disinfected. The presence of the model bacteria in the endophytic community, was targeted by culture-dependent method. Disinfected sprouts were smashed with 9 mL of sterile saline solution (0.85% (w/v) NaCl) and the sprouts slurry was plated in the respective culture medium.

To confirm the ability of model bacteria to enter the plant, two different seeds were used. Alfalfa seeds germinated in the presence of *E. coli* A2FCC14, *L. plantarum* NCFB 1752 or in absence of bacteria were disinfected using 70% ethanol and 25% bleach. After sprouts surface disinfection with the latter process, the sprouts were smashed, and the slurries

obtained were plated in the appropriate culture medium. The no smashed sprouts, after disinfection treatment, were also plated directly onto Plate Count Agar medium to test the efficiency of the disinfection process. Bacterial growth was observed suggesting the inefficiency of this process to disinfect the plant surface.

A second plant-surface disinfection treatment using 30% formaldehyde and 1 M sodium hydroxide was applied to the Adzuki bean sprouts that were germinated in the presence of test bacteria. This process proved to be an efficient method to disinfect the surface of the plant with no bacterial growth being observed when the no smashed sprouts were plated directly onto Plate Count Agar medium. Although *L. plantarum* NCFB 1752 was not detected as endophyte in the disinfected adzuki bean sprouts, *E. coli* A2FCC14 was detected in the sprout slurry suggesting the ability of this strain to enter the plan (**Figure IV.1**).

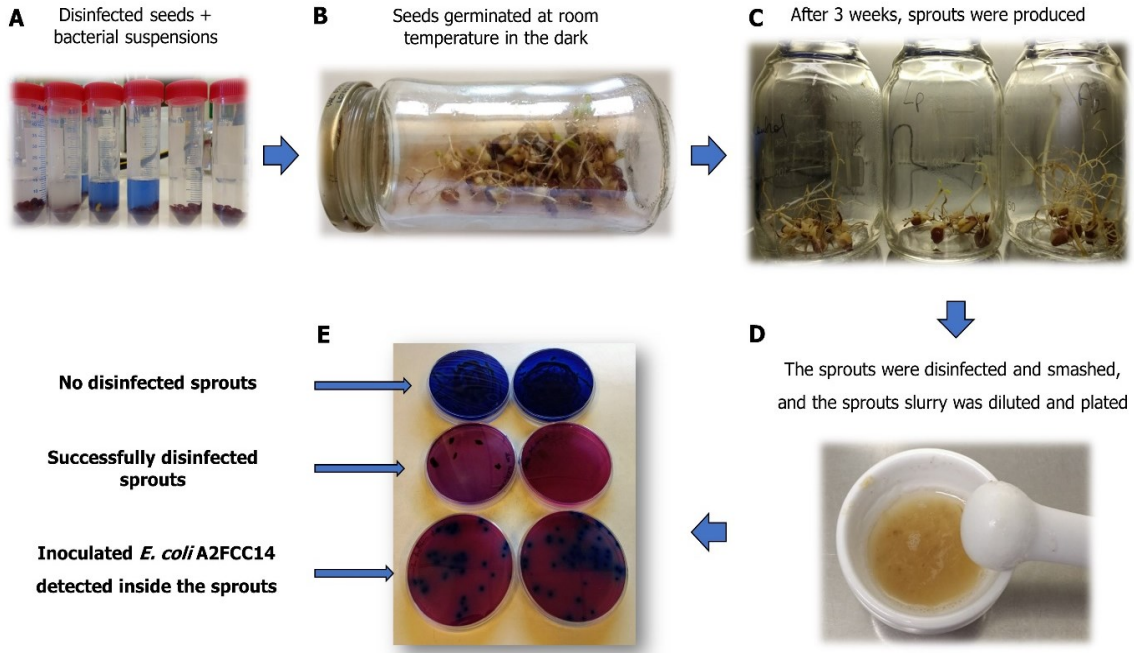

Figure IV.1. Schematic representation of the sprouting system approach. The picture E shows the presence of *E. coli* A2FCC14 colonies detected as endophytic bacteria.

To test whether bacterial isolates such as *E. coli* A2FCC14 and *L. plantarum* NCFB 1752 may enter the plant, a sprouts germination system was developed as a model tool. We observed that *E. coli* A2FCC14 was able to grow onto the sprout surface and entered the plant, using the azuki beans seeds. Although the disinfection procedure of the sprouts was a critical step, the application of 30% formaldehyde and 1 M sodium hydroxide proved to be an efficient method for plant-surface disinfection. Alcohols and hypochlorite are usually used as a disinfectant in food processes and both disinfectants are effective against a broad

spectrum of microorganisms and easy to use (Wirtanen and Salo, 2003). However, they are microbiostatic and do not prevent bacterial adhesion. We used a high bacterial inoculum (around 10^7 bacterial cells) in our studies to explore the ability of bacteria uptake by the plant. The test bacterium used (*E. coli* A2FCC14) was able to survive on the surface of the plant for at least a month and enter it. In the near future, further experiments are needed to establish this system and to confirm the ability or not of *L. plantarum* NCFB 1752 to enter the plant. Once the disinfection method was established and due to limited time, we focused our efforts to study the "worst" case of plant-bacteria interaction such as human pathogen uptake by edible plants. Albeit plant-bacteria interactions are highly species specific and a myriad of other factors influence the interplay among bacteria and plants, this approach may aid to further explore the possible role of plants, particularly raw-eaten vegetables, as vehicles of harmful bacteria transmission to humans.

9. References

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