The risk of antibiotic-resistance transmission through endophytic bacteria

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Abstract

Antibiotic resistance is a global human health threat distributed across humans, animals, plants and the environment. Under the One-Health concept (humans, animals and environment), the contamination of water bodies and soil by antibiotic resistant bacteria cannot be dissociated from its potential transmission to humans. Edible plants can be colonized by a vast diversity of bacteria, representing an important link between the environment and humans in the One-Health triad. Based on multiple examples of bacterial groups that comprise endophytes reported in edible plants and that have close phylogenetic proximity with human opportunistic pathogens, we argue that plants exposed to human-derived biological contamination may represent a path of transmission of antibiotic resistance to humans.

Edible plants as potential vectors of antibiotic resistance

Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are distributed across all the One-Health interconnected compartments, comprised by humans, animals, plants, and the natural environment [1]. ARB and ARGs have been considered contaminants of emerging concern, whose origin is due to the extensive use of antibiotics in human medicine and animal production, and whose control is impaired by the ubiquity and fast-propagation of bacteria that are able to thrive and adapt to a wide array of environmental conditions [1-4]. Although ARB pathogens are mostly reported in clinical episodes, mostly in association with other morbidities, the human colonization by ARB may occur before and outside the health care context [5-7]. Although much investigation is still needed to unveil the role of the environment on the transmission of ARB to humans, the human-food chain has been regarded as a crucial path of transmission [1, 8-10]. Plant-based products, mainly when consumed without processing (e.g., raw vegetable salads) have been suggested as possible sources of transmission of pathogens, including ARB, from the environment to humans [11-14]. An emblematic example was the deathly outbreak caused by the ingestion of bean sprouts that emerged in Germany and spread to several other countries. This outbreak was caused by an *Escherichia coli* strain that presumably suffered genetic recombination in the environment and possibly originated in the farm where the sprouts were being produced [15].

Given the fact that plants are naturally colonized by bacteria thriving in the surrounding water and soil environment (Box 1), it is arguable that the enrichment of the environmental resistome with ARB and ARGs contributes to the uptake of these contaminants by plants. Some practices, such as soil amendment with manure or reuse of wastewater for irrigation (Box 2), may contribute to enrich the environmental resistome and consequent transfer to plants, mainly when adequate safety recommendations are disregarded [16, 17]. Previous studies have discussed the risks of ARB and ARGs occurring in irrigation water or manure-based fertilizers being transferred to plants [17-19], leading to the hypothesis that from edible plants these biological contaminants can reach humans through ingestion. This hypothesis has particular relevance for vegetables that are usually consumed uncooked or minimally processed [20]. Although bacteria thriving on the surface of the plant can be removed by adequate washing procedures, this does not apply to endophytic bacteria, whose persistence after washing, or even disinfection, does not prevent the potential transmission to humans [21, 22]. Once ingested, exogenous ARB may colonize the digestive tract of the host, integrating the gut microbiome, where even a transient presence may offer the opportunity of transferring ARGs to the native microbiota (Box 3) [10, 13]. Considering this hypothesis, we explored the diversity of endophytic bacteria that have been reported in edible plants, with a special focus on bacterial groups that have been described as part of the human microbiome and that harbor acquired ARGs. As a major link between plants and humans we also considered the wastewater microbiome. This option relies on the fact that wastewater is simultaneously a repository of the human microbiome resistome [23] and, mainly in world regions under water stress, it is increasingly used in agriculture for irrigation, after duly treatment [11, 19, 24].

Based on this rationale, we searched the literature and databases for bacterial groups and associated ARGs occurring simultaneously in wastewater, as plant endophytes, and in the human microbiome. The goal was the identification of bacterial groups that might represent possible vectors of transmission of antibiotic resistance from the environment to humans, through edible plants. Because such risks of transmission are higher in plants consumed unprocessed, we paid special attention to plants that, based on the information available, represent likely vectors of ARB transmission from the environment to humans.

Context and approach

We searched the public databases PubMed and Google Scholar for scientific publications meeting the keywords “endophytic bacteria”, “bacterial endophytes”, “communities of endophytes”, or “bacterial communities and vegetables”, published between 1995 and 2017. Additional scientific publications were retrieved from that references list. Because endophytic bacteria is the focus of this opinion, we only considered research papers where microbiological surveys followed the surface-sterilization of the parts of the plants to be examined. From this preliminary literature screening we compiled 67 publications, most describing the identification of bacterial isolates based on 16S rRNA gene sequence analysis and other six reporting the 16S rRNA gene amplicon-based (pyrosequencing) community analysis in lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), tomato (*Solanum lycopersicum* L.), grapevine (*Vitis vinifera* L.), soybean (*Glycine max*), wheat

(*Triticum aestivum*) or populus (*Populus deltoides*) (see online Supplemental Information Table S1). Based on this procedure we built a list of endophytic bacteria belonging to 22 distinct phyla and reported in a total of 49 plant varieties (see online Supplemental Information Table S1). We narrowed this list based on two additional criteria - the plant list comprised only varieties in which at least a part of the plant is edible, and for which endophytic bacteria were reported in at least two publications. This filter shortlisted 11 edible plants (banana (*Musa* sp.) cv. Grand Naine), bell pepper (*Capsicum anuum*), black pepper (*Piper nigrum*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), ginseng (*Panax ginseng* C.A. Meyer), lettuce, onion (*Allium cepa*), radish (*Raphanus sativus*), sugarbeet (*Beta vulgaris*), and tomato (*Solanum lycopersicum*), where we searched for endophytic bacterial groups that appeared in at least 5 publications, to attest that it was not an occasional event (see online Supplemental Information Table S2). Based on these criteria we compiled a list of eighteen endophytic bacterial groups, whose presence was further screened in edible plants that can be consumed raw (Figure 1). Considering our aim of studying bacteria that plants can uptake from the environment, we decided to focus on lettuce, carrot, radish, cucumber, and tomato, all edible plants frequently consumed raw, in which the uptake of contaminants of emerging concern was reported before [25]. Once we had defined the bacterial groups found in the edible plants of interest, we searched for the same bacterial group in wastewater (raw, treated wastewater or activated sludge) and in the human microbiome (Human Microbiome Project Catalog https://www.hmpdacc.org/catalog/). In parallel, we investigated the bacteria belonging to those groups regarding the ARGs (comprehensive antibiotic resistance database, CARD - https://card.mcmaster.ca/analyze/rgi and antibiotic resistance genes database, ARDB - https://ardb.cbcb.umd.edu/index.html) and the potential to cause disease (based on the literature) (Figure 1; Table S2). Aiming to get additional insight into the virulence and resistance potential of endophytic bacteria of the groups under study, we examined the whole genome sequences of endophytic strains of *Acinetobacter* sp., *Enterobacter* sp., *Pseudomonas* sp. and *Staphylococcus* sp. (available at the genome database of the National Center for Biotechnology Information -

https://www.ncbi.nlm.nih.gov/) (Figure 1). The genomes were inspected using the databases CARD and ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) and RAST (http://rast.theseed.org/FIG/rast.cgi) for annotation and metal resistance genes (MRGs) screening.

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

Our literature survey demonstrated that endophytic bacteria comprises an impressive phylogenetic diversity. Not surprisingly, the groups most commonly reported as endophytes comprise those described as among the dominant in soil and wastewater habitats [16, 26]. Those were represented by members of the phyla *Proteobacteria* (classes *Gamma*-, *Alpha*- and *Betaproteobacteria*), *Firmicutes* (classes *Bacilli* and *Clostridia*), *Actinobacteria* (class *Actinobacteria*), and *Bacteroidetes* (classes *Flavobacteriia* and *Chitinophagia*) (see online Supplemental Information Table S1).

Endophytic bacteria in edible plants

According to our search criteria, endophytic bacteria observed in edible plants belong to 17 bacterial genera (*Enterobacter*, *Serratia*, *Erwinia*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Burkholderia*, *Agrobacterium*, *Bacillus*, *Staphylococcus*, *Paenibacillus*, *Microbacterium*, *Kocuria*, *Arthrobacter*, *Micrococcus, Chryseobacterium*) and one bacterial family (*Oxalobacteraceae*) (Figure 1, see online Supplemental Information Table S2). These groups include bacteria that can be described as mesophilic, facultative aerobes, non-fastidious, with fast growth, wide metabolic versatility, and recognized genome plasticity [27, 28]. These features explain the ubiquitous character that justifies their occurrence in soil, plants, waters, sewage, or animal and human bodies [27, 28]. These same features explain the role of these bacteria as plant symbionts, growth promotors, xenobiotic degraders, as well as human, animal, or phyto-pathogens [27, 28]. Different roles can indeed be observed in the same genus, whose members can share ecophysiological properties and, therefore, the range of habitats where they can thrive. The diversity and characteristics of endophytic bacteria observed in edible plants suggest that depending on the pristine or polluted nature of the environment where the plant is growing it can uptake innocuous or ARB/pathogenic bacteria, respectively. Although it has been demonstrated that the uptake of bacteria is influenced by the interplay between soil-plant-nutrients [29], highlighting the possible selective role of the plant [30], the understanding of the uptake mechanisms is still scarce [31]. It is however important to note that most studies on endophytic bacteria explore the beneficial effects for the plant, leading to the consensus that these bacteria may provide stress tolerance, and pathogen and disease resistance [32]. However, the fact that bacteria host human-clinically relevant features such as ARGs or virulence genes, is not *per se* an impediment of a beneficial role for the plant. Indeed, both roles are in principle compatible in the same organism, which brings an interesting contraposition that has been also discussed for probiotic dietary supplements [33]. The uptake of bacteria harboring ARGs due to irrigation with wastewater or other types of soil contamination has been consistently demonstrated [34-36]. The factors that rule the uptake of ARB or ARGs as endophytic bacteria and the risks they may represent for human health are novel and timely topics to which the multidisciplinary insight from plant science, microbiology, ecology, physiology, and genomics may bring relevant clarifications and control measures.

Potential overlaps between endophytic and wastewater bacterial communities

Wastewater results from human activities, where human excreta is the major microbial source [37]. Wastewater treatment, till recently regarded only as a major protection for the environmental and human health, is nowadays also considered a pivotal process to simultaneously meet “human water security and ecosystem sustainability” [38]. The recycling of wastewater, especially for agriculture, has important economic and environmental benefits. For example, treated wastewater reuse can supply agriculture crops with organic and inorganic matter and micronutrients, which can reduce the use of fertilizers as required to meet sustainability goals. However, if adequate safety and quality standards are not met [16, 39], water reuse may lead to the contamination of water bodies, soil, or plants by contaminants of emerging concern, in which ARB and ARGs are included (Box 2) [16]. Treated wastewater holds a wide phylogenetic and ecological diversity of environmental-, human- and animal-derived bacteria, where *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia* are reported as predominant community members [16, 26]. 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* are also among the most cited endophytic bacteria we observed in our study (Figure 1; see online Supplemental Information Table S1). Curiously, our search showed that, also at lower taxonomic ranks, the most common endophytic bacteria are also detected in wastewater [26, 40-55] (Figure 1; see online Supplemental Information Table S2). This observation supports our hypothesis that wastewater can be a source of bacteria of human origin that directly through irrigation or indirectly through soil contamination can reach plants, where they become part of the endosphere. Considering that endophytic bacteria survive in harvested or non-actively growing plants, an assumption that receives good support from the literature [56, 57], the next question in our reasoning is if they can be transmitted to humans.

Potential overlaps between endophytic and human microbiota

Considering the human microbiome, the digestive tract holds the richest and most diverse assembly of microorganisms, with essential roles in the host metabolism or disease control [58, 59]. The human gut microbiome is shaped mainly through diet, in which water, fermented foods, or raw vegetables are important sources of bacteria (Box 3) [60, 61]. Giving support to our hypothesis we observe that 15 out of 18 endophytic bacteria reported in edible plants have been also described in the human microbiome (i.e., gastrointestinal and urogenital tract, oral cavity, skin, and airways) (Figure 1; see online Supplemental Information Table S2). Specifically, when we screened the human microbiome catalogue (https://www.hmpdacc.org/catalog/) for the 18 endophytic bacteria genera and family reported above, we retrieved most of the groups - *Enterobacter*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Burkholderia*, *Bacillus*, *Staphylococcus*, *Paenibacillus*, *Microbacterium*, *Kocuria*, *Arthrobacter*, *Micrococcus*, *Chryseobacterium*, and *Oxalobacteraceae*. Hence, we have evidences that members of these groups can thrive in both plants and humans, supporting our original hypothesis that endophytic bacteria have the potential to colonize the human body. Although this is an issue deserving further research, we realize that the same rationale has been discussed in different contexts. Lang *et al.* [61] estimated that during a vegan meal (fruits, vegetables, and whole grains) 10^6 bacterial cells can be ingested and listed the 50 most abundant bacterial families present in those vegan meals. Curiously, the bacterial endophytes that we report above are part of the Lang's *et al.* list, which includes members of the families *Enterobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Bacillaceae*, *Staphylococcaceae*, *Paenibacillaceae*, *Micrococcaceae*, and *Flavobacteriaceae* [61].

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

The antibiotic resistome of the plant microbiome has been described as the set of ARGs present in endophytic and phyllosphere bacteria [10]. Despite the scant information available, the role of the plant microbiome in the spread of antibiotic resistance has been discussed recently [10, 14, 62]. For example, bacteria from the lettuce phyllosphere have been reported as a source of multidrug resistant bacteria, transferable plasmids and/or ARGs [12-14, 17]. Also, the use of antibiotics to control food crops disease has been suggested as a driver for the occurrence of acquired antibiotic resistance in plant-associated bacteria [62, 63]. For instance, the long-term application of streptomycin in agriculture has been suggested as a possible selective pressure for the development of plant-associated pathogenic bacteria, such as members of the species *Erwinia amylovora*, *Pseudomonas syringae* or *Xanthomonas campestris* that harbour streptomycin resistance mechanisms either associated with the transposon Tn*5393* or due to chromosome mutations [62]. However, antibiotic resistance may be intrinsic in bacteria, as is frequently observed in environmental bacteria. Therefore, while intrinsic resistance represents a minimal risk of intercellular dissemination, acquired antibiotic resistance, which is frequent in wastewater, represents a high risk of dissemination and propagation, as ARGs may be propagated through horizontal gene transfer in a microbial community [64]. This effect may be enhanced in the presence of antibiotics, leading to the overgrowth of bacteria harbouring ARGs that represent a competitive advantage in a given environment [64]. As the genetics and ecology of antibiotic resistance have shown, acquired resistance traits may become stable in the microbial genome without representing additional costs for the cell, even in the absence of any selective pressures [4]. This process can occur in any environment where bacteria can survive, including the plant endosphere [10, 64]. The array of antibiotic resistance genes that we observed in bacteria living as endophytes suggest their possible role as vectors of transmission of ARGs from plants to humans (see online Supplemental Information Table S2). Particularly relevant in this respect is the finding of members of the genera *Enterobacter*, *Acinetobacter* or *Pseudomonas* as endophytes when the role of members of these groups as ARGs carriers is very well known, comprising resistance against different classes of antibiotics, specifically aminoglycosides, beta-lactams, macrolides, quinolone, sulfonamides or tetracyclines (see online Supplemental Information Table S2). An important fraction of ARGs described in bacteria belonging to the group of endophytes we report above, may be part of the intrinsic resistome. However, this seems to be not the case for all reported ARGs. Interesting examples are the ARGs associated with **mobile genetic elements (MGEs)** (e.g., *amp*C, bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC}, bla_{OXA48} [65]). The latter include essential platforms of ARGs dissemination and are particularly rich in bacteria with dynamic genomes, as is the case of members of the genera *Enterobacter*, *Acinetobacter* or *Pseudomonas,* or others of the endophytic bacterial groups that we reported above. Aligned with our hypothesis, we find support to consider that endophytic bacteria present in edible plants may transfer their ARGs to the bacteria of the human microbiome. To further support this hypothesis, we investigated the genome of selected endophytic bacteria.

Inspection of the genome of endophytic bacteria

To get additional insight into the invasion and resistance potential of endophytic bacteria, we analysed several whole genome sequences. Because they were among the most cited in the literature, present in humans and in wastewater and also closely related with priority pathogens, we selected members of the genera *Enterobacter*, *Acinetobacter*, *Pseudomonas* and *Staphylococcus* (Figure 1; see online Supplemental Information Table S2). Specifically, we analysed 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 (Table 1) in which we screened ARGs and metal resistance genes (MRGs). This analysis reveals 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 bacteria/plant interactions such as plant colonization, plant toxin resistance or plant bacterial virulence [66]. Examples of efflux pumps observed in endophytic bacteria were the MexAB-OprM, MdtABC and MdtUVW under the resistance–nodulation–cell division (RND) superfamily, in *Pseudomonas syringae* or in *Erwinia amylovora*, respectively [66]. We also identified the ARGs *bla*$_{\mathrm{z}}$, *erm*C, *fos*B and *mph*C encoding resistance to beta-lactams, macrolides, and fosfomycin in *Staphylococcus hominis* RIT-PI-k (Table 1). The association of these genes to plasmids in *Staphylococcus* spp. was reported before [65, 67]. Additionally, we also observed that the genomes of strains of the genera *Staphylococcus*, *Enterobacter* and *Acinetobacter* presented MRGs encoding arsenic and chromium resistance (Table 1). Although these MRGs might be responsible for intrinsic resistance, the co-localization of ARGs and MRGs on the same mobile genetic element may promote horizontal transfer under the crossed selective pressure exerted by metals or antibiotics, respectively [67].

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

Some endophytic bacterial groups that we highlight in this opinion are phylogenetically closely related to the ESKAPE group (acronym for ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* spp.) which comprises human pathogens responsible for difficult-to-treat infections, frequently due to multidrug resistance [68, 69]. The demonstration that the same bacterial groups may exist in edible plants, wastewater and the human microbiome is only the first preliminary step to discuss the possible associated risks. The risk of edible plant endophytes to humans is influenced by a complex interplay of factors, such as (but not limited to) the ARB fitness, either in the crop and in the human body, the infective dose, and the health condition of the host [13, 16]. Risk assessment procedures evaluate the risk resulting from 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 the consumption of raw vegetables has been established [13, 70] but the effect of environmental contamination is still unknown. Quantitative microbial risk assessment (QMRA) models that include the food chain supply and consider all the intervention points that can be mitigated are still a major challenge [70]. While QMRA are designed for pathogens, ARB are not pathogens, their infectious dose is unknown, and they may occur at very low abundance, with increased risks only when the conditions for proliferation are favourable. This scenario makes it almost impossible to estimate the probability of infection [71]. 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 in humans asymptomatically for a long time, eventually without being responsible for any disease or symptom [5, 13]. This is one of the limitations to develop a reliable risk assessment for ARB. Another limitation refers to the quantitative analysis of exposure because risk-relevant exposure doses can be far below those that common microbiology and molecular biology tools can detect. In fact, another limitation is the difficulty to determine the ARB infectious dose, which refers to the minimal number of cells needed to develop a successful colonization and infection in the human body [5, 9, 13]. This is also related to the limited understanding of the mechanisms of colonization and infection by ARB and the processes that underlie the transfer of ARGs to commensal and pathogenic bacteria [5, 9, 13]. Despite the uncertainty on the quantification of risks posed by environmental ARB, different types of evidence recommend that barriers must exist to minimize the transmission to humans via food chain. Further insights in this field may lead to recommending novel food-safety controls, in a farm-to-fork perspective. 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.

Concluding Remarks and Future Perspectives

It was hypothesized that endophytic bacteria occurring in edible plants that are consumed unprocessed may act as possible antibiotic resistance vectors from the environment to humans. In our opinion, we conclude that leafy and root vegetables (e.g., lettuce, carrot, radish) and fruit vegetables (e.g., tomato and cucumber), may host bacteria of genera such as *Enterobacter*, *Acinetobacter*, *Pseudomonas* or *Staphylococcus.* Members of these genera include priority human pathogens and have a recognized capability to acquire ARGs, representing a potential human health risk. We argue that plants grown in environments contaminated with ARB of these same genera may represent a (silent) vector of transmission to humans. Indeed, members of the same genera thrive in wastewater, plants endosphere, and human microbiomes. Through evidence available in the scientific literature and public databases, we demonstrate that plant endophytic bacteria, like the ones cited above and also members of the genera *Burkholderia*, *Serratia*, *Stenotrophomonas* or

Bacillus, comprise bacteria that have the potential to move and survive across different One-Health compartments. Edible plants that are normally consumed raw, like lettuce, carrot, radish, cucumber or tomato can be relevant vectors of ARB. Our key message is that depending on the quality and safety of water and soil where edible plants are produced, the products may be safe or represent a microbiological hazard. This opinion aims to be an alert for the need of further research on the risks posed by water or soils contaminated regarding the transmission of ARB to humans.

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Supplemental information

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Glossary

Mobile genetic elements (MGEs): are genetic elements that allow the mobility of DNA fragments, for instance from chromosomes to plasmids or between plasmids, which are responsible for the dissemination of antibiotic resistance and other genes.

Plant microbiome: includes all the microbial genomes that interact either outside (rhizosphere and phyllosphere) or inside (endosphere) of the plant.

Box 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 [72, 73]. The microbial community on the above-ground part of the plant is generally inhabited by phyllosphere bacteria which live on the phylloplane (leaves) or carposphere (fruits) microenvironments, which are exposed to the air microbiota. Phyllosphere bacteria may also originate from soil or water through agricultural practices, such as irrigation or manure application. Endophytic bacteria do not visibly cause any injury to plants and can be isolated from the internal part of plants [74]. Endophytic bacteria enter plants mainly from the below-ground environment, and originate from the soil microbiota and root-associated bacteria (rhizosphere bacteria) [75]. Particularly, soil bacteria can enter the plant through plant root system suggesting the role of soil as main reservoir of plant-associated bacteria [10]. In addition, agriculture practices (irrigation procedure or manure application) may impact both soil and plant microbiota [16].

Box 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 [16]. 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 increasing. Treated wastewater refers to wastewater that has been processed through a physical, chemical and biological procedure to reduce its organic matter (C, N, P) content and should meet the quality criteria to be discharged in the environment [16, 37]. Water reuse can contribute to recycling the natural resources, and it may represent a valid alternative to overcome the unbalance between water demand and water supply [24, 37]. Indeed, treated wastewater is reused in different countries. For instance, Israel, Cyprus, Malta, Gulf Cooperation Council (GCC) countries (e.g., Qatar) have a high reuse rate of treated wastewater mainly for agricultural purposes [16, 24]. Despite all the benefits of wastewater reuse, if adequate practices are not adopted, environmental and human health risks may emerge in the short or long-term. For instance, the high content of salts in wastewater may be responsible for soil salinization, which would be deleterious for agricultural crops [16, 37]. With respect to the human health, if wastewater contains ARB or pathogens, such as bacteria, viruses, protozoa or helminths, the reuse of wastewater can represent a human threat via food chain supply, even if the consequences are not perceived in the short time [37]. The broad diversity of human- and animal-derived bacteria present in treated wastewater can harbour ARGs that might be associated with mobile genetic elements and may disseminate among bacteria [76]. Although the impacts of environmental ARB on humans are still poorly characterized, the likelihood of transmission from treated wastewater to humans, via wastewater-irrigated crops cannot be discarded.

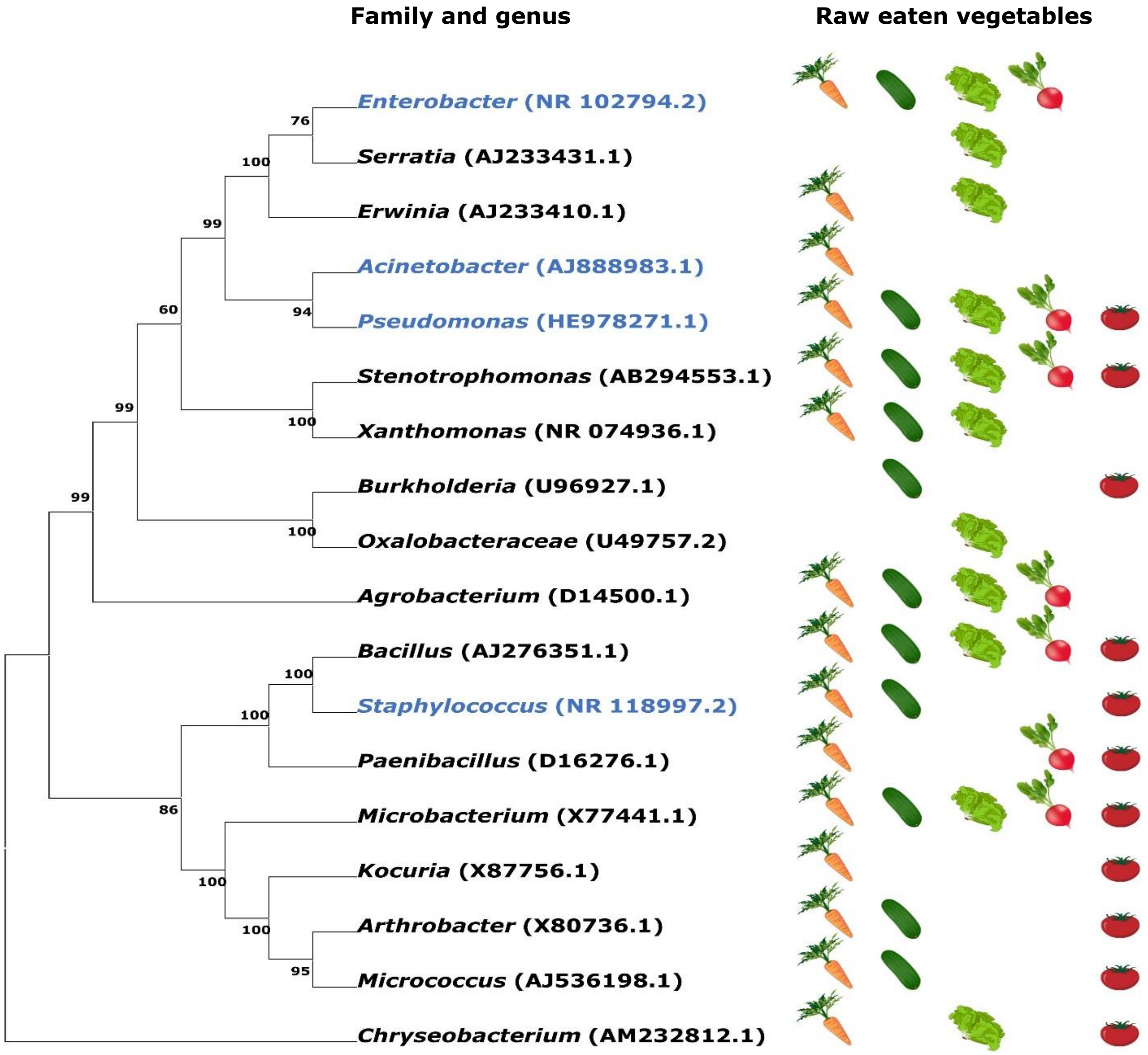
Box 3. A possible link between crops and the human resistome

"Gut microbiota" refers to all microorganisms living within the gastrointestinal tract. This microbial community plays a pivotal role in human health and disease [77]. The gut 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* [58, 59]. The gut microbiota compromises the native (indigenous or autochthonous) members, resident in the gastrointestinal tract and the exogenous (or allochthonous) microorganisms that can transiently colonize the intestine [60]. Diet is an important source of exogenous bacteria which can influence the gut microbiota. For instance, plant-based food products may promote the microbial diversity of the gut or, stimulate the prevalence of beneficial gut-associated bacteria [78]. 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 gut colonization 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 [60]. 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 (e.g., decrease of pH, niche competition or bacteriocins production) and iii) impact the gut microbiota by host response stimulation [60]. Given the high density and nutrient abundance, horizontal gene transfer is a likely process in the gut, with possible implication on ARGs dissemination, for example from exogenous bacteria to the native community [79].

Figure 1. 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. Indicated for the different taxa are the occurrence of antibiotic resistance genes (ARGs) or of members described as human pathogens (P).

Indicated in blue are bacteria closely related to human pathogens.

Note: For the construction of the dendrogram we used sequences representative of each genus/family. Abbreviation: n.i., no information was found.



Family and genus	Habitat	AR and pathogenicity	References
***Enterobacter* (NR 102794.2)**	HM, WM	ARGs, P	[40, 80]
***Serratia* (AJ233431.1)**	HM, WM	ARGs, P	[41, 81]
***Erwinia* (AJ233410.1)**	WM	ARGs	[42]
***Acinetobacter* (AJ888983.1)**	HM, WM	ARGs, P	[43]
***Pseudomonas* (HE978271.1)**	HM, WM	ARGs, P	[44, 82]
***Stenotrophomonas* (AB294553.1)**	HM, WM	ARGs, P	[45, 83]
***Xanthomonas* (NR 074936.1)**	n.i.	ARGs	n.i.
***Burkholderia* (U96927.1)**	HM, WM	ARGs, P	[46, 84]
***Oxalobacteraceae* (U49757.2)**	HM, WM	ARGs	[55]
***Agrobacterium* (D14500.1)**	WM	ARGs	[47, 85]
***Bacillus* (AJ276351.1)**	HM, WM	ARGs, P	[26, 86]
***Staphylococcus* (NR 118997.2)**	HM, WM	ARGs, P	[48, 87]
***Paenibacillus* (D16276.1)**	HM, WM	ARGs, P	[49, 88]
***Microbacterium* (X77441.1)**	HM, WM	n.i.	[50]
***Kocuria* (X87756.1)**	HM, WM	P	[51, 89]
***Arthrobacter* (X80736.1)**	HM, WM	ARGs, P	[52, 90]
***Micrococcus* (AJ536198.1)**	HM, WM	P	[53, 91]
***Chryseobacterium* (AM232812.1)**	HM, WM	ARGs, P	[54, 92]

Table 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.	Refs.	Assembly level	ARGs[1]	Antibiotic	% Identity	MRGs[2]	Metal	% Identity
Staphylococcus hominis RIT-PI-k	Poison Ivy	LHPB00000000.1	[93]	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	[94]	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	[95]	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			
					_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	[96]	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	[97]	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			
					_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	[98]	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	[99]	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**			
					ydhE/norM***	**Q, AG**	**89.4**			
Pseudomonas syringae B301D	Sugarcane	CP005969.1	[100]	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			
					_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**			
					ydhE/norM***	**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.