UNIVERSITY OF MILAN

PHYLOSOPHY DOCTORATE IN

FOOD SYSTEMS

XXXIV CYCLE

Department of Food, Environmental and Nutritional Sciences

ANTIBIOTIC RESISTANCE SPREAD MEDIATED BY HORIZONTAL GENE TRANSFER IN THE AGRI-FOOD ECOSYSTEM

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Academic year 2020-2021

ABSTRACT

Antibiotic resistance (AR) is a public problem for human health and food safety. Globalization has contributed to create an intense connection among human and animal health and the environment, allowing bacteria and their genes to move among all these compartments, making a “one-health approach” necessary to counteract this phenomenon. Horizontal gene transfer (HGT), which contributes to AR determinants diffusion, is mediated by three main mechanisms: i) conjugation, ii) transduction, and ii) natural transformation. Several environments linked to the agri-food system are both sources of AR determinants and hot spots of HGT.

One of the main routes of AR spread in the agri-food system could be represented by the use of treated wastewater as irrigation source: the reuse of water is indeed a common practice in several countries, including Europe, to fight the water crisis exacerbated by global warming. Wastewater treatment plants (WWTPs) could be one of the main sources of free antibiotic resistance genes (ARGs) which could be released in freshwater bodies. AR determinants present in the treated wastewater would thus enter in the food production through irrigation and could be acquired by pathogenic strains, potentially posing a risk to human health. Even if the presence and the related issues about AR determinants in the environment are well known, there are many aspects which have to be understood e.g. the relative contributions of different sources of AR determinants in the environment, also considering HGT events. Since information about the relationship between environmental HGT and spread of AR determinants is limited, the aim of this PhD thesis was to evaluate the diffusion of ARGs through natural transformation and conjugation in environment or in environmental-like conditions to describe several possible routes of AR spread in the agri-food system.

Zooplankton plays a crucial role in waterbodies, being closely linked to bacteria inhabiting aquatic environments in several ecological function, and it

establishes a connection with bacterial communities that are inhabitant of the environment in which it lives. Due to the interaction between bacteria and zooplankton, together with the presence of *Escherichia coli* in waterbodies, derived from human and animal faecal waste, I first evaluated the relationship established between zooplankton, with the model *Daphnia obtusa*, and *E. coli*, isolated from it, suggesting that *Daphnia* could help the bacterium to adapt to the harsh condition that could be found in the freshwater bodies, highlighting the possible role of zooplankton in the diffusion of antibiotic resistant bacteria (ARB) in the agri-food system.

The interaction observed between *Daphnia* and *E. coli* in the first part of this thesis, together with the knowledge of the presence of ARGs in aquatic environment and the moderate ability to *E. coli* to acquire DNA through natural transformation, have thus led to the study of natural transformation in zooplankton-associated bacteria, also in terms to unveil the animal influence. Indeed, I studied the natural transformation of the environmental *E. coli* strain ED1, isolated from *D. obtusa*, mimicking environmental conditions which could be found in the agri-food system. ED1 ability to acquire exogenous DNA, with a higher frequency than the one of a laboratory strain, together with its ability to thrive in lettuce rhizosphere, underlined the importance to investigate the spread of AR determinants in the agri-food system, especially in the rhizosphere of plants which are usually raw-consumed. Moreover, the possible influence of the zooplankton on natural transformation was investigated through the use of *D. obtusa* and *Acinetobacter baylyi* BD413, known to be naturally competent to acquire DNA. A decrease of transformation frequency was observed in presence of *Daphnia*, due to the degradation of exogenous DNA, highlighting the need of further investigations on zooplankton involvement in ARGs diffusion in aquatic environments.

Considering possible routes of diffusion of AR determinants in the agri-food system, i.e. from WWTPs to freshwater bodies and their inhabitant community, to crops and plants, I then devoted my attention on HGT by conjugation in rhizosphere of lettuce, used as model of raw-eaten vegetables.

The aim of this work was the construction of a donor strain belonging to the Enterobacteriaceae family and isolated from treated wastewater. Specifically, *Klebsiella variicola* subsp. *variicola* was genetically manipulated through the chromosomal tagging with a mCherry gene and a constitutively expressed $LacI^q$ gene, and the insertion of the broad host range plasmid pKJK5::gfp::Kan^R, carrying a green fluorescent protein gene (Gfp) under the control of $lacI^q$ repressible promoter, and thus resulting in the absence of gfp expression in the donor. The gfp was expressed only in recipient strains, following the mobilization of the plasmid through conjugation. The strain ability to donate the plasmid within the bacterial community of lettuce rhizosphere and its ability to colonize the plant root system were verified, making *K. variicola* subsp. *variicola* EEF15::$lacI^q$-pLppmCherry-Gm^R with plasmid pKJK5::gfp a perfect candidate for the study of conjugation in plants microniches.

Finally, I contributed to prepare a critical review on microbial assisted phytodepuration and the use of plant growth promoting bacteria in Constructed Wetland (CW) systems, with a focus on HGT events and the possible spread of AR determinants in the rhizosphere of plants used in phytodepuration.

Data collected in this PhD project underline the importance to study the diffusion of AR determinants trough HGT events in the agri-food system, in order to create a rank risk and a risk assessment map to mitigate the diffusion of AR.

RIASSUNTO

La diffusione dei determinanti genetici che rendono i batteri resistenti agli antibiotici è un problema pubblico per la salute umana e la sicurezza alimentare. La globalizzazione ha contribuito ad abbassare le barriere tra ambienti antropizzati e no, permettendo ai batteri di spostarsi liberamente tra questi comparti, rendendo così necessario un approccio indicato come "one health" per affrontare questa problematica. Il trasferimento genico orizzontale (*horizontal gene transfer*, HGT) è mediato da 3 meccanismi principali: i) coniugazione, ii) trasduzione e iii) trasformazione naturale. Diversi ambienti collegati al sistema agroalimentare sono sia fonti di determinanti dell'antibiotico resistenza, sia *hot spot* di HGT.

Una delle vie principali di diffusione dell'antibiotico resistenza nel sistema agroalimentare è rappresentata dall'uso dell'acqua depurata (acqua reflua depurate in un depuratore) per l'irrigazione dei campi. Il riutilizzo di acque reflue depurate per fini irrigui è una pratica comune in molti Paesi, inclusa l'Europa, al fine di contrastare la mancanza di acqua dovuta al surriscaldamento globale. I depuratori potrebbero essere una delle fonti principali di geni che conferiscono la resistenza agli antibiotici (*antibiotic resistance genes*, ARGs), che rilasciati nelle acque superficiali potrebbero poi entrare nel sistema agroalimentare (attraverso l'irrigazione dei campi) e rappresentare quindi un possibile rischio per la salute umana. Nonostante la presenza dei determinanti dell'antibiotico resistenza nell'ambiente e le relative problematiche associate siano ben conosciute, alcuni aspetti relativi a questo fenomeno devono ancora essere compresi, come ad esempio l'identificazione delle principali fonti di ARGs nell'ambiente, considerando anche i fenomeni di HGT. Dato che la diffusione dei determinanti dell'antibiotico resistenza dovuta all' HGT non è ancora completamente chiara, lo scopo di questo progetto è stato quello di valutare la diffusione di ARGs, attraverso trasformazione naturale e coniugazione, in ambienti o in condizioni simili a quelle ambientali,

per descrivere delle possibili vie di diffusione dei determinanti dell'antibiotico resistenza nel sistema agroalimentare.

Lo zooplancton ha un ruolo fondamentale negli ambienti acquatici e interagisce attivamente con la sua comunità batterica. Vista l'interazione che si viene a stabilire tra la comunità batterica degli ambienti acquatici e lo zooplancton, e considerando la presenza di *Escherichia coli* di origine umana (fecale) nelle acque superficiali, per prima cosa in questa tesi è stata valutata l'interazione tra il crostaceo *Daphnia obtusa* e diversi *E. coli* isolati dallo stesso animale, scoprendo che lo zooplancton potrebbe aiutare il microrganismo ad adattarsi all'ambiente acquatico (caratterizzato da condizioni sfavorevoli di crescita per i batteri): ciò inoltre sottolinea un possibile ruolo dello zooplancton nella diffusione dei determinanti dell'antibiotico resistenza nel sistema agroalimentare, che potrebbe portare ad una maggiore diffusione di questi determinanti genici. Una volta verificata la relazione esistente tra *Daphnia* e *E. coli* nella prima parte della tesi, considerando la diffusione di ARGs nelle acque superficiali, e l'abilità di *E. coli* di acquisire DNA mediante trasformazione naturale (anche se moderata), la seconda parte di lavoro di questo progetto è stata dedicata allo studio della trasformazione naturale legata ai batteri assoiciati allo zooplancton e al suo ruolo durante la trasformazione. Inizialmente è stata quindi studiata la trasformazione naturale di un ceppo di *E. coli* (ED1) isolato da *D. obtusa*, riproducendo alcune condizioni che potrebbero essere paragonabili a quelle trovate nel sistema agroalimentare. Lo studio ha dimostrato l'abilità di ED1 di acquisire DNA mediante trasformazione naturale con una frequenza maggiore rispetto a quella riscontrata in un ceppo di laboratorio che, unita alla sua abilità di colonizzare la rizosfera di lattuga, evidenzia l'importanza di studiare la diffusione dei determinanti dell'antibiotico resistenza nel sistema agroalimentare, specialmente nella rizosfera delle piante solitamente consumate crude. Inoltre, l'influenza dello zooplancton sulla trasformazione naturale è stata studiata attraverso l'uso di *D. obtusa* e *Acinetobacter baylyi* BD413, noto per essere costitutivamente competente. In questa parte di

lavoro è stato osservato come lo zooplancton influenzi la trasformazione naturale diminuendone la frequenza, per via della degradazione del DNA esogeno. Questo studio evidenzia la necessità di ulteriori approfondimenti relativi al ruolo che lo zooplancton potrebbe avere nella diffusione degli ARGs in ambienti acquatici.

Considerando le possibili vie di diffusione dei determinanti dell'antibiotico resistenza nel sistema agroalimentare, come ad esempio dai depuratori ai campi e alle piante di interesse agrario, una parte del progetto di dottorato è stata pertanto focalizzata sullo studio della coniugazione a livello della rizosfera di lattuga (usata come organismo modello per i vegetali consumati crudi). Lo scopo è stato quello di costruire un ceppo "donatore" appartenente alla famiglia delle Enterobatteriacae e isolato dalle acque trattate di un depuratore. Specificamente, *Klebsiella variicola* subsp. *variicola* è stato ingegnerizzato, inserendo sul cromosoma una cassetta genica contenente un gene codificante per una proteina rossa fluorescente (mCherry) e il gene $LacI^q$ costitutivamente espressi. Il ceppo inoltre è stato trasformato per l'inserimento del plasmide pKJK5::gfp::Kan^R contenente un gene codificante per una proteina verde fluorescente (Gfp) sotto il controllo di un promotore represso dal prodotto del gene $LacI^q$: comportando la mancata espressione della Gfp nel donatore, la quale invece viene attivata nel batterio ricevente una volta avvenuta la coniugazione. L'abilità di questo ceppo di trasferire il plasmide nella comunità batterica della rizosfera, insieme alla sua abilità di colonizzarla, permette di considerare *K. variicola* subsp. *variicola* EEF15::$lacI^q$-pLppmCherry-Gm^R pKJK5::gfp un perfetto candidato per lo studio della coniugazione nel sistema-pianta.

Infine, nell'ultima parte del progetto di dottorato ho contribuito alla stesura di una revisione critica della letteratura sugli impianti di fitodepurazione e sull'uso di batteri promotori della crescita della pianta, con un approfondimento sul trasferimento genico orizzontale e sulla diffusione dei determinanti dell'antibiotico resistenza nella rizosfera delle piante usate per la fitodepurazione.

In conclusione, i dati ottenuti in questo progetto di dottorato hanno evidenziato l'importanza dello studio della diffusione dei determinanti dell'antibiotico resistenza, attraverso meccanismi di HGT, nel sistema agroalimentare. Tutto questo potrebbe contribuire a creare una mappa di valutazione del rischio relativo alla diffusione dell'antibiotico resistenza.

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Chapter I

Introduction

Antibiotics have revolutionised modern medicine over the past 80 years due to their ability to control bacterial infections. Their use in medicine, plant production and livestock has led microbial communities to the exposure of sub-inhibitory concentrations of these molecules, which could have ultimately driven the rise of resistance in bacteria, trough the onset of mutations and horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs) (Smalla et al., 2018). Due to the continuously increase of antibiotic resistance (AR), nowadays approximately 700,000 people die every year and 10 million death per year are estimated by 2050 (Lim et al., 2019).

Globalization has contributed to create an intense connection among human and animal health and the environment allowing bacteria and their genes to move among all these compartments. Due to the intense interaction between all these compartments, the challenge against AR requires a “one-health approach”. This strategy has been suggested in the action plan of WHO and has been already adopted by several nations, e.g. Sweden and India, and by several pharmaceutical industries. Many ARGs founded in pathogens have been originated from bacteria normally thriving in the environment, which indeed acts as a reservoir and a route of transmission of the pathogens and ARGs (Larsson et al., 2018). AR determinants generated from environmental compartments are now common in human-dominated ecosystems, which have become themselves a source of AR determinants (Zhu et al. 2017; Gillings, 2018).

HGT, which is associated with AR determinants and pathogenicity, is fundamental for bacterial adaptation and evolution. There are three main mechanisms of HGT: i) conjugation, which is the direct cell to cell exchange of plasmidic DNA through specialized conjugal pilus; ii) transduction, which is the DNA transfer mediated by a virus; and ii) natural transformation, which is the acquisition of exogenous free DNA by bacteria in a specific physiological state (Aminov., 2011; Blokesh 2016). HGT events, especially conjugation and transduction, happen with a frequency which depends on several factors: they are usually enhanced in environments with high cell density and high

metabolic activity (Ulrich et al., 2015) that can be found especially in several spots, defined as hot spots (Smalla et al. 2018), characterized by the presence of dense aggregates of cells in which their contact is boosted (Riva V et al., 2020), e.g. biofilm and mycelia (Qui et al., 2018; Berthold et al., 2015).

Several environments linked to the agri-food system are known to be sources of AR determinants (which could reach humans) and hot spots of HGT (Riva et al., 2020a). ARGs in the environment are divided in two different fractions, i.e. internal antibiotic resistant genes (iARGs) and extracellular antibiotic resistant genes (eARGs), which could be free, particle associated or absorbed. HGT of iARGs may happen through conjugation and transduction, while the diffusion of free eARGs through HGT mechanism may occur by natural transformation (Zarey-Baigi and Smith. 2021). Usually, in environments rich of nutrients that allow cell growth, e.g. manure, influent and bioreactor of wastewater treatment plants (WWTPs), iARGs are present with a higher frequency in comparison than free eARGs (Yuan et al., 2019; Dong et al., 2019), while free eARGs are more concentrated than iARGs in environments characterized by a low availability of nutrients, such as aquatic environments and sediments (Mao et al., 2014). Since cells' lysis may be induced, for instance, in the effluent of WWTPs in consequence of the disinfection procedures (Yuan et al., 2019). Due to the different distribution of iARGs and eARGs, the movement of iARGs among bacteria through conjugation and transduction takes place with a higher frequency in "solid environments" such as soil, manure, and sewage sludge, while the spread of free eARGs happens with a higher frequency in water environments e.g. marine and river sediments due to the lysis of cells and because of the possible absorption and inactivation of free eARGs by soil and other solid particles (Zarey-Baigi and Smith 2021).

One of the main routes of AR spread in the agri-food system could be the use of reclaimed water for irrigation purpose. Nowadays the reuse of water is a common practice in several countries, including Europe, to fight the water crisis exacerbated by global warming (Riva et al., 2020). At least 20 million

hectares of croplands worldwide are irrigated with urban treated wastewater (Bouaroudj et al., 2019). Several studies underline that WWTPs could be one of the main sources of cell bound and free ARGs for the main aquatic system (Czekalski et al., 2014; Amos et al., 2015; Zhang et al., 2018); AR determinants present in the treated wastewater would enter in the food production through irrigation and could contribute to the diffusion of ARGs which could be acquired by pathogenic strains, including *Escherichia* coli, which is one of the predominant species forming the microbial community of gut of vertebrates (Martinson and Walk 2020), which can enter and survive in freshwater bodies (Espinosa-Urgel and Kolter 1998),e.g. in lake sediments and phytoplankton (Power, et al. 2005; Ishii, Ksoll, et al. 2006; Ishii, Yan, et al. 2006; Walk, et al. 2007). Daphnia sp. and other zooplakton plays a crucial role in aquatic ecosystem linking phytoplankton and bacteria to higher trophic levels such as fish (Xiong et al., 2020), and they can be used by bacteria as refuge, dispersal agent or as bioreactor (Tang et al., 2010). Interestingly Daphnia continuously filter water, including DNA and bacteria (Eckert & Pernthaler, 2014). The crucial role of zooplankton in freshwater bodies, toghether with the presence of possible pathogens derived form anthropogenized envrionement in freshwater bodies could be considered as a risk in AR diffusion also because it is known that WWTPs can promote the generation and the stabilization of a resistome in the water bodies in which they are released (Corno et al., 2019). Moreover a recent study revealed that crop irrigation, including the use of treated wastewater, could enhance the spread of ARGs in agricultural soil (Kampouris et al., 2021), especially for the ones treated with chicken manure and sewage sludge (Chen et al., 2016). Known to carry AR determinants, possible pathogens such as bacteria of genus *Klebsiella*, of which the multidrug resistant pathogen *Klebsiella pneumoniae* is one representative, have been found in agri-food environments such as soil, water, vegetation and also in domesticated mammals and insects (Wareth and Neubauer 2021). The ability of *Escherichia coli* to survive in environments such as water, soil, manure and microhabitats associated to

plants e.g. roots, endosphere and leaf surface have been demonstrated (Van Elsas et al., 2011; Wright et al., 2017; Eissenberger et al., 2020) and ARBs belonging to the pathogenic species *E. coli* and *Salmonella enterica* have been already reported in farm environments and fresh products. A study showed that the irrigation of lettuce with treated wastewater increased the presence of *E. coli* cells resistant to ampicillin and cephalothin on their leaves (Nüesch-Inderbinen et al., 2015; Araújo et al., 2017; Schierstaedt et al., 2019; Perera et al., 2020; Yang et al., 2020; Summerlin III et al., 2021). Moreover, HGT events are very common in environments related to the agri-food system: for instance, conjugation has been studied in activated sludge biofilm of a WWTP (Qui et al., 2018), in soil (Klumper et al., 2015) and mycelia (Berthold et al., 2016). Moreover, the acquisition of exogenous DNA by different strains of *E. coli* through transduction and transformation has been studied in environmental conditions and in conditions encountered in the food industry such as in the freeze-thaw treatment (Hasegawa et al., 2018). Interestingly many HGT hot spots have been detected in plant microenvironments, such as rhizosphere, phyllosphere and spermosphere (Aminov 2011; Chen et al., 2019; Van Elsas et al., 2003). However natural transformation linked to the spread of ARGs is underestimated (Winter et al., 2021).

Even if the presence and the issues about AR determinants in the environment, including the ones linked to the agri-food system, are well known, there are many aspects which have to be understood in order to help to manage the AR diffusion (Smalla et al., 2018), i.e.:

i) The relative contributions of different sources of AR determinants in the environment. The abundance, the distribution and the activities of microbial communities have been extremely altered by anthropogenic activity in the last 50 years. Therefore, the presence and the dissemination route of AR determinants, e.g. through dust, water, wind and anthropogenic activities such as tourism, agriculture and water management, through different environments are still unclear

and their description and quantification are mandatory to assess a risk model and a rank risk (Smalla et al., 2018; Larsson et al., 2018).

ii) The relationship between antibiotic exposure and the selection of AR in bacterial communities. The identification of high risk environments in which the generation of ARB is favoured, due to HGT and the presence of antibiotic, is mandatory to understand the possible barriers which limit the movement of AR determinants from harmless environmental bacteria to pathogens (Larsson et al., 2018). Several studies suggested that antibiotic selective pressure results in a higher frequency of transduction and natural transformation, the impact of which, linked to the spread of AR in the environments, was underestimated (Zarey-Baigi and Smith., 2021).

iii) The magnitude of consequences on human health due to the exposure to ARB.

iv) The study of different technologies and different behaviours able to counteract the spread of AR (Larsson et al., 2018).

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Rationale and aim of the work

As mentioned in the last part of the **Introduction**, the magnitude of the spread of ARGs through HGT in the environment is not clearly understood (Smalla et al., 2018; Larsson et al., 2018). Since information about the relationship between environmental HGT and spread of antibiotic resistance (AR) determinants is limited, **the aim of this PhD** thesis was to evaluate the diffusion of ARGs through natural transformation and conjugation in environment or in environmental-like conditions in order to identify and describe several possible routes of AR spread in the agri-food system and that may affect human health.

Due to global warming, water reuse is mandatory to minimize water consumption (WEF, 2015). Agriculture is one of the main human water consumption activities (FAO, 2015), therefore the reuse of treated wastewater for irrigation has become a common practice. Wastewater treatment plants (WWTPS) are one of the main sources of AR determinant in water bodies (Corno et al., 2019) and, indeed, treated wastewater used for irrigation could enhance the diffusion of AR in agricultural soils (Kampouris et al., 2021), representing a possible risk for food production.

Treated wastewaters are usually released in rivers and lakes: these freshwater bodies are inhabited by zooplankton that closely interacts with the bacteria of the ecosystems (Tang et al., 2010). Through faecal route *Escherichia coli*, one of the predominant species of the gut microbial community of vertebrates (Martinson and Walk, 2020), is commonly released in aquatic habitats. In the perspective of AR diffusion, the **second chapter** has been dedicated to investigate the relationship established between *E. coli*, an opportunistic pathogen, often resistant to different antibiotics (Araújo et al., 2017), and the model zooplankton animal *Daphnia obtusa*. I tested the hypothesis that the association with zooplankton animals could help the faecal indicator bacterium *E. coli* to adapt to the harsh conditions of a freshwater lake.

Considering the interconnection between *Daphnia* and *E. coli* demonstrated in the second chapter, the **third chapter** has been then devoted to the study

of natural transformation of one of the environmental *E*. *coli* strains previously isolated from *D. obtusa*. Natural transformation is a HGT mechanism, which is less studied than transduction and conjugation, and the magnitude of which could be thus underestimated (Smalla et al., 2018; Zarey-Baigi and Smith 2021). Considering that recent studies have also underlined the moderate ability of different strains of *E. coli* to acquire exogenous DNA in conditions which could be found in different environments (Hasegawa et al., 2018), the aim of this chapter was the study the acquisition of exogenous DNA by the environmental *E. coli* strain ED1 in environmental-like conditions that may occur in the agri-food system. The frequency of natural transformation of strain ED1 was investigated changing different parameters i.e. cell growth phase, amount of DNA and exposure to artificial lake water (ALW) and treated wastewater. Then, the ability of strain ED1 to thrive in the rhizosphere of lettuce, a model plant representative of raw consumed vegetables, was verified.

Taking into account the connection established between zooplankton and bacteria (as reported in the third chapter; Tang et al., 2010), the ability of zooplankton-associated bacteria to acquire exogenous DNA (third and fourth chapter), as well as the presence of eARGs in freshwater bodies and the underestimation of natural transformation magnitude in ARGs spread (Smalla et al., 2018; Zarey-Baigi and Smith 2021), the **fourth chapter** has been devoted to ascertain the influence of zooplankton on the natural transformation frequency of *Acinetobacter baylyi* BD413, well known for the capability to acquire exogenous DNA (Borin et al., 2008; Rizzi et al, 2008; Santala and Santala 2021). Transformation experiments were thus performed in presence and absence of *D. obtusa* in ALW microcosms and were supported by the evaluation of free DNA concentration variation and proteomic analyses on the water of the microcosms to explain differences among treatments.

Considering possible routes of diffusion of AR determinants in the agri-food system, i.e. from WWTPs to freshwater bodies and their inhabitant

community, to crops and plants, in the **fifth chapter** the study has been focused on HGT by conjugation, which is the most favourite HGT mechanism in soil (Zarey-Baigi and Smith 2021). Specifically, I explored gene transfer between a donor strain isolated from treated wastewater and the inhabitant community of the rhizosphere of lettuce, a plant model of raw-consumed vegetables. Aim of this chapter was indeed the construction of a conjugative donor strain of environmental origin and the assessment of its ability to conjugate in environmental like condition (Klumper et al., 2014) and to survive in lettuce rhizosphere.

Finally, in the context of water reuse for irrigation purposes, I co-authored a critical review, presented in the **sixth chapter**, on microbial assisted phytodepuration, a promising technology for water reclamation, and the use of plant growth promoting bacteria in Constructed Wetland (CW) systems, with a focus on HGT events and the possible spread of AR determinants in the rhizosphere of plants used in phytodepuration procedures.

Lastly, the **last chapter** of this PhD thesis presents the conclusions and the future perspectives of this work.

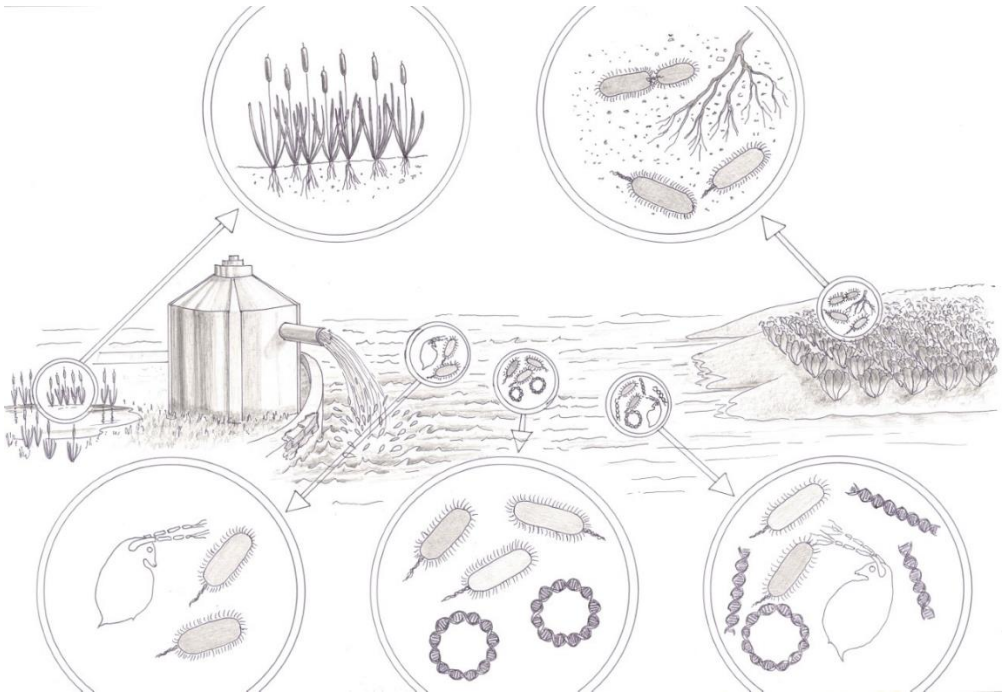

Figure 1: Graphical summary of the study conducted in this PhD project. The main body of the picture represents the journey of treated wastewater, from the WWTPS to the crops. The lenses in the picture represent the different chapters of the PhD project.

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Chapter II

Zooplankton as a transitional host for *Escherichia coli* in freshwater

Abstract

This study shows that *Escherichia coli* can be temporarily enriched in zooplankton in nature and that these bacteria can belong to different phylogroups and sequence types in which are included environmental as well as clinical and animal isolates. We isolated 10 *E. coli* strains and sequenced the genomes of two of them. Phylogenetically the two isolates clustered rather with *E. coli* isolated from poultry meat than with freshwater *E. coli*, albeit their genomes were smaller than those from poultry. After isolation and fluorescent protein tagging of strains ED1 and ED157 we show that *Daphnia* sp. can take up these strains and release them alive again, thus forming a temporary host for *E. coli*. In a chemostat experiment we show that association does not prolong their long-term survival, but that at low abundances it does also not significantly reduce the bacterial numbers. We demonstrate that *E. coli* does not belong to the core microbiota of *Daphnia*, and suffers from competition by the natural microbiota of *Daphnia*, but profit from the presence of pieces of its carapax to survive in water. All in all this study suggests that the association of *E. coli* is only temporary but that the cells are viable therein and this might allow encounters with other bacteria for genetic exchange and potential genomic adaptations to the freshwater environment.

1. Introduction

Faecal bacteria can enter aquatic environments by different routes, e.g., sewage discharge or direct faecal deposition (Korajkic, 2019). Although faecal bacteria are tendentially seen to rapidly drop in abundance once outside host, some aquatic environments might allow their long-term survival or growth (Korajkic, 2019). *Escherichia coli* is one of the predominant species forming the microbial community that shapes the commensal gut of vertebrates (Martinson and Walk 2020). Thus it is commonly released, by faecal route, into aquatic environments (Espinosa-Urgel and Kolter 1998), and it is therefore used as a faecal indicator bacterium (FIB) to evaluate anthropogenic water pollution (Jang, et al. 2017). Clinically relevant *E. coli* strains (Vignaroli, et al. 2013; Jørgensen, et al. 2017), including antibiotic resistant isolates (Vignaroli, et al. 2012; Baniga, et al. 2020; Fernandes, et al. 2020), can be found in waters. Moreover, there is evidence that *E. coli* can adapt to a freshwater lifestyle shown for example through differential gene expression once incubated in water (Espinosa-Urgel and Kolter 1998).

Naturalized *E. coli*, e.g. *E. coli* that entered the aquatic environment from the gut and then adapted to this lifestyle, were isolated from lake sediments and phytoplankton and different studies showed the capability of *E. coli* to genetically adapt and persist in environment for example through encapsulation (Power, et al. 2005; Ishii, Ksoll, et al. 2006; Ishii, Yan, et al. 2006; Walk, et al. 2007). Several *E. coli* isolated from freshwaters had a small genome size and other peculiarities at the genomic level that suggested an evolutionary adaptation to this habitat (Touchon, et al. 2020). This is particularly interesting since genome reduction has been proposed repeatedly as an adaptation to aquatic environments in common environmental bacteria (Grote, et al. 2012; Salcher, et al. 2019) and in experimental systems (Lee and Marx 2012; Baumgartner, et al. 2017). Thus the aquatic environment may contribute to the genetic evolution of *E. coli* (Touchon, et al. 2020).

However, mammal associated faecal bacteria usually persist badly in cold habitats, such as deep lakes. Particularly, pelagic cold waters are a very

hostile environment for such gut symbionts. If they are not grazed by flagellated predators upon arrival, they are surely not favoured by resource competition (González, et al. 1992; Wanjugi and Harwood 2013; Eckert, et al. 2019). Since evolution, as seen in the genome reduction of *E. coli*, takes time, a certain long-term persistence of vertebrate commensal strains in the aquatic habitat is crucial, and the question remains: In which niche does this take place? In clinical settings these bacteria thrive better in biofilms (Costerton, et al. 1999) and they might persist in a similar niche in aquatic habitats (Hall-Stoodley and Stoodley 2005; Eckert, et al. 2018). In lake environment biofilms can be formed on dead organic and inorganic material, sediments and stones or animals, and FIB have been found in sediments (Haller, et al. 2009; John, et al. 2009), macrophytes (Quero, et al. 2015) and on fish (Declerck, et al. 2007; Abgottspon, et al. 2014) for example. Much less attention has been devoted to small invertebrates, i.e. zooplankton, as a potential host for such bacteria. Such animals are interesting since their microbiota seems to be composed by many transient microbes and thus likely more prone to be invaded by allochthonous bacteria (Grossart, et al. 2010; Hammer, et al. 2019; Callens, et al. 2020; Eckert, et al. 2021). In fact, antibiotic resistant bacteria were easily removed from the surrounding water in a laboratory experiment but persisted in the crustacean *Daphnia obtusa* (Eckert, et al. 2016) and FIB have even been shown to even exchange genetic material in *Daphnia pulex* (Olanrewaju, et al. 2019). Despite the presence of *Daphnia* sp. reduces *E. coli* abundance in the water (Burnet, et al. 2017b; Ismail, et al. 2019) we found in a study based on 16S amplicon sequencing data from a lake (Eckert, et al. 2020) that the 16S rRNA gene related *to E. coli/Shigella* made up for a large percentage of the copepod and *Daphnia* microbiota, but was present only with low abundances on stones, water and sediments. In the present study we thus want to investigate the nature of the relationship between *E. coli* and *Daphnia* in freshwater to clarify the possible role that *Daphnia* might have on the persistence of *E. coli* in waters from an ecological point of view.

Here we tested the hypothesis that an association with zooplankton animals of the genus *Daphnia* could help a FIB, *E. coli*, to survive in the harsh conditions of a freshwater lake. It was shown that the presence of *Daphnia pulex* in a few hours reduced the abundance of surrounding *E. coli* (Burnet, et al. 2017a), but here we were interested in the longer-term association of the bacteria with the animal under natural conditions. Our hypothesis is that such an association might help the bacterium to adapt to this environment and even lead to changes in its genome. Therefore, we quantified *uid*A, an indicator gene of *E. coli*, in DNA extracted from various potential niches for FIB from a freshwater lake, including stone biofilms, zooplankton, sediment and compared it to the pelagic water. Moreover, we searched for the occurrence of *E. coli* related reads in a large dataset of zooplankton associated microbiota. We then isolated *E. coli* strains from a *Daphnia* host, typed them and tagged two of the strains with fluorescent proteins and sequenced their genome. This allowed us to conduct experiments on the association of these strains with an invertebrate host. Our hypothesis was that despite *Daphnia* would reduce the abundance of *E. coli* in the water it would still allow for a better survival of the FIB over a longer time thanks to a short term refuge of part of the population within its gut. Moreover, we speculate that such an association might in a long term help *E. coli* to adapt to freshwater over physiological and/or genetic adaptations.

2. Materials and Methods

2.1 *E. coli* abundance in zooplankton microbiomes

In the dataset of zooplankton (*Daphnia* gr. *galeata/longispina* and copepod) associated microbiota obtained from Lake Maggiore (Eckert, et al. 2020) we observed a large number of *E. coli/Shigella* related reads in Illumina MiSeq data, associated with the animals, but not in the surrounding water neither in other benthic environments. In order to confirm the presence of *E. coli*, quantitative PCR (qPCR) assays were conducted using *E. coli* specific primers for the *uid*A gene (1-CAATGGTGATGTCAGCGTT and 2-

ACACTCTGTCCGGCTTTTG, Srinivasan et al., 2011) using the RT-thermocycler CFX Connect (Bio-Rad). The standard calibration curve for the quantification of *uid*A was carried out as described elsewhere (Sabatino, 2015) and the gene concentration was expressed as gene copy/daphnia or mL of water. The same was done for *Daphnia obtusa* isolated from a small pond in the garden of CNR-IRSA (Eckert, et al. 2016). Twenty individuals were washed in autoclaved *MilliQ* water, recollected per triplicate and introduced in DNA Isolation Kit in Ultra Clean Microbial or Power Soil DNA Isolation Kit (Qiagen) for DNA extraction. The right size of all qPCR products was evaluated by electrophoresis (30 min at 80 V, 1% agarose gel). The efficiency of reaction was 87.5% and R^2 was 0.99. The limit of quantification (LOQ) was determined (Bustin, 2009) and was 45 gene copy/uL.

Furthermore, we checked a large dataset of microbial communities associated with zooplankton, from many natural freshwater habitats and cultures published elsewhere (Eckert, et al. 2021) looking for the presence of *E. coli*/*Shigella* affiliated reads. The dataset is composed of cladocerans (*Daphnia magna, Daphnia obtusa, Diaphanosoma brachyurum, Simocephalus* sp*.* and *Mesocyclops leukarti)* and, rotifers (*Adineta vaga, Keratella serrulata, Lecane elsa, Lecane inermis, Epiphanes senta, Keratella quadrata* and *Polyarthra* sp*.)* (see also Figure 2)*.*

2.2 *E. coli* isolates

2.2.1 Isolation

Individuals of *Daphnia obtusa* were collected in the field between May to July and October to November, two or three times per week from a rainwater fed pond in the garden of the CNR-IRSA in Verbania (Italy). Thirty individuals of *Daphnia obtusa* were washed in autoclaved *MilliQ* water (Millipore), crushed and sonicated (3 times, 1 minute each cycle with vortex within cycles) in 1 ml of physiological solution. Serial ten-fold dilutions were performed (from 1:10 to $1:10^6$). 1 ml of each dilution was filtered onto nitrocellulose membrane filters

(type GSWP, 25 mm diameter, 0.22 µm pore size, Millipore) and filters were plated onto mFC agar plates (Biolife) and incubated for 48 h at 37 ºC.

2.2.2 Identification and genetic characterization of *E. coli* isolates

Aliquots of presumptive *E. coli* colonies were introduced in 1 mL of physiological solution, centrifuged (5000 rcf, 4ºC, for 10 minutes), boiled for 15 minutes, frozen for 2-4 hours and centrifuged as before. DNA from presumptive *E. coli* colonies and from *Daphnia*, were tested for the presence of the *uid*A gene using the primers above mentioned by PCR. For the PCR assays, 5 µl of Buffer 5X, 0.5 µl dNTPs (10 mM), 0.2 µl Taq-polimerase (5U/ µl), 0.25 µl per each primers (100 µM), and water that it was added to arrive to final volume of 23 µl. PCR reactions were: denaturation 3 min at 95ºC, 35 cycles of 30 s at 95ºC, 1 min at 58ºC and 1 min at 72ºC, and a final extension step of 7 min at 72ºC. PCR products were separated by agarose gel electrophoresis (1%) and visualized with GelRed (Midore Green Advance DNA Stain). In order to assign a specific phylogroup or clade to the 10 *E. coli* strains isolated we used the PCR-based method described by Clermont et al., 2013 (Clermont, 2013). PCR products were separated by agarose gel electrophoresis (1%) and visualized with GelRed (Midori Green Advance DNA Stain). The 10 *E. coli* isolates were further analyzed to obtain an unambiguous DNA fingerprint by Enterobacterial Repetitive Intergenic consensus (ERIC-PCR) as previously described (Versalovic, 1991). ERIC-PCR products were separated by electrophoresis for 8 h at 40 V/cm, in 2% agarose Tris borate-EDTA (TBE) gel stained with GelRed (Midori Green Advance DNA Stain).

The strains ED1, ED4, ED8, ED157 and ED166 were chosen to be typed by Multi Locus Sequence typing (MLST) by sequence analysis of internal fragments of seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA, recA) according to the Achtman scheme (http://enterobase.warwick.ac.uk/species/index/ecoli). The allelic profiles of the seven genes and the resulting Sequence Type (ST) were determined from

the submission of sequence data on the PubMLST database (https://pubmlst.org).

2.2.3 Pathogenicity assay

The hemolytic activity of the strains was evaluated as described by Ghosh et al., (2014) with some modifications. Briefly, 4 mL of freshly drawn, heparinized human blood was diluted with 25 mL of phosphate buffered saline (PBS), pH 7.4. After washing three times in 25 mL of PBS, the pellet was resuspended in PBS to 20 vol %. A 100 µL amount of erythrocyte suspension was added to 100 µL of bacterial strains. PBS and 0.2% Triton X-100 were used as the negative and positive controls, respectively. After 1 h of incubation at 37 °C each well was centrifuged at 1200 × g for 15 min, the supernatant was diluted 1:3 in PBS and transferred to a new plate. The OD350 was determined using the Synergy HT microplate reader spectrophotometer (BioTek, Winooski, VT, USA). The hemolysis (%) was determined as follows:

$$[(A - A0)/(Atotal - A0)] \times 100$$

where A is the absorbance of the test well, A0 the absorbance of the negative control, and A total the absorbance of the positive control; the mean value of three replicates was recorded.

To detect the biofilm development, the strains were grown in LB medium (Oxoid), adjusted to 5×10^6 CFU/mL and inoculated (100 µL) in 24-well polystyrene plates (VWR). After 24 h of incubation at 37 °C, and 24°C the wells were washed with PBS to eliminate unattached cells and covered with 0.1% (v/v) crystal violet (CV) dissolved in H2O for 15 min, washed in PBS and air-dried. The remaining CV was dissolved in 85% ethanol for 15 min at room temperature and 200 µL from each well was transferred to a 24-well plate for spectrophotometric quantification at 570 nm (Multiscan Ex Microplate Reader, Thermo Scientific, Waltham, MA, USAThe strains were classified as strong, moderate, or weak biofilm producers based upon the ODs of the bacterial biofilms (Stepanović, 2007). Quantification of biofilm in microtiter plates:

overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci (Stepanović, 2007). All assays were performed in triplicate using independent cultures.

2.2.4 Genome sequencing and analysis

Two of the successfully isolated strains, namely strains ED1 and ED157, were chosen for genome sequencing. These two strains were selected because they were both affiliated with the D phylotype and because of their respective sequence type: ED1 belonged to a sequence type that contained many bacteria isolated from mammals including humans. The sequence type of ED157 only contained one other *E. coli* strain that was isolated from water. The strains were grown in Luria Broth (LB, Merck Life Science) overnight and DNA extraction was performed using the ultraclean microbial DNA extraction kit (QIAGEN).

Purified DNA was sequenced on a NovaSeq Illumina Platform (IGA technologies, Padova, Italy), providing a total of 10 and 15 million reads of output for ED1 and ED157 strains, respectively. One of the two genomes was already mentioned in a previous article (Riva et al., 2020). Briefly, reads were first trimmed using Trimmomatic (Bolger et al., 2014) and genomes were assembled using SPAdes default parameters (Prjibelski et L., 2020), obtaining a total of 54 and 59 assembled contigs >1 Kb, respectively.

To verify the phylotype of *E. coli* strains ED1 and ED157, we submitted the genome sequences to the website ClermoTyper (Beghain et al., 2018). Plasmids' presence in the genomes was examined through the platform PlasmidFinder (Carattoli et al., 2014), while the identification of antimicrobial resistance genes was done using ResFinder4.0 (Bortolaia et al., 2020). Virulence genes were instead found using the VirulenceFinder 2.0 platform (Joensen Katrine et al., 2014) and phage genome sequences were recognized using PHASTER (Arndt et al., 2016).

In order to evaluate whether the *Daphnia* associated isolates were similar to other freshwater *E. coli* we compared them to other genomes from the D

phylotype mentioned in Touchon et al. (2020): i) C4_38 and C2_45 strains, isolated from poultry meat, and ii) E5895 and E6003 strains, isolated from freshwater (Touchon et al., 2020; Tab. 1). Strains E5895 and E6003 were indeed randomly selected as representative *E. coli* strains adapted to the freshwater environment, owing a reduced genome, while the other two strains (C4_38 and C2_45) were randomly selected as representatives of strains from poultry meat which are known to have the largest average genome within the *E. coli* species (Touchon et al., 2020).

Strain	Accession number	Total length (bp)	Isolation source	Reference
ED1	JAAWVB000000000	5159712	*Daphnia obtusa*	Riva et al., 2020
ED157	JABEXY000000000	5273211	*Daphnia obtusa*	This study
C4_38	ERS3883848	5511727	Poultry meat	Touchon et al., 2020
C2_45	ERS3883832	5623389	Poultry meat	Touchon et al., 2020
E5895	ERS3883463	4825729	Water	Touchon et al., 2020
E6003	ERS3883339	4771985	Water	Touchon et al., 2020

Table. 1. *Escherichia coli* genomes included in the study.

Phylogenetic analysis considering the whole genome sequences was performed through the MICROSCOPE platform (http://www.genoscope.cns.fr/agc/microscope;(Vallenet, et al., 2009; Blondel Et al., 2008). Phylogenetic three was built thanks to the tool “genome clustering “of MICROSCOPE. Genomic similarity is estimated using Mash. This distance is correlated to the ANI like: D ≈ 1-ANI. From all the pairwise distances of the genomes set, a tree is constructed dynamically using the neighbor-joining javascript package. The tree displays clustering annotations. This clustering has been computed from all-pairs distances ≤ 0.06 (≈94% ANI)

that correspond to the ANI standard to define a species group. The clustering has been computed using the Louvain Community Detection.

In order to evidence differences and examine the distribution of protein families across the *E. coli* genomes indicated in Tab. 1, we used the "Protein Family Sorter" tool of PATRIC (https://www.patricbrc.org/), setting Genus-specific families (PLfams) (Davis et al., 2020 ;Davis et al., 2016).

2.2.5 GFP and DsRed tagging of strains

Competent cells of *E. coli* ED1 and ED157 strains were prepared in LB medium following the protocol described in Favia et al. (2007). Sixty microliters of competent cells (~10^{10} cells ml^{-1}) were mixed with 100-200 ng of plasmid DNA, transferred to a cold 0.1-cm-diameter cuvette and pulsed at 1,700 V in the Electroporator 2510 apparatus (Eppendorf, Milan, Italy). Plasmids used were pHM2-Gfp (Favia et al., 2007) and pKan (DsRed) (Crotti et al., 2009). Following the pulse, cells were immediately supplemented with 1 ml of LB medium and incubated at 37°C for 1 h. Transformants were selected by plating on LB agar medium added with i) 100 $mg \cdot ml^{-1}$ kanamycin, 40 $mg \cdot ml^{-1}$ 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) and 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) in case of plasmid pHM2-Gfp or ii) 100 $mg \cdot ml^{-1}$ kanamycin (KMY) in case of plasmid pKan(DsRed). Verifications of the presence of pHM2-Gfp or pKan(DsRed) plasmids were done observing the bacterial cells by fluorescent microscopy. Furthermore, the identity of *E. coli* transformants was confirmed by BOX-PCR amplification (Urzì et al., 2001) by comparing the BOX-PCR profiles with those of wild type ED1 and ED157 strains.

2.4 Experiments

All laboratory experiments were carried out using *Daphnia obtusa* from the garden of CNR-IRSA. The animals were collected 2 days before the experiment to adapt them to lab conditions, washed with artificial lake water medium (ALW, inorganic compounds in composition described here (Zotina et al., 2003)) and fed with a small amount of washed *Kirchneriella* sp. and kept

in the dark before experimental use. The animals were washed again in ALW before each experiment and the experiments were conducted in the same medium if not specified differently. The *E. coli* strains ED1 and ED157 tagged with the fluorescent proteins which were used in the experiments were grown overnight at 37°C in liquid LB containing 100 mg·ml^{-1} kanamycin to maintain the plasmid. The strains were centrifuged and washed twice with ALW before inoculation in experimental treatments the next day. All experiments were carried out at room temperature in the dark. All figures of the experiments set up were drawn in *R* with help of the packages *ggplot*2(Wickham 2009), *reshape*2 (Wickham 2012) and *cowplot* (Wilke 2020).

2.4.1. Localisation of *E. coli* on *Daphnia*

We verified where *E. coli* attached to *Daphnia* by incubation of *E. coli* with live individuals *Daphnia*. Animals were then dissected and different body parts were subjected to qPCR amplification of the *gfp*-gene to verify the presence and quantify *E. coli*. qPCR assay was carried out in a volume of 20 uL containing 2 uL of DNA, 0.5 uM of each primer (1-GAAGATGGAAGCGTTCAA and 2-AGGTAATGGTTGTCTGGTA, (Hale et al., 2015)), 10 uL of SsoAdvanced universal SYBR Green supermix (Bio-Rad), and filtered and autoclaved MilliQ water (Millipore) to the final volume. The program of qPCR was 95°C for 2 min, 35 cycles of 95 °C for 15 s, 54°C for 30 s and 72 °C for 15 s. Melting curve was performed from 60 °C to 95 °C with increments of 0.5 °C/5 s. The right size of all qPCR amplicons was evaluated by electrophoresis run (carried out as described above for *uid*A gene). The standard curve for the *gfp-gene* quantification was carried out by the dilution of the purified and quantified amplicon, as made for *uid*A gene and previously described in Sabatino et al. (2015). The efficiency of reaction was 91% and R2 was 0.99. The LOQ (determined as described above for *uid*A gene) was 9.85 gene copy/uL. The concentration of the *gfp*-gene was expressed as gene copy/daphnia (Figure 1A).

2.4.2 Release of *E. coli* after gut passage

We then tested whether *E. coli* was a food source for *Daphnia*, if *Daphnia* functioned as a refuge for *E. coli* or if they simply passed through the gut. First we incubated ED1-gfp and ED157-gfp separately with and without *Daphnia* in 50ml ALW for 2h in the dark at room temperature, then we transferred 50 µl of only water or water with a single *Daphnia* (+D treatment or control) and, after another 2h of incubation, we compared the amount of ED1-gfp transferred in the surrounding water. ED1-gfp was counted on a flow cytometer as green fluorescent events (BD C6, Accuri). Differences between treatments were evaluated using a linear model of log-transformed count data conducted in *R* (Figure 1B).

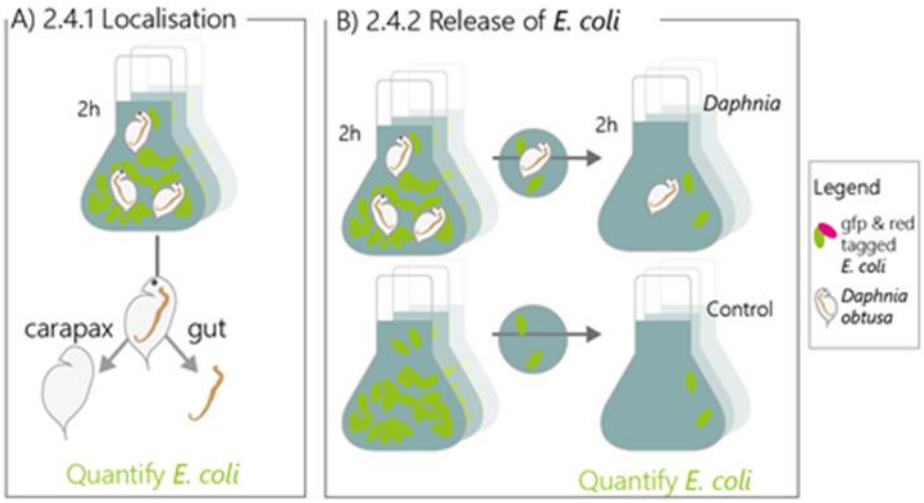

Figure 1: Experimental set-up of Localization (A) and food source experiments (B) involving *E. coli* and *Daphnia obtusa*.

2.4.3 Persistence of *E. coli* with *Daphnia*

The chemostat is a continuous culturing system with three medium tanks containing ALW attached to six vessels containing 700 ml of medium and a non-axenic *Kirchneriella* culture was added to both medium and vessels at a density of around 20'000 cells per ml (T-5). The system was kept in the dark. After three days (T-2) the chemostat pumps were switched on with a daily water replacement rate of 10%. After one day (T-1) ED1-gfp was added to the

vessel at a concentration of 10^6 cells ml^{-1} as well as Algae to maintain around 20'000 cells ml^{-1}. This experiment was conducted with strain ED1 because according to its sequence type it is a more relevant potential contamination from mammalian origin into freshwaters. After another day (T0) *Daphnia obtusa* was added to the vessels, which were randomly assigned with a quantity of animals in a gradient with the following number of animals per vessel: 0, 2, 6, 8, 10 and 15. Samples of 40ml were taken every 2-3 days over the outflow of the chemostat vessel and *Kirchneriella* solution was always added after sampling to maintain food for *Daphnia*. These samples were used for CFU counts for ED1-gfp and microscopy counts for both Algae and ED1. For phytoplankton counts 10ml solution was filtered on 0.45um pore-size polycarbonate filters and at least 10 fields and 500 cells were counted at a magnification of 80'000x at an epifluorescence microscope (Zeiss). For CFUs of day 0 and day 2 spots of 5ul of a gradual dilution between 1 and 10^{-4} were spotted on LB+KMY plates and grown for 24h at 37°C and counted. The presence of the gfp and thus univocal identification of ED1-gfp was done by placing the plate on a transilluminator (UV light) and observation of green fluorescence of the colonies. Due to the strong reduction of *E. coli* numbers of CFU for T 5 and 7 were counted by filtration of 1ml of undiluted and 1:10 diluted sample and on T 9 by filtration of 1ml and 10ml of sample, on a 0.2um pore-size nitrocellulose membrane filter that was placed on the plates and colonies were counted as described above. All spots and filters were done in triplicate per sample. On T12 *Daphnia* numbers had strongly reduced (see Supplementary Table 1) thus the experiment was considered finished. Individuals of *Daphnia* were extracted from the vessels, washed 3 times with sterile ALW and then dissected; the different body parts were placed in 200ul LB+Km in a black multiwell-plate in a plate reader (GlowMax, Promega). Growth of *E. coli* was detected by monitoring fluorescence over 48h every 30 min. The total number of adult dissected *Daphnia* was 12 (3 from vessel 6, 4 from vessel 5, 4 from vessel 3 and 1 from vessel 1) and the number of juvenile *Daphnia* was 9 (1 from vessel 6, 4 from vessel 5, 2 from vessel 3); 7 negative

controls were included. We checked the influence of the original gradient of abundance of *Daphnia* on the CFU of *E. coli* by a generalised linear model assuming a negative binomial distribution of the data. The model was evaluated using *check_model* from the package *performance* (Lüdecke, et al. 2019) and the output depicted as a type II analysis of variance table using the *car* package (Fox, et al. 2012).

2.4.4 Coexistence experiment

In order to test whether ED1 and ED157 reacted similarly to the presence of *Daphnia* and its associated bacteria we conducted a batch experiment where we incubated both strains together with no *Daphnia*, alive *Daphnia*, and dissected *Daphnia* for which we made one treatment containing the *Daphnia* guts and one containing the *Daphnia* carapax and filtration apparatus and their associated bacteria. Each replicate was amended with either 3 alive *Daphnia* or dissection pieces from 10 *Daphnia* in 2ml in 1:100 diluted LB with ALW and in triplicate. Moreover each treatment was conducted twice once using ED1-*gfp* + ED157-DsR*ed* and once using ED1-DsR*ed* and ED157-*gfp* to account for potential differences in fitness reduction by the two different fluorescence markers (total treatment n= 2 stainings x 3 replicates x 4 treatments = 24). In fact, in both cases ED1-DsR*ed* had a fitness advantage, thus the numbers presented here are averages between the CFUs counted for gfp and red of the same strain in the same treatment. CFUs were counted over 10 days starting from T4 on by spotting of 5ul diluted up to 10^{-8} in triplicates and green and red colonies were counted on a trans-illuminator (UV). The experiment was stopped after 10 days due to major mortality of *Daphnia* in the alive treatment (>90%). To evaluate the long-term differences between treatments data from March 24 and 26 were used (6 and 8 days). A generalised linear model assuming a negative binomial distribution of the data was made to evaluate the effect of the treatment and the date on the abundance of bot *E. coli* ED1 and ED157 (*glm.nb* in R with model: CFU ~ treatment (4 levels: alive,

carapax, gut, no *Daphnia*) + strain (2 levels: ED1 or ED157) + date (2 levels: March 24 and 26)). The model was evaluated using *check_model* from the package *performance* (Lüdecke, et al. 2019) and the output is from the *Anova* function from the *car* package (Fox, et al. 2012) and pairwise differences between treatments were evaluated with a post-hoc using *emmeans* from the homonymous package (Lenth, et al., 2020).

2.5 Data accessibility

All scripts and raw data are deposited at https://github.com/EsterME/E_coli_Daphnia. ED1 genome was deposited into the NCBI-Genbank database under accesion numbers JAAWVB00000000 (RIva et al., 2020). ED 157 Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JABEXY000000000. The version described in this paper is version JABEXY010000000.

3. Results

3.1 Abundance of *E. coli* in Lake Maggiore

By screening for the presence/abundance of the *E. coli* specific marker gene *uid*A in DNA extracted from three different locations in Lake Maggiore, we found that the gene was absent in the sediments, epilithic biofilms and water samples but could be found in both *Daphnia gr. galeata/longispina* and copepods, showing between 144-976 (mean 580) copies per animal (Figure 2A).

3.2 E. coli in other zooplankton microbiomes

We screened a large dataset of zooplankton related microbiomes and could find the presence of *E. coli/Shigella* related 16S rRNA gene sequences in samples from other cladocerans (*Daphnia magna, Daphnia obtusa, Diaphanosoma brachyurum)* and rotifers (*Adineta vaga, Keratella serrulata, Lecane elsa, Lecane inermis* and *Polyarthra* sp.*)* (Figure 2B). The genotype was not found in other rotifers *(Epiphanes senta, Keratella quadrata),*

Mesocyclops leukarti, a large calanoid copepod, and the cladoceran *Simocephalus* sp. (Figure 2B). We quantified *uid*A in *Daphnia obtusa* sampled from a rainfed ponds, because there *E. coli/Shigella* related reads were particularly high: we also confirmed the presence of *E. coli* by Real Time PCR.

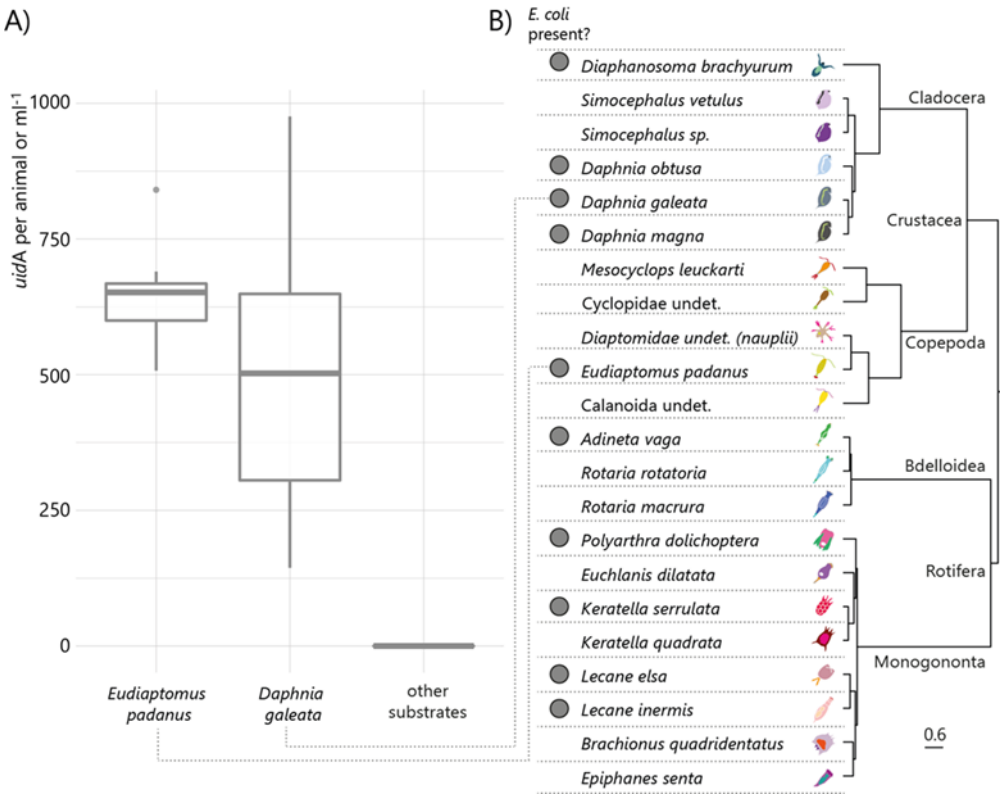

Figure 2: A) Boxplots of the abundance of the *E. coli* specific uidA gene copies in DNA isolated from animals and other substrates that include sediments, stones and water from 10 and 40m depth from Lake Maggiore. For each plot, the tick horizontal line represents the median value, the box includes 50% of the data from the first to the third quartile, the whiskers extend to the minimum and maximum data within the 1.5 interquartile range and the dots represent single outlier data points outside such range. B) Occurence of *E. coli* in various zooplankton species, a grey dot means *E. coli* was found in the microbiome of at least one sample. Images and phylogeny of animals are given as reference and are modified from Eckert et al, 2021.

3.3 E. coli Isolates

3.3.1 Isolation of *E. coli* from *Daphnia obtusa*

We attempted to isolate *E. coli* from *Daphnia obtusa* to further investigate which phylogroups of *E. coli* were affiliated with zooplankton. Through multiple isolation campaigns we retrieved ten *E. coli* strains and identified their phylogroup: strains ED1, ED2, ED3, ED4, and ED5 formed one cluster and were affiliated with phylogroup D/E and strains ED157, ED158 and ED166 a second cluster affiliated with phylogroup D/E (we did not succeed in the discrimination between these two phylogroups for these strains), strains ED8 and ED12 to phylogroup B1 (Figure 3A). Five of these strains were further chosen for MLST (ED1, ED4, ED8, ED157 and ED166) and pathogenicity assays: None of the strains showed traits of pathogenicity except for weak biofilm formation for the isolates ED1, ED4 and ED166 and they were classified in four different sequence types (ST38, ST1727, ST3573 and ST4166) (Figure 3A and Supplementary Table 2). We then analysed the other strains in the MLST database affiliated with these STs and most of the ST38 isolates were of human origin, whereas ST1727 included strains mostly isolated from animals (41%) than from humans (10%). The other two STs (ST3573 and ST4166) have been rarely described and they included isolates from non-human sources (Figure 3B).

ED1 and ED157 were selected for further analysis. The rationale behind the selection of these two isolates was their ST: ED1 was affiliated with ST38 where many other *E. coli* seem to be associated with mammals or even pathogens, whereas ED157 was affiliated with ST3573, including only one *E. coli* strain isolated from water. Genomes of ED1 and ED157 strains were sequenced, and the strains were then successfully marked with gfp and DsRed proteins, for interaction studies.

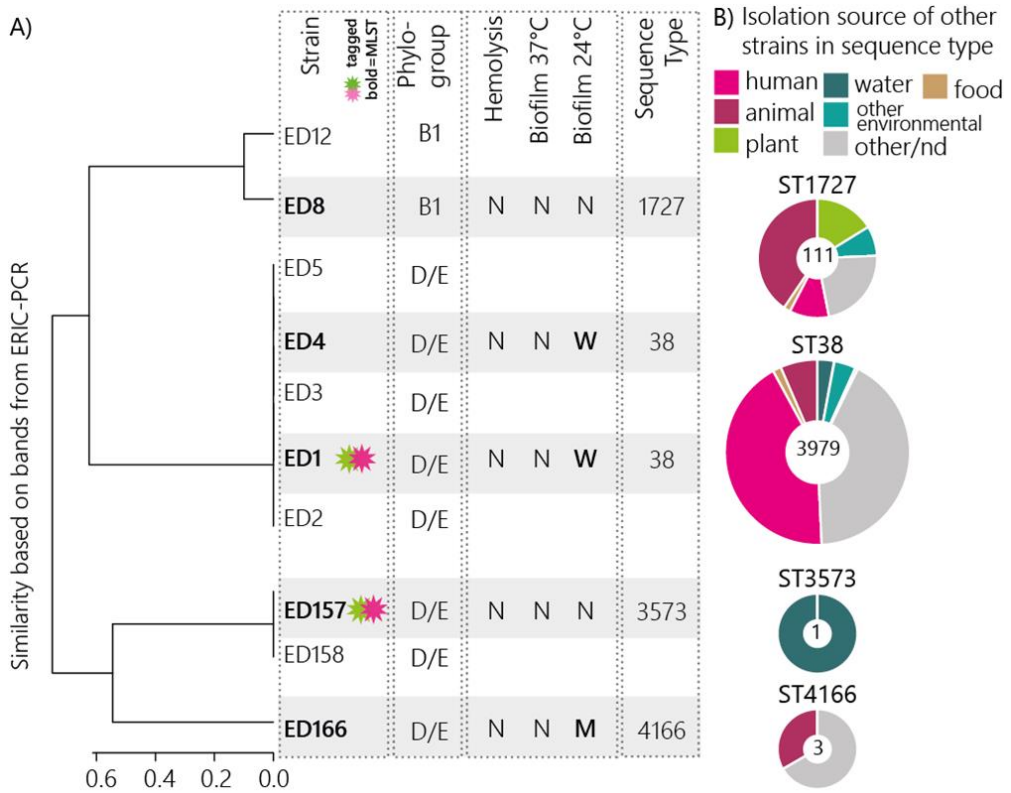

Figure 3 A: (fLTR) Cluster dendrogram of dissimilarity of the ERIC profile of the different *E. coli* strains isolated from D. obtusa and their phylogroup according to the ERIC profile. Strains that are grey shaded also present data for their phenotype in the pathogenicity assay (N=none, M=medium and W=weak), their sequence type according to Multi-locus sequence typing. **B**. Pie-charts summarising the isolation source of the deposited E. coli strains of the same sequence type as the E. coli strains from daphnids strains; the number in the middle of the pie-charts denotes the number of strains deposited per each sequence type.

3.3.2 Genome analysis of ED1 and ED157

Genome sequencing and analysis performed by ClermoTyper and pylogenetic tree construction with MICROSCOPE showed that ED1 and ED157 genomes belong to the D phylotype (Figure 4).

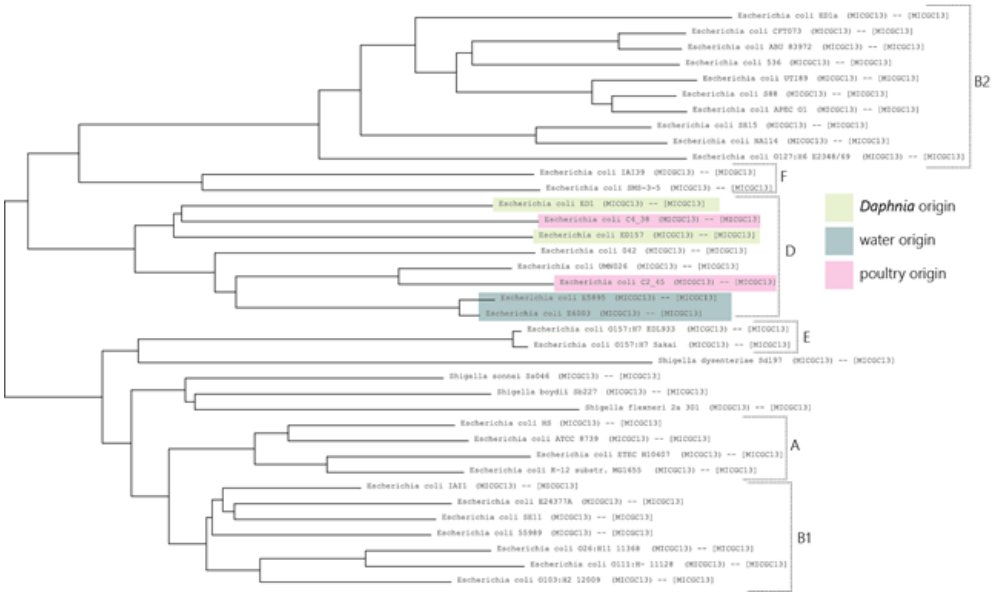

Figure 4 *Phylogenetic tree of* E. coli *genomes included in tab. 1. The tree was constructed with MICROSCOPE tool.*

Phylogenetic analysis performed on the whole genome sequences of the strains and genomes of water and poultry isolated *E. coli*, showed that *E. coli* strains isolated from *Daphnia* sp. did not cluster with the water isolates. Conversely, strains isolated from poultry meat clustered with the genomes of the *Daphnia* isolates, whereas the actual water *E. coli* clustered in a sister group (Figure 4). Comparing genome size, we could observe that ED1 and ED157 genome size were bigger than the genome sequences of strains from water, but smaller than the ones originated from poultry meat (Table 1). We found the presence of a higher number of plasmid replicon sequences in *E. coli* strains originated from poultry meat (C4_38: 5 plasmid replicon sequences; C2_45: 3 plasmid replicon sequences) than in the strains obtained from water (E5895: 1 plasmid replicon sequence; E6003: no plasmid replicon sequence detected) or from *Daphnia obtusa* (ED1: 2 plasmid replicon sequences; ED157: no plasmid replicon sequence detected) (Supplementary file 1). Poultry meat strains had a higher number of virulence genes (C4_38: 32; C2_45: 29) than the *Daphnia* strains (ED1: 12; ED157: 16) and the water

strains (E5895: 10; E6003: 12). Our analysis revealed that more phage sequences were present in ED1 and ED157 genomes than in the other genomes analysed here. Specifically, we found 7 phage sequences in ED1 and 5 in ED157, whereas only three and 0.5 phage sequences were found in poultry and in water strains, respectively. To compare which genes were different in the *Daphnia* isolates compared to other *E. coli*, pangenomic analyses was done with the strain listed in table 1 by the protein family sorter tool of PATRIC (Supplementary Table 3). The pangenome was composed by 7108 protein families while the core genome of 3789 protein families (53,7%). *E. coli* isolated from poultry meat and from *Daphnia* shared 57.5% protein families (from a total of 6787 protein families), while *E. coli* isolated from *Daphnia* and from freshwater bodies shared 65% (from a total of 5993 protein families).

The genomes from all groups shared a very high number of protein families that can help the strains to thrive in the different habitats: we found, for instance, the presence of protein families related to the production of the capsular polysaccharides, or the presence of protein families linked to the Type I fimbriae system. Interestingly, we detected the presence of RhS protein families, which are supposed to inhibit the intercellular growth as primary function (Koskiniemi et al., 2013) and the presence of some protein families linked to sucrose utilization only in isolates from *Daphnia* and poultry meat. Focusing on the Accessory genome of *E. coli* isolated from *Daphnia* (i.e. protein families that were not found in the other two groups), specific groups included i.e. ii) the presence of xanthosine related protein families, which allow bacteria to utilize purine nucleoside as carbon and energy source; iii) the presence of poly-beta-1,6-N-acetyl-D-glucosamine (PGA) protein families, which are involved in the synthesis, the export and the localization of PGA polymer, a necessary component for the formation of biofilms, which protect the bacteria to adverse environmental conditions.

3.4 Interaction of *E. coli* with *Daphnia obtusa*

3.4.1 Attachment

First we verified where *E. coli* localised in the animal by incubating ED1-gfp and ED157-gfp strains separately with *Daphnia*, dissecting the animals and performing a qPCR assay targeting the *gfp*-gene to verify the presence of *E. coli* on the various body parts (Figure 1A). We found that 70 ± 8% of the administered ED1-*gfp* and ED157-*gfp* were found in the gut compared to filter apparatus and carapax (data in Supplementary Table 4). We then tested whether *E. coli* was digested by *Daphnia* or if *Daphnia* functioned as a refuge for *E. coli* or if they simply passed through the gut. First we incubated ED1-gfp and ED157-gfp with *Daphnia* and transferred them to new clean water (Figure 1B). Compared to the control (transferred water without *Daphnia*) we found a significantly higher abundance of both *E. coli* strains in the surrounding water of *Daphnia* (Figure 5).

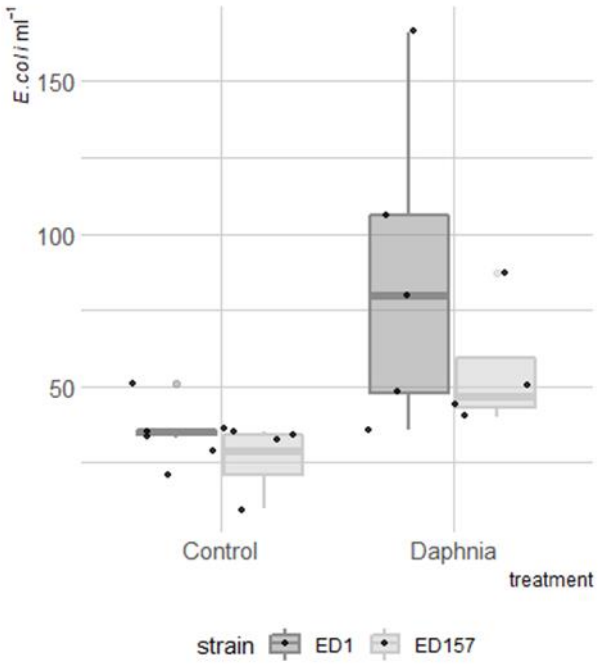

Figure 5 E. coli density in the treatment where only water was transferred (Control) and where animals were transferred (Daphnia) to sterile water after feeding on E. coli strains ED1-gfp (dark grey) or ED157-gfp (light grey). For each plot, the tick horizontal line represents the median value, the box includes 50% of the data from the first to the third quartile, the whiskers extend to the minimum and maximum data within the 1.5 interquartile range and the grey dots represent single outlier data points outside such range. The original data points for each treatment are superimposed on the plots as jittered black dots.

3.4.2 Persistence with and without *Daphnia*

We investigated how the presence of *Daphnia* impacted on the general survival of ED1-*gfp* in freshwater systems in continuous culture experiments (chemostat). We therefore filled eight chemostat vessels with ALW, phytoplankton, *E. coli* and incubated *Daphnia* at different densities (0, 2, 4, 8, 10, 15 animals per vessel, Figure 6).

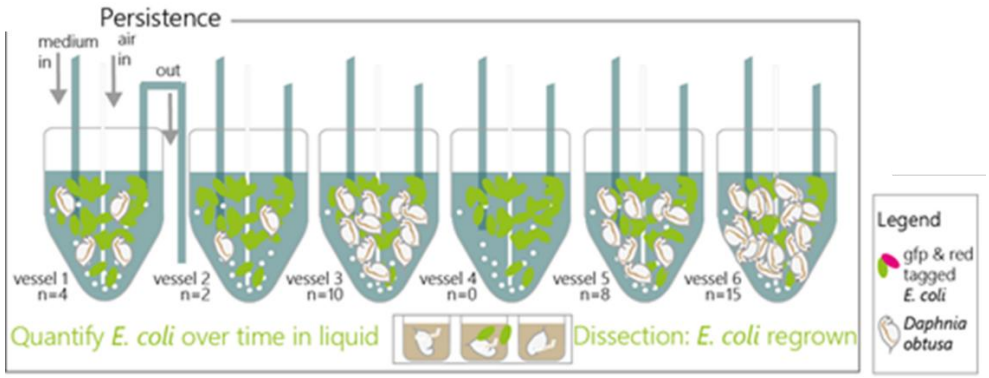

Figure 6: Experimental setup of persistence study conducted in a chemostat

We monitored the abundance of *E. coli* overtime and found that the abundance of *Daphnia* had a slightly significant negative effect on the abundance of *E. coli* (glm: Estimate = -0.11±0.06, z = -2, p = 0.048). However, *E. coli* abundances were in the same order of magnitude with only 1-6 CFU detected per ml of surrounding water in all treatments after 10 days of incubation even without animals (Figure 7).

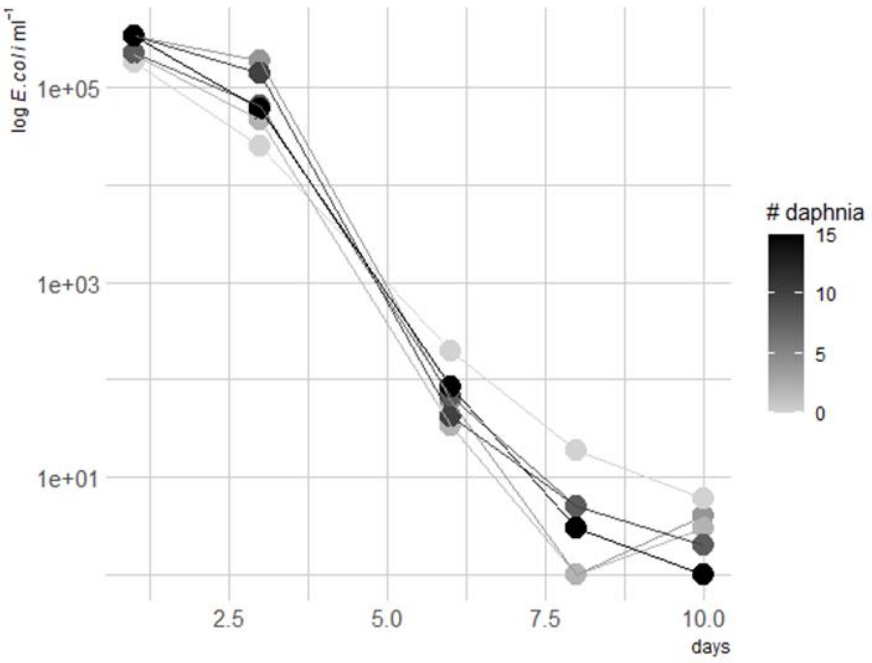

Figure 7: Log transformed cell density of *E. coli* in chemostat experiment over time. The color of the dots and lines indicates the starting number of *Daphnia obtusa* added to the vessels.

Some animals were then washed and guts dissected and *E. coli* cells were regrown in a plate reader to see whether there were culturable *E. coli* cells in the gut of the animals. One fourth of the total 12 dissected adult *Daphnia* (2 from vessel 5 and 1 from vessel 3, 0 from vessel 6 and 1) resulted in growth of *E. coli* within the first 48h of incubation, whereas no growth was detected from the gut of juvenile animals.

3.4.3 Coexistence of ED1 and ED157

We then conducted an additional experiment where we combined both strains ED1-*gfp* with ED157-DsRed and ED157-*gfp* with ED157DsRed, respectively, and incubated them either without animals, alive animals, dissected guts or dissected carapax (Figure 8).

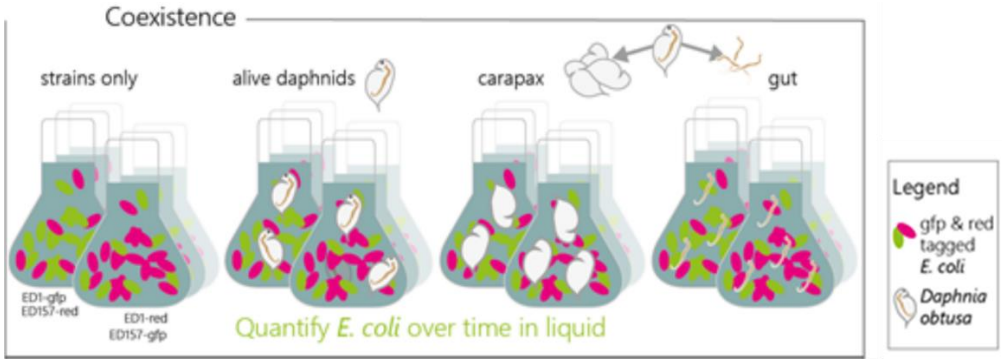

Figure 8 Setup of coexistence experiment

At day 6 and 8 the abundances of ED1 and ED157 (average of both tag combinations) were not statistically different (Figure 9, Table 2) whereas differences in treatments were visible: all treatments were different from each other except for the treatment with no *Daphnia* and the treatment with carapax pieces (Table 2).

A.	Chi-square value	degrees of freedom	p value	
treatment	52.2	3	<0.0001	***
strain	1.2	1	0.2830	
date	4.8	1	0.0284	*

B.	Carapax	Gut	Strains only
Alive	***	***	***
Carapax		*	n.s.
Gut			***

Table 2: A Statistical output from the generalised linear model made for the coexistence experiment to evaluate the dependence of the abundance of *E. coli* on the treatment (four levels: alive, carapax, gut, and strains only), the strain (two levels: ED1 and ED157) and the sampling date (two levels: May 24th and 26th). The table is a type-II analysis-of-variance table with Wald chi-square tests for predictors. B. Significance of the differences in the pairwise comparisons between the four treatments from a Tukey post hoc test. *** means p value < 0.001, * p value < 0.05 and n.s. p value > 0.05.

Living animals caused a faster reduction of *E. coli* abundances than the other treatments. *E. coli* growth with carapax pieces increased in numbers notwithstanding the presence of other bacteria and reached numbers that were very similar to the treatment that contained only the strains. In the presence of gut pieces and flora the abundances of both strains was reduced much more rapidly and at the end their abundance was similar to the one with live *Daphnia* (Figure 9).

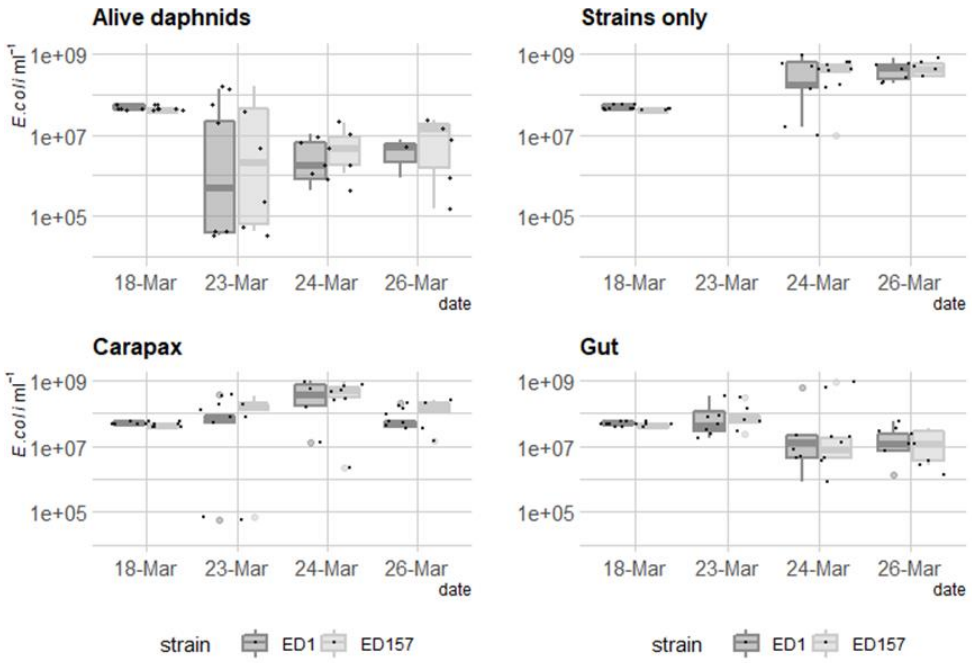

Figure 9: *E. coli* cell density over time in a batch experiment with the addition of Daphnia obtusa (Alive daphnids), no daphnids (strains only), pieces of Daphnia carapax (Carapax) and gut

pieces (gut). Dark grey shows ED1 and light grey ED157 strains labelled with fluorescent proteins.

4. Discussion

In this study we tackled the question whether *Daphnia* was a host for *E. coli* in freshwaters. While on one hand these bacteria are known to not be competitive in such environments and to be grazed easily when entering freshwaters through faecal pollution (i.e. González, et al. 1992; Eckert, et al. 2019), the evolution of freshwater *E. coli* (Touchon, et al. 2020) and renaturation of these bacteria have been observed (Ishii, Ksoll, et al. 2006; Ishii and Sadowsky 2008), showing that at least part of the incoming *E. coli* must survive over longer time periods in water. In a previous study we found that lake zooplankton could show remarkable quantities of *E. coli* related 16S rRNA gene sequences and we confirmed this here by quantifying the abundance of *uid*A gene, an unambiguous indicator for *E. coli* presence (Figure 2) (Eckert et al., 2020). Indeed, this is not the first time that *E. coli* is found associated to zooplankton (Ishii et al., 2006; Yan, et al. 2006). Evidence is accumulating that freshwater zooplankton microbiota is rather flexible in terms of composition (Callens, et al. 2015; Macke, et al. 2020; Eckert, et al. 2021, Pfenning-Butterworth, 2021), meaning that the association with zooplankton might be an interesting potential niche for the short term survival of FIB. Such a habitat offers protection from protistan grazing and higher nutrient concentrations thanks to filtration feeding from the animal (Eckert and Pernthaler 2014). Furthermore, surface attachment is generally considered favourable for the survival of such bacteria compared to a planktonic lifestyle (Costerton, et al. 1999; Allen, et al. 2010). We thus tested here whether the association with *Daphnia* allowed *E. coli* to survive longer in aquatic habitats compared to when the animal was not present. In this study we found that *Daphnia* can function as a short term and transitional host for *E. coli*: As expected *Daphnia* did reduce abundances of *E. coli* in the surrounding waters, but it was not responsible for the complete removal of *E. coli*, since many

bacterial cells survived gut passages (Figure 5) and *E. coli* was still detected after 10 days of co-culturing with *Daphnia* (Figure 5 and 7). In this study we confirm that the main presence of *E. coli* is in the gut of *Daphnia* and that at least part of its population survives the gut passage (Burnet, et al. 2017b; Ismail, et al. 2019). However, the association with *Daphnia obtusa* did not seem to give a long term advantage in the survival of *E. coli* (Figure 7 and 9). Many studies have recently suggested to use *Daphnia* as a biological controlling mechanism for *E. coli* contaminations in water: through experiments using very large densities of animals and bacteria these studies showed that the abundance of *E. coli* reduced with *Daphnia* (Nørgaard and Roslev 2016; Burnet, et al. 2017b; Ismail, et al. 2019). This is surely feasible to reduce large abundances of *E. coli*, but here we showed that *E. coli* persisted in low abundance even in the presence of *Daphnia* in abundances that were more similar to that found in nature. In fact, we also showed that *E. coli* was still culturable from the gut, even if the bacterium was in very low abundance in the surrounding water. In our environmental survey we found it associated with different zooplankton hosts, especially cladocera and rotifera (Figure 2), but particularly abundant at one time point in zooplankton from Lake Maggiore (Figure 2). However, also other samples from Lake Maggiore were analysed in the large study of zooplankton-associated microbes (Eckert, et al. 2021), and there we did not find any *E. coli*. This shows that this short-term association can also occur in nature and the short-term association to the animals might consequently also spread *E. coli* that enter the system through superficial contamination due to the animals vertical and horizontal migration (Grossart, et al. 2010). However, there does not seem to be an actual persistence of these bacteria in there indicating that these occurrences in the gut are rather stochastic events and that *E. coli* does not form part of the general *Daphnia* microbiota.

In the experiment where we incubated *E. coli* with dissected guts, we observed a similar reduction of the bacteria (after 8 days) as was seen with alive *Daphnia* (Figure 9). This data strongly indicates that the competition of

the gut microbiota was the main reason for reduced abundances of *E. coli*. Another interesting finding was that *E. coli* seemed to profit from the presence of carapax pieces of *Daphnia,* which are composed mostly of chitin. Both strains grew better in the presence of carapax, despite they did not have chitinolytic enzymes in its genome. It is more likely that the two strains indirectly profited from chitin degradation since such degradation is usually more efficient when done by multiple species (Corno, et al. 2015) and many bacteria are known to profit from these compounds without being directly involved in the primary degradation (Beier and Bertilsson 2013). The presence of poly-beta-1,6-N-acetyl-D-glucosamine (PGA) protein families, which are involved in the synthesis, the export and the localization of PGA polymer, shows that these *E. coli* strains might also be involved in multispecies biofilm formation (Kang, et al. 2018).

We isolated *E. coli* from *Daphnia* collected from a small pond, however with major difficulty. Despite multiple isolation campaigns and a clear presence of *E. coli*, confirmed by amplification of the *uid*A gene, we only isolated ten strains (Figure 3). This could mean that *E. coli* associated with *Daphnia* were in a viable but nonculturable state (VBNC) which has been observed in other freshwater environments (Liu, et al. 2008) or when exposed to sunlight (Pommepuy, et al. 1996). For *Enterococcus feacalis* it was shown that much higher numbers are detected attached to plankton with culture independent methods, compared to the culturable fraction of these bacteria, and it has been suggested that this attachment in a VBNC state is a mode of survival of this species in freshwater (Signoretto, et al. 2004). A similar situation might also be true for *E. coli*.

The *E. coli* strains isolated from *Daphnia* in this study belonged to phylogroups D/E or B1. The analysis of our *Daphnia* deriving *E. coli* strains themselves did not give strong indication that these were environmental strains. Touchon and colleagues have shown that freshwater *E. coli* strains had a reduced genome (Touchon, et al. 2020) a typical form of adaptation to oligotrophic environments (Baumgartner, et al. 2017; Salcher, et al. 2019). In an

experimental system Baumgartner and colleagues showed that such genome reduction was rather fast, when bacteria were under predation (only few hundred generations, Baumgartner, et al. 2017). In the case of our *E. coli* strains their genome was of intermediate size, meaning that they were smaller than the genomes found in poultry meat derived *E. coli*, but larger than those of freshwater *E. coli*, which could indicate a certain transition to adaptation of the genome. The two here analysed *Daphnia*-associated *E. coli* genomes did not cluster with the freshwater isolates but with those isolated from poultry meat. In fact, also the *E. coli* strains isolated from freshwater might derive from avian faeces (Meerburg, et al. 2011) and survive associated with zooplankton for a short time. The *E. coli* genomes also showed some traits that were considered important to adapt to different environments and for survival in freshwater, e.g., the presence of protein families related to the production of the capsular polysaccharides, which protect the bacteria from several environmental stress factors (Walk, et al. 2007; Azurmendi, et al. 2020), or the presence of protein families linked to the Type I fimbriae system, which allows bacteria to attach to several eukaryotic cells (Gally, 1993). Whether or not zooplankton is a place of genetic adaptation of FIB to the environment is an interesting question arising from this study.

Overall our results showed that the FIB *E. coli*, when released into the aquatic environment, can form a short-term association with zooplankton, e.g. *Daphnia.* We demonstrated that *E. coli* does not belong to the core microbiota of *Daphnia*, and suffers from competition by the natural microbiota of *Daphnia,* but some resist passages through its gut and profit of its carapax to survive in water. This association does not prolong their long-term survival in our experiments but might provide a niche where these bacteria can encounter other aquatic bacteria, a possible spot for horizontal gene transfer, and a possible spot for genomic adaptation.

Supplementary Materials

Supplementary table 1: Numbers of surviving *Daphnia* in the chemostat vessels over time.

Vessel	day 1	day 7	day 13
V1	4	2	1
V2	2	0	0
V3	10	6	4
V4	0	0	0
V5	8	4	3
V6	15	13	3

Supplementary table 2: Details of genes and sequence types found in MLST screening of *E. coli* strains isolated from *Daphnia obtusa*.

	adk	***fumC***	***gyrB***	***icd***	***mdh***	***purA***	***recA***	**ST**
ED1	4	26	2	25	5	5	19	ST38 (ST38 Cplx)
ED4	4	26	2	25	5	5	19	ST38 (ST38 Cplx)
ED8	6	19	3	16	11	8	6	ST1727
ED157	54	66	17	27	286	4	4	ST3573
ED166	83	331	42	44	1	2	2	ST4166

Supplementary table 3: Accessory genome of each genome group. PFs indicate protein families.

	N° of PFs
Accessory genome of *Daphnia* strains vs Genomes of freshwater ones	1369
Accessory genome of *Daphnia* strains vs Genomes of poultry meat ones	873
Accessory genome of Freshwater vs Genomes of *Daphnia* strains	492
Accessory genome of poultry meat vs Genomes of *Daphnia* strains	1284
Accessory genome of *Daphnia* strains vs Genomes of the other *E. coli* groups	818

Supplementary Table 4: Percentage of the gfp gene found in the gut and carapax samples of dissected *Daphnia*

	ED1-gfp		ED157-gfp	
% carapax	%gut	samples	% carapax	%gut
88.20586	11.79414	1	30.55211	69.44789
66.67754	33.32246	2	36.37496	63.62504
73.28058	26.71942	3	28.15056	71.84944
80.6547	19.3453	4	44.26287	55.73713
74.56091	25.43909	5	22.66621	77.33379
78.94122	21.05878	6	30.60439	69.39561
52.08777	47.91223	7	31.78352	68.21648
53.51601	46.48399	8	15.38194	84.61806
69.41398	30.58602	9	32.33105	67.66895

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Chapter III

An environmental Escherichia coli strain is naturally competent to acquire exogenous DNA

Riva, F., Riva, V., Eckert, E. M., Colinas, N., Di Cesare, A., Borin, S., Mapelli, F. & Crotti, E. (2020). An Environmental *Escherichia coli* Strain Is Naturally Competent to Acquire Exogenous DNA. *Frontiers in microbiology*, *11*, 2131.

Abstract

The diffusion of antibiotic resistance determinants in different environments, e.g. soil and water, has become a public concern for global health and food safety and many efforts are currently devoted to clarify this complex ecological and evolutionary issue. Horizontal gene transfer (HGT) has an important role in the spread of antibiotic resistance genes (ARGs). However, among the different HGT mechanisms, the capacity of environmental bacteria to acquire naked exogenous DNA by natural competence is still poorly investigated. This study aimed to characterize the ability of the environmental *Escherichia coli* strain ED1, isolated from the crustacean *Daphnia* sp., to acquire exogenous DNA by natural competence. Transformation experiments were carried out varying different parameters, i.e. cell growth phase, amount of exogenous DNA and exposure to artificial lake water (ALW) and treated wastewater to mimic environmental-like conditions that may be encountered in the agri-food system. Results were compared with those showed by the laboratory *E. coli* strain DH5α. Our experimental data, supported by genomic sequencing, showed that, when exposed to pure water, ED1 strain was able to acquire exogenous DNA with frequencies (10^{-8} - 10^{-9}) statistically higher than the ones observed for DH5α strain (10^{-10}). Interestingly, higher values were retrieved for ED1 than DH5α strains exposed to ALW (10^{-7} *vs* 10^{-9}, respectively) or treated wastewater (10^{-8} *vs* 10^{-10}, respectively). We tested, therefore, ED1 strain ability to colonize the rhizosphere of lettuce, a model plant representative of raw-consumed vegetables of high economic importance in the ready-to-eat food industry. Results showed that ED1 strain was able to efficiently colonize lettuce rhizosphere, revealing a stable colonization for 14 days-long period. In conclusion, ED1 strain ability to acquire exogenous DNA in environmental-like conditions by natural competence, combined with its ability to efficiently and stably colonize plant rhizosphere, poses the attention to food and human safety showing a possible route of diffusion of antibiotic

resistance in the agri-food system, sustaining the “One Health” warnings related to the antibiotic spread.

1. Introduction

Antibiotic Resistance (AR) is a public concern for global health. About 700,000 people die every year from antibiotic resistant bacteria-infections and 10 million annual deaths caused by antibiotic resistant pathogens are estimated by 2050 (Lim et al., 2019). In the last century, antibiotics have been widely used in medicine, plant production and livestock industries, imposing a strong selective pressure on the environmental microbial communities (Van Hoek et al., 2011). The exposure of bacteria to a sub-lethal concentration of antibiotics has led to the generation and diffusion of antibiotic resistant bacteria (ARB), through mutations and horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs) (Smalla et al., 2018). This can be particularly enhanced in specific hot spots of natural and engineered ecosystems, such as mycosphere, residuesphere, rhizosphere and wastewater treatment plants (WWTPs) (Riva et al., 2020; Eckert et al., 2018). The spread of ARGs in different environments linked to anthropogenic activities has been largely demonstrated: for example, long-term applications of sewage sludge and chicken manure can improve the abundance and the diversity of ARGs and ARB in soil (Chen et al., 2016), while WWTPs can be considered as one of the main ARGs' contaminated aquatic systems for both ARB and free DNA (Amos et al., 2015; Czekalski et al., 2014). Despite several studies have described the presence and spread of ARGs and ARB in the environment, some gaps of knowledge about the selection, evolution, persistence and HGT of ARGs remain to be unveiled (Smalla et al., 2018; Larsson et al., 2018).

HGT is crucial for bacterial adaptation to new environments and, consequently, for bacterial evolution. DNA transfer is generally accomplished by three "classical" mechanisms, namely transduction, conjugation and transformation (Sun, 2018). While in transduction and conjugation specific apparatuses are required to transfer DNA from donor to recipient cells, i.e. phage virions and conjugative pili, respectively, in transformation the acquisition of DNA is usually transient and linked to the capability of the

bacterial cells to express competence at a specific physiological phase. Concerning the environmental ARG diffusion through HGT mechanisms, researchers have highlighted that many aspects have yet to be clarified, e.g. the contribution of the different mechanisms to ARG spread or the drivers of gene transfer (Smalla et al., 2018). For instance, since conjugation-based experiments are more feasible in laboratory and field conditions than those based on the other HGT mechanisms, this might have underestimated the importance of transformation or transduction (Smalla et al., 2018).

Natural competence for transformation is a specific physiological state in which bacteria are able to acquire genetic material from their surroundings. The acquired DNA can be then integrated into the bacterial genome or be maintained as a plasmid in the cell (Blokesch, 2016). There are more than 80 prokaryotic species described to be naturally transformable and different species and strains can show peculiar traits: for instance, *Vibrio cholerae* has been described to acquire DNA in presence of chitin (Meibom et al., 2005), while *Acinetobacter baylyi* is constitutively competent for transformation with frequency rates depending on the bacterial growth phase (Blokesch, 2016; Domingues et al., 2019). For a long time, *Escherichia coli* has not been considered a naturally transformable bacterium. *E. coli* is routinely forced to acquire exogenous DNA by artificial laboratory treatments i.e. following the exposure to i) solutions with high concentrations of divalent metal ions followed by heat shock, ii) polyethylene glycol solutions, or iii) electrical shock pulses (Hasegawa et al., 2018). Nonetheless, in some specific conditions, not related to the artificial transformation, *E. coli* has been demonstrated capable to acquire exogenous DNA e.g. in contact with environmental waters (Baur et al., 1996; Woegerbauer et al., 2002; Ishimoto et al., 2008), in food extracts (Maeda et al., 2003) or after freeze-thaw processes. Besides the “classical” exogenous DNA uptake machinery of natural transformation, based on conserved proteins for the transport of single-stranded DNA (ssDNA) into the cell cytoplasm, two new routes of DNA acquisition by transformation have been recently identified in this species. In the first way, double-stranded DNA

(dsDNA) is internalized into *E. coli* cells on agar plates, while the second DNA uptake mechanism depends on a cell-to-cell contact, not related to conjugation, and occurs in a colony on agar plates (Sun et al., 2006; Sun et al., 2009; Etchuuya et al., 2011; Sun, 2018). While the latter mechanism has been recently reported to be induced by a P1*vir* bacteriophage (Sugiura et al., 2017), the former foresees the participation of several proteins, among which researchers have so far identified *ydcS* and *ydcV* genes, encoding for a putative periplasmic protein and a putative inner membrane protein, respectively (both located on the putative ABC transporter *ydcSTUV* operon for putrescine transport; Sun, 2016) and the general stress response regulator factor RpoS (Sun, 2016; Zhang et al., 2012).

One of the main recognized routes that could allow AR spread in environments related to the agri-food system is the use of reclaimed water for irrigation purposes. Nowadays the water reuse represents a common practice in several countries and is considered a priority also by the European water management policy to combat the water crisis exacerbated by global warming (Riva et al., 2020). Indeed, at least 20 million hectares of croplands worldwide are irrigated with urban treated wastewater (Bouaroudj et al., 2019). WWTPs have been indicated as one of the main contributors of both cell bound and free ARGs for the aquatic systems (Amos et al., 2015; Czekalski et al., 2014; Zhang et al., 2018; Li et al., 2018); the reuse of treated wastewater for irrigation purposes would enter the food production and could contribute to the diffusion of ARGs that finally could potentially be acquired by pathogenic strains. Indeed, it has been found that WWTPs can promote, in the water in which the effluents are released, the stabilization of a resistome derived principally from treated wastewaters (Corno et al., 2019), making the freshwater bodies reservoirs of ARGs (Di Cesare et al., 2015). The ability of *E. coli* to acquire and transfer exogenous DNA (Hasegawa et al., 2018; Sun, 2018), together with its capability to survive and thrive in different habitats (i.e. water, rhizospheric soil or human gut; Van Elsas et al., 2011; Raimondi et al., 2019), where the presence of ARGs has been reported (Osińska et al., 2020;

Du et al., 2020) and HGT can be enhanced (i.e. rhizosphere, Chen et al. 2019), could pose a risk for the food safety and public health (Krzeminski et al., 2019). This risk could be high for fresh products such as spinach, sprout and lettuce, which are generally consumed as raw vegetables (Shen et al., 2019). Indeed, antibiotic resistant bacteria belonging to the pathogenic species *E. coli* and *Salmonella enterica* have been already reported in farm environments and fresh products, including lettuce and ready-to-eat food (Perera et al., 2020; Araújo et al., 2017; Yang et al., 2020; Nüesch-Inderbinen et al. 2015; Schierstaedt et al., 2019).

In the framework of 'One Health' approach, this study aimed to i) characterize the possible acquisition of exogenous DNA by an environmental strain of *E. coli* mimicking the conditions that may be encountered in the agri-food system, and to ii) study the *E. coli* strain capacity to colonize plant rhizosphere, using soil potted lettuce as model system.

2. Materials and methods

2.1 Strains and media

Escherichia coli strain ED1 was isolated from individuals of *Daphnia* sp. collected from a small rainwater-fed pond in the garden of the CNR-IRSA, Verbania, Italy. Thirty daphnids (in triplicates) were washed in sterile Milli-Q water, crushed and sonicated (3 times, 1 minute each cycle with a shaking application by vortexing between cycles) in 1 ml of 2M NaCl. Serial ten-fold dilutions were prepared and filtered on nitrocellulose membrane filters (type GSWP, 25 mm diameter, 0.22 µm pore size, Millipore) which were placed onto agar plates of the selective medium mFC (Biolife) and incubated for 24-48 hours at 37 ºC. Once colonies of presumptive *E. coli* (blue color on mFC agar) appeared on plates, they were purified by streaking three times and then stored in 25% glycerol solutions at -80°C. A small amount of the bacterial biomass was then introduced in 1 mL of 2M NaCl, centrifuged (5000 g, 10 minutes, 4ºC), boiled 15 minutes, frozen for 2-4 hours and finally centrifuged

as before. One of the isolate, named ED1, was identified as an *E. coli* strain due to positive amplification of the *uid*A gene (Srinivasan et al., 2011) by PCR as described elsewhere (Sabatino et al., 2015).

2.2 Preparation of transforming exogenous DNA

Transformations were carried out by using pCR™II-TOPO® (Invitrogen) plasmid carrying ampicillin and kanamycin resistance genes. The plasmid was extracted from the strain *E. coli* Mach1™ T1 Phage-Resistant pCR™II-TOPO® using the QIAPrep® Spin Miniprep Kit (Qiagen, Milan, Italy) following the manufacturer instructions. The plasmid was quantified by measuring the optical density at 260 nm wavelength in a spectrophotometer (BIO RAD SmartSpec™ 3000).

2.3 Natural transformation protocol

Precultures of ED1 and DH5α strains were firstly grown in 25 ml of LB liquid medium overnight at 37°C with shaking. Then, 1 ml of cultures were diluted in 100 ml of LB and incubated at 37°C until the cells reached early exponential or stationary growth phases, i.e. at optical densities at 600 nm (OD_{600nm}) of 0.4-0.5 or 2, respectively. Forty ml of cells were then centrifuged twice with Milli-Q water for 10 minutes at 2700 g and finally resuspended in 500 µl of the same washing buffer. All centrifugation steps were performed at room temperature (RT) between 20-23°C. Four aliquots of 100 µl of cells were prepared, and the proper quantities of plasmidic DNA were added and gently mixed, without pipetting (the mixture is hereafter named as transformation mixture). The remaining 100 µl-cells aliquot was used as negative control (no DNA was added). Samples were incubated at RT for 1 hour: three aliquots were then plated on LB plates added with ampicillin (100 µg/ml), while the fourth aliquot was serially diluted (from the undiluted sample to -8) and used to evaluate (in triplicate) the total cell count on LB agar plates without the antibiotic selection. Aliquot of negative control was plated as well on LB plates added with ampicillin (100 µg/ml). All the plates were kept at 37°C overnight. Experiments were performed with three biological replicates. Putative

colonies of transformants, retrieved by ampicillin selection, were then streaked on LB plates added with kanamycin (100 µg/ml). Both ED1 and DH5α strains are sensitive to 100 µg/ml ampicillin, 100 µg/ml kanamycin and 50 µg/ml rifampicin. To further confirm the plasmid acquisition, kanamycin-resistant colonies were also subjected to PCR amplification. Transformation frequencies were calculated as the ratio between the number of transformants and the total number of culturable cells (about 10^9 cell/mL in case of cells harvested at the exponential phase and 10^{10} cell/mL in case of cells harvested at the stationary phase). Bacterial transformation was performed using 0.25 µg, 0.5 µg, 1 µg and 2 µg of plasmidic DNA.

Transformation protocols were then carried out using cells collected at the early exponential phase and exposing them to 2 µg of plasmidic DNA in two different types of water as washing and incubation buffers: besides Milli-Q water (pH 6.23) we used i) artificial lake water (ALW, pH 7.69) prepared modifying the protocol of Zotina et al. (2003) in regard to the inorganic medium components (Supplementary Table 1), and ii) water collected from the effluent of a WWTP located in Verbania (pH 6.84; water sampled on December 10th, 2019; Supplementary Table 1), serving 51,000 population equivalent and equipped with chlorination as disinfection process (Di Cesare et al., 2016). In order to reduce the presence of environmental bacteria, water samples were filtered through nitrocellulose membrane filters with 0.22 µm pore size (Millipore).

DNase sensitivity was tested by adding DNase I to the transformation mixture at different times, e.g. immediately after the transformation mixture preparation and after 1, 3, 4, 6 and 18 hours from the preparation of the transformation mixture. Then, the transformation mixtures containing DNase I were incubated 1 hour at RT before plating on LB agar plates added with ampicillin (100 µg/ml) (Sun et al., 2006).

To verify the acquisition of pCR™II-TOPO® plasmid, DNA was extracted from putative transformants by boiling lysis (Ferjani et al., 2015) and used as template to amplify a plasmid sequence fragment of about 250 bp with primer

M13f (-20) (5'-GTA AAA CGA CGG CCA G-3') and M13r (5'-CAG GAA ACA GCT ATG AC-3') according to manufacturer's (Invitrogen) instruction. Thermal protocol was set up as follows: 94°C for 5 minutes, followed by 34 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes and the last step at 72°C for 10 minutes.

2.4 Generation of rifampicin resistant mutants of *E. coli* strains ED1 and DH5α

Rifampicin mutants of ED1 and DH5α strains were obtained by plating stationary-phase cultures on LB plates added with 50 µg/ml of rifampicin. Plates were then incubated at 37°C overnight. Upon appearance, rifampicin resistant (RIF-R) colonies were selected and initially re-streaked on LB added with 50 µg/ml rifampicin and, finally, on LB added with 100 µg/ml rifampicin.

2.5 Root colonization by *E. coli* strains

RIF-R ED1 and RIF-R DH5α strains were used for the bacterization of *Lactuca sativa* (var. Canasta) seedlings to verify their ability to colonize plant rhizosphere. Lettuce seeds were sterilized with 0.7% sodium hypochlorite for 5 min followed by 5 rinsing steps in sterile distilled water (Bonaldi et al., 2015) and grown in pots filled with non-sterile soil under greenhouse conditions. Three days after sowing, lettuce seedlings (n=3 for each strain) were inoculated with 5 ml of bacterial suspensions obtained by growing the RIF-R ED1 and RIF-R DH5α strains in LB medium supplemented with rifampicin (100 µg/ml) for 24 h at 37°C, centrifuging twice the bacterial cultures at 4000 rpm for 10 min and re-suspending the pelleted cells in physiological solution (NaCl 0.9%) to obtain a final bacterial concentration of 10^8 cell/g of soil. Six lettuce seedlings were irrigated with 5 ml of distilled water and considered as negative control. One week after bacterization, lettuce seedlings were harvested and the rhizosphere soil was separated from the root by vortexing for 5 minutes the root system in physiological solution. To evaluate the number of colony-forming units (cfu) per gram of soil, rhizosphere samples (n=3 for each strain; n=6 for negative control) were serially diluted in physiological solution, plated

in triplicate on LB medium supplemented with rifampicin (100 µg/ml) and cfu were counted after 24 hours of incubation at 30°C. In order to confirm the identity of the isolates, after the visual check of colony morphology on the Petri dishes, ten bacterial colonies isolated from the rhizosphere of each bacterized lettuce seedlings were picked. The DNA was extracted through boiling cell lysis and the 16S-23S rRNA Intergenic Transcribed Spacer (ITS) region was amplified by ITS-PCR fingerprinting (Mapelli et al., 2013), comparing the ITS profiles of the bacteria re-isolated from the rhizosphere at the end of the experiment with those of RIF-R ED1 and RIF-R DH5α strains used for lettuce bacterization.

The colonization experiment was repeated to investigate the stability of ED1 and DH5α strains in the lettuce rhizosphere over time (14 days). For this experiment, lettuce seeds were sterilized as reported above and grown in soil previously sterilized through tindalization process. One week after sowing, lettuce seedlings were inoculated with 5 ml of bacterial suspensions (10^8 cell/g of soil) prepared as described above. The presence of RIF-R ED1 and RIF-R DH5α strains in lettuce rhizosphere was verified one week (t1) and two weeks after bacterization (t2). As previously described, rhizosphere soil samples (n=3 for each strain and each experimental time) were serially diluted and plated in triplicate on LB medium supplemented with rifampicin (100 µg/ml). Assessment of cfu/g of soil and strain identity were performed as described above.

2.6 DNA extraction, genome sequencing and analysis

Genomic DNA from *E. coli* strain ED1 was extracted from an overnight culture in LB liquid medium using the UltraClean Microbial DNA extraction kit (Qiagen), according to the manufacturer's protocol. DNA quantity was assessed using fluorometry (Qubit, Invitrogen) according to the manufacturer's protocol. Sequencing was performed on an Illumina NovaSeq platform using paired-end sequencing of 150 bp fragments at IGA Technologies (Udine, Italy). The genome was assembled as described by

Cabello-Yeves et al. (2018): briefly, *Trimmomatic* was used for read trimming and filtering and *SPAdes* for the genome assembly, while a preliminary gene annotation was done using NCBI (Johnson et al., 2008a). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAAWVB000000000. The version described in this paper is version JAAWVB010000000.

Genome assemblies of *E. coli* strains ED1 and K12 NEB DH5α (Accession Number CP017100; Anton and Raleigh, 2016) were submitted to the RAST Service (http://rast.nmpdr.org/) and compared taking advantage of the RAST function-based comparison tool. Plasmid presence in ED1 genome was investigated through the platform PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/; Carattoli et al., 2014). VirulenceFinder 2.0 platform (https://cge.cbs.dtu.dk/services/VirulenceFinder/; Joensen et al., 2014) was used to identify virulence genes in the genomes of *E. coli* strains ED1, K12 NEB DH5α, O157:H7 Sakai (Accession Number BA000007, Makino et al., 1999) and O157:H7 EDL933 (Accession Number AE005174, Perna et al., 2001). Genomic islands, insertion sequences (IS) and phage genome sequences were searched in ED1 and K12 NEB DH5α genomes by IslandViewer4 (Bertelli et al., 2017), ISfinder (Siguier et al., 2006) and PHASTER (Arndt et al., 2016). Details on RAST and NCBI annotation can be found in Supplementary Table 2.

2.7 Statistical analyses

Statistical analyses were conducted with R 3.1.2 (R Core Team, 2013) through RStudio (RStudio Team, 2015) and with Calc Statistical Function of Microsoft® Office Excel. Linear model was applied to assess the relation between transformation frequency and quantities of DNA added during transformation protocols. Student's t-test was employed to verify differences between ED1 and DH5α strains concerning transformation frequencies

(considering growth phase and types of water) and root colonization efficiency.

3. Results

3.1 Influence of different growth phases on transformation

The capability to acquire exogenous DNA by the environmental *E. coli* strain ED1, compared with the laboratory *E. coli* strain DH5α, was initially tested in pure water on resting cells harvested at different phases of the growth curve: Milli-Q water was used as washing and incubation buffer (to avoid the presence of interfering cations) and a large amount of transforming DNA plasmid (2 µg) was added to minimize any possible interference on transformation frequencies linked to a limiting quantity of DNA. First, we used cells harvested from early exponential phase cultures (OD_{600nm} between 0.4 and 0.5) (Supplementary Figure 1), observing a transformation frequency of 4.26×10^{-8} ($\pm 2.26 \times 10^{-8}$) and 4.44×10^{-10} ($\pm 7.70 \times 10^{-10}$) for ED1 and DH5α strains, respectively (Supplementary Table 3). ED1 cells in early exponential growth phase demonstrated a significantly higher transformation frequency in comparison with DH5α cells (Student's t-test, p=0.032, Figure 1). When cells were harvested at the stationary phase (OD_{600nm} between 2.1 and 2.2), a transformation frequency of 3.95×10^{-9} ($\pm 3.91 \times 10^{-10}$) was obtained with ED1 strain, resulting however statistically higher than the value recovered for DH5α strain ($1.93 \times 10^{-10} \pm 1.56 \times 10^{-10}$; Student's t-test, p=0.0001, Figure 1; Supplementary Table 3).

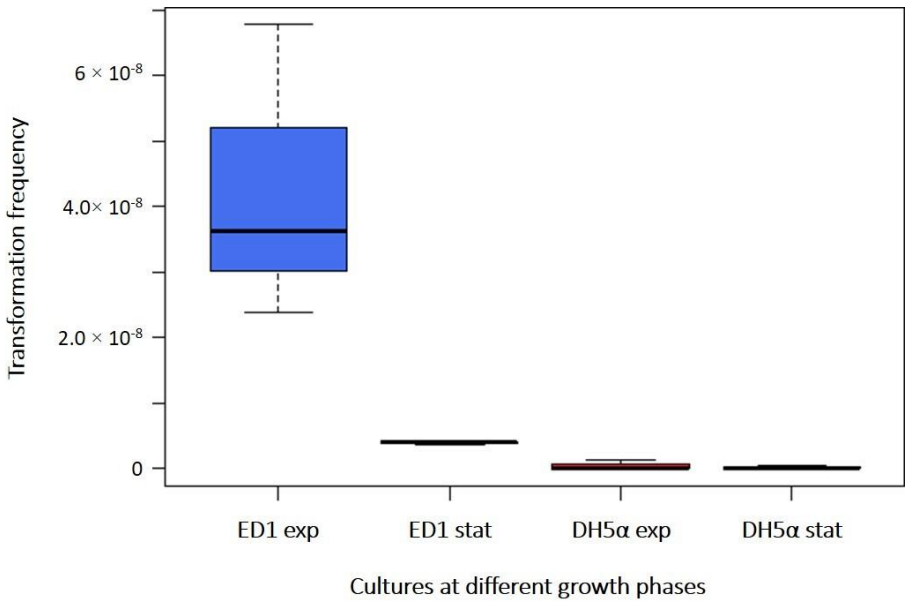

Figure 1: Transformation frequencies of *E. coli* strain ED1 with cells collected at early exponential ("exp") and stationary ("stat") phases. Transformations were performed in Milli-Q water with 2 µg of plasmidic DNA.

While transformation frequencies of DH5α strain were not significantly different between both growth phases (Student's t-test, p=0.609), statistical analysis indicated that ED1 natural competence is significantly higher in the early exponential phase than in the stationary one (Figure 1; Student's t-test, p=0.0415). All the following transformation assays were therefore run with cells at the early exponential phase.

In order to confirm the occurrence of natural transformation (which is a DNase-sensitive mechanism differently from the DNase-resistant mechanisms i.e. conjugation and transduction), we checked the sensitivity of ED1 uptake of DNA to the addition of DNase I. Since no transformation events were retrieved, unveiling thus the DNase sensitivity of the mechanism, we confirmed ED1 cells' ability to uptake DNA by natural competence (Hasegawa et al., 2018).

3.2 Influence of exogenous DNA quantity on transformation frequency

Transformation frequencies of ED1 and DH5α strains were analysed in Milli-Q water with increasing quantities of plasmid pCR®II-TOPO® as exogenous DNA, by adding 0.25 µg, 0.5 µg, 1 µg and 2 µg of plasmidic DNA to the cells harvested at the early exponential phase. As shown in Supplementary Table 4, transformation frequency for DH5a strain was estimated to be ≤ 4.44×10^{-10}, while increasing transformation frequencies were reported for ED1 strain, ranging from 5.48×10^{-9} to 4.26×10^{-8} when increasing quantities of plasmid from 0.25 µg to 2 µg, respectively, were added. Statistical analysis revealed a statistical difference for ED1 strain exposed to 2 µg or 0.25 µg of plasmidic DNA (Student's t-test, p=0.0480 between 2 µg or 0.25 µg). As shown in Figure 2, transformation frequency of ED1 strain was significantly related to the amount of plasmid added (linear model: t=3.9, p=0.003), whereas this was not the case for DH5a strain (linear model: t=0.55, p=0.6) (Baur et al, 1996).

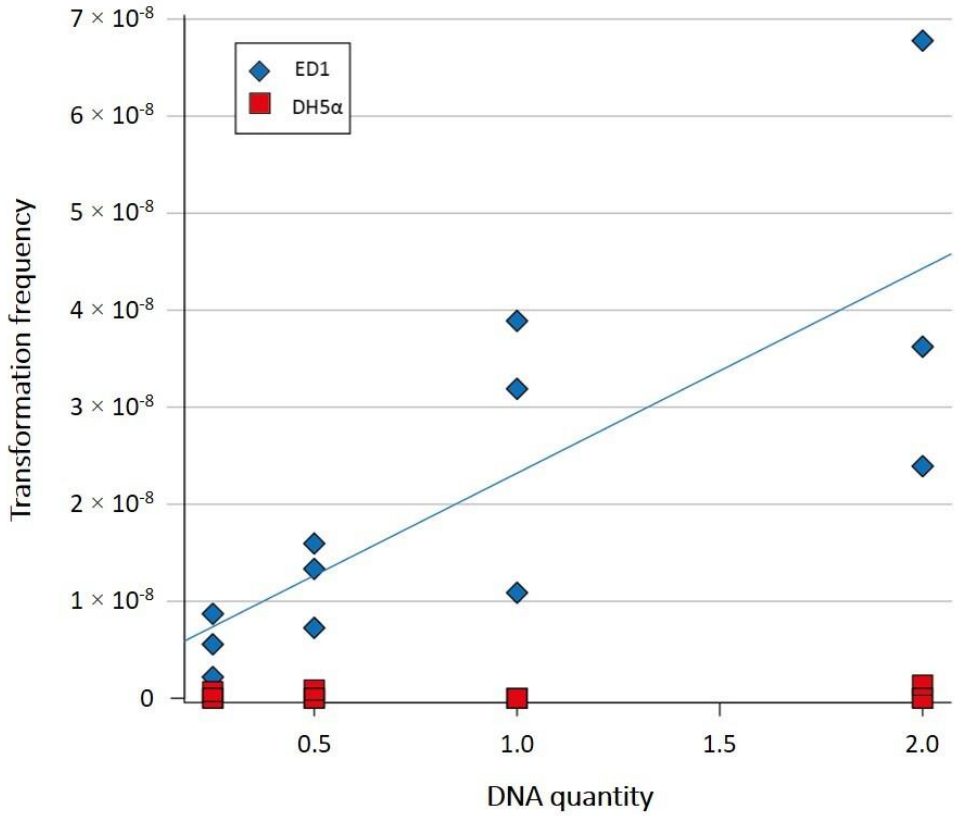

Figure 2: Transformation frequencies of *E. coli* strains ED1 and DH5α in Milli-Q water with increasing quantities of plamidic DNA. Transformation frequency of ED1 strain was significantly related to the amount of plasmid added (linear model: t=3.9, p=0.003).

3.3 Bacterial transformation in different types of waters

Transformation of ED1 and DH5α strains was assessed in natural and artificial water solutions considered as representative of environmental habitats, i.e. the artificial lake water (ALW) and the water collected from the effluent of Verbania WWTP. Milli-Q water was used as control and the transformations were carried out with a not limiting quantity of transforming DNA (2 µg). Statistical analysis showed that the transformation frequencies of ED1 strain were significantly higher than the ones observed for DH5α strain considering all the types of water used (Student's test; p=0.0295, 0.0226 and 0.0364 with ALW, Milli-Q water and treated wastewater, respectively; Figure 3).

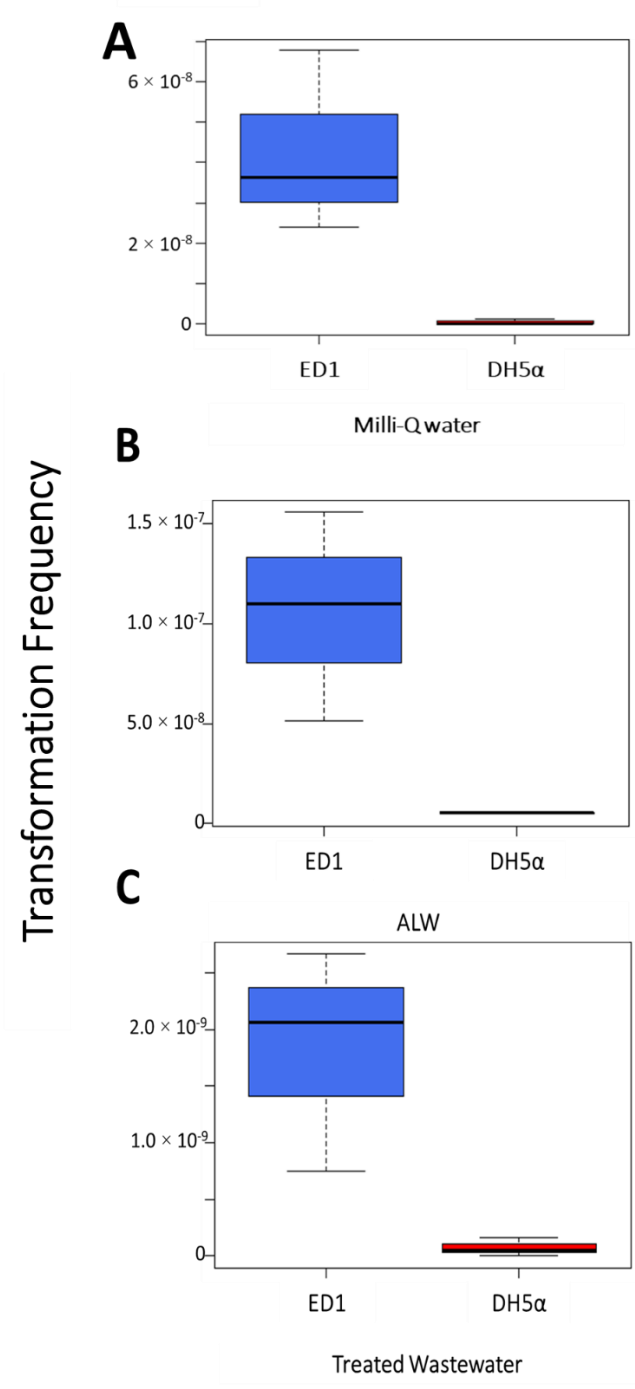

Figure 3. Transformation frequencies of *E. coli* strains ED1 using different types of water: (**A**) Milli-Q water, (**B**) ALW and (**C**) treated wastewater (WW).

Transformation frequencies ≤ 5.19×10^{-9} were obtained for DH5α strain in the different types of water (Supplementary Table 5). Moreover, transformation frequencies of ED1 strain were significantly higher in ALW than in the other types of water (Figure 3; Student's t-test p values: between Milli-Q water and ALW, p=0.029; between ALW and treated wastewater, p=0.047): specifically, we obtained for this strain transformation frequencies values of 1.06×10^{-7} ($\pm$ 5.26×10^{-8}) in ALW and 1.83×10^{-8} ($\pm$ 9.80×10^{-9}) in the effluent water released into the environment from Verbania WWTP, whereas for the control in pure water a value of 4.26×10^{-8} ($\pm$ 2.26×10^{-8}) was retrieved (Supplementary Table 5).

3.4 Plant colonization by *E. coli* strains

The ability of ED1 and DH5α strains added to the soil to colonize plants' rhizosphere was verified using the correspondent RIF-R strains and lettuce seedlings as model system. The experiment was firstly conducted in short term conditions in non-sterile soil to check the rhizocompetence of *E. coli* strains in presence of the competing soil dwelling microbial community. Seven days after *E. coli* addition to the 3 days-old plantlets surrounding soil, the rifampicin resistant bacteria re-isolated from the rhizosphere of the lettuce seedlings amounted to 1.59×10^{9} ($\pm$ 8.29×10^{8}) cfu/g rhizospheric soil for RIF-R ED1 strain and resulted statistically higher in comparison to rifampicin resistant bacteria isolated from the rhizosphere of both non-bacterized lettuce seedlings ($1.97 \times 10^{5} \pm 1.62 \times 10^{5}$cfu/g soil; p=1.61 x 10^{-7}) and seedlings bacterized with RIF-R DH5α strain ($4.23 \times 10^{8} \pm 4.45 \times 10^{8}$ cfu/g soil; p=1.14 x 10^{-3}), as shown in Figure 4A. Ten randomly picked colonies isolated from each bacterized plant (n=30 per ED1 strain bacterization; n=30 per DH5α strain bacterization) were subjected to ITS-PCR fingerprinting. The ITS profiles detected for all colonies corresponded to those of the *E. coli* strains used for plants bacterization, as shown in Supplementary Figure 2A and Supplementary Figure 2B for ED1 and DH5α respectively. Although both the tested *E. coli* strains were able to colonize in 7 days the lettuce rhizosphere

under non-sterile soil condition, the environmental *E. coli* strain ED1 showed a higher colonization performance of this microhabitat compared to the laboratory strain DH5α ($p=1.92 \times 10^{-3}$).

Similar results were obtained when the experiment was repeated with lettuce plants older (7 days-old) than those used in the first colonization assay in sterile soil and for a longer period, to verify the stability of the strains in the rhizosphere microhabitat, without any competition with the soil residing microbiota. As shown in Figure 4B, one week after plant bacterization with ED1 strain, 1.35×10^6 ($\pm$ 8.39×10^5) cfu/g soil of RIF-R cells were recovered, whereas plants exposed to DH5α strain led to isolate from the lettuce rhizosphere a significant lower RIF-R titer ($3.86 \times 10^5 \pm 2.38 \times 10^5$ cfu/g soil; p=0.0043). The RIF-R isolated colonies in all the assays demonstrated to belong to the inoculated *E. coli* strains by evaluating their ITS-PCR fingerprinting on representative colonies (Supplementary Figure 3). Two weeks after bacterization the presence of both ED1 and DH5α *E. coli* strains remained stable in plants rhizosphere, amounting respectively to 1.01×10^6 ($\pm$ 1.03×10^6) cfu/g soil and 2.44×10^5 ($\pm$ 3.33×10^5) cfu/g soil (p=0.048; Figure 4B).

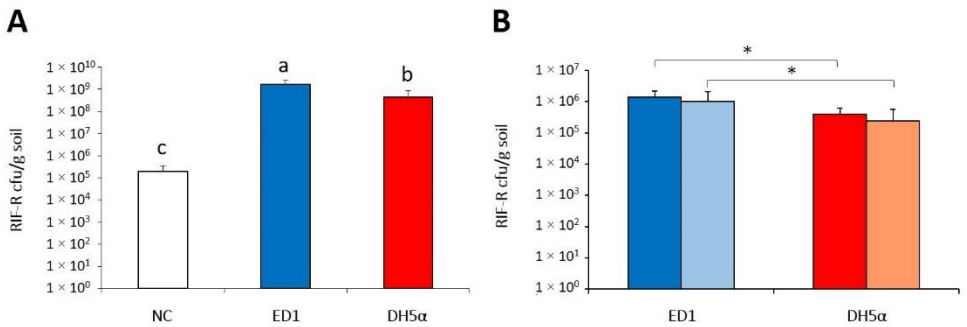

Figure 4: Evaluation of *E. coli* strains ED1 and DH5α rhizocompetence. (**A**) Bacterial abundance (cfu/g soil) of rifampicin-resistant bacteria in the lettuce rhizosphere after a 1-week colonization experiment performed with non-sterile soil. (**B**) Assessment of the stability of the rifampicin-resistant bacteria abundance in the rhizosphere of lettuce seedlings grown in sterile soil. Dark- and light-coloured columns indicate cfu/g of soil 1–week and 2-week after the bacterization, respectively. NC = non-bacterized lettuce seedlings; ED1/DH5α = lettuce

seedlings bacterized with ED1 and DH5α strains, respectively. Different letters or asterisks indicate statistically significantly differences according to Student t-test (p value < 0.01).

3.5 Genome analysis

Taking advantage of RAST function-based comparison tool, genomes of *E. coli* strains ED1 and K12 NEB DH5α (Anton and Raleigh, 2016; Accession Number CP017100), composed of 5,159,712 bp and 4,583,637 bp respectively, were compared. *E. coli* strain K12 NEB DH5α has been chosen as reference strain for genomic analysis since it is a fhuA2 derivative of *E. coli* K12 DH5α, the genome sequence of which is not currently available.

Both genomes shared a high percentage of metabolic functions: indeed, the presence of all the main basic metabolic functions, such as, for instance, carbohydrate metabolism or respiration, was assessed. Differences in the genomes' size are primary reflected in the fact that ED1 strain owns a larger number of genes (included in the below reported metabolic pathways) than K12 NEB DH5α strain. We detected the presence of the "propanediol metabolic pathway" and the "methylcytrate cycle" in ED1 genome, which were absent in K12 NEB DH5α genome (Table 1). Furthermore, in ED1 genome we retrieved genes encoding proteins related to the osmoregulatory choline-glycine betaine system e.g. the high-affinity choline uptake protein BetT, a choline dehydrogenase and a betaine aldehyde dehydrogenase (Table 1).

Role	CDS ID in ED1 genome*	ED1
IncF conjugative transfer genes	HBA78_21285-HBA78_21370, HBA78_21380-HBA_21390, HBA78_21405-HBA78_21425, HBA78_21435-HBA78_21455,	+
Propapendiol metabolism pathway	HBA78_16485-HBA78_16525, HBA78_16535-HBA_7816560, HBA78_16570-HBA78_16580	+
Type I secretion system LapB, C, E	HBA78_15000, HBA78_15005, HBA78_15015	+
CFA/I fimbriae encoding system	HBA78_08810, HBA78_08825	+
Type III secretion system	HBA78_04615, HBA78_04630, HBA78_04645, HBA78_04655-HBA78_04665, HBA78_04680, HBA78_04690, HBA78_04695, HBA78_20315, HBA78_20335	+
Choline and Betaine Uptake and Betaine Biosynthesis	HBA78_08680, HBA78_08695	+
Hydroxyaromatic non-oxidative decarboxylase protein	HBA78_20830, HBA78_20835	+

"+" indicates presence of functions; "-" indicates absence of functions.

*Details on RAST and NCBI annotation can be found in Supplementary Table 2.

Table 1: Main subsystems revealed in the genome of *E. coli* strain ED1.

Only in the genome of ED1 we found several genes classified by RAST as involved in the bacterial adhesion and secretory systems, i.e. CFA/I pili, the secretion system type I and the type III secretion injectosome (Table 1).

Considering genes related to the acquisition of exogenous DNA, the automatic annotation revealed in both genomes the presence of several genes homologous to those required for the DNA uptake in species that are known to be naturally competent: *pilQ/HofQ* (HBA78_15695 and NEB5A_17330; HBA78 code refers to ED1 strain, while NEB5A one refers to K12 NEB DH5α strain), encoding for a transmembrane channel allowing dsDNA to cross the outer membrane; *pilA* (HBA78_09875; NEB5A_00545), *pilB* (HBA78_09880

and NEB5A_00540), *pilC* (HBA78_09885; NEB5A_00535), related to the construction of the pseudopilus; *dprA* (HBA78_16130; NEB5A_16795), also called *smf*, responsible of the DNA processing and *ycaI/ComEC* (HBA78_03445; NEB5A_04210) related to the uptake of exogenous DNA (Cameron and Redfield, 2009; Chen and Dubnau, 2004; Sun, 2018). We detected in both genomes the presence of genes involved in one of the two *E. coli*-specific mechanisms of natural transformation i.e. the general stress response regulator factor RpoS (HBA78_20820; NEB5A_05530) (Zhang et al., 2012; Sun, 2018), as well as the RpoS-regulated genes *ydcS* and *ydcV* (HBA78_24185 and HBA78_24200) in ED1; NEB5A_07355 and NEB5A_07370 in K12 NEB DH5α) (Sun, 2016). Additional analysis was performed submitting ED1 genome to the PlasmidFinder platform (Carattoli et al., 2014; Yang et al., 2015; Table 1): we found the presence of i) a IncFII plasmid replicon sequence (with an identity of 96.55% against the one of the reference sequence AY458016) and ii) a IncX1 plasmid replicon sequence (with an identity of 95.23% against the one of the reference sequence JN935898). The replicon sequences were located on two separate contigs of 79,647 bp and 25,889 bp, respectively, and allowed us to speculate the presence of two plasmids in ED1 chromosome.

In order to identify virulence factors, we further analysed the genomes of strains ED1 and K12 NEB DH5α through the platform VirulenceFinder 2.0 (Joensen et al., 2014). We found a higher number of virulence factors in ED1 than in DH5α genome (Table 2).

		ED1	K12 NEB DH5α
air	enteroaggregative immunoglobulin repeat protein	+	-
astA	heat stable enterotoxin-1	+	-
eilA	hilA-like regulator in enteroaggregative *E. coli*	+	-
gad	glutamate decarboxylase	++	++
iss	increased serum survival	-	+

"+" indicates presence of functions; "-" indicates absence of functions. Number of "+" indicates the number of sequences of virulence factors detected in genomes.

Table 2: Virulence genes revealed by the analysis of the genomes of *E. coli* strains ED1 and K12 NEB DH5α using the platform VirulenceFinder 2.0.

Both genomes showed the presence of the glutamate decarboxylase (GAD) system which contributes to acid resistance in the human gut (Vanaja et al., 2009). Conversely, we detected only in ED1 genome the presence of genes encoding the adhesin *air*, an enteroaggregative immunoglobulin repeat protein involved in bacterial aggregation and colonization (Sheikh et al., 2006), *astA*, a heat stable enterotoxin-1 (Yatsuyanagi et al., 2003) and *eilA*, a putative activator of the type three secretion system (T3SS), which contributes to the pathogenicity of enteroaggregative *E. coli* (EAEC) strains (Sheikh et al., 2006). Moreover, from the comparison with DH5α genome we found that ED1 genome lost *iss* virulence factor, defined as a serum survival gene (Johnson et al., 2008b). When we included in our analysis the genomes of two pathogenic strains of *E. coli*, i.e. *E. coli* strains O157:H7 Sakai (Accession Number BA000007, Makino et al., 1999) and O157:H7 EDL933 (Accession Number AE005174, Perna et al., 2001), we could observe that a conspicuous higher number of virulence factors was retrieved in the latter than in ED1 or K12 NEB DH5α genomes (Supplementary Table 6). Whereas similar numbers of genomic islands are present in both genomes, the number of IS sequences predicted in ED1 genome is higher than the one retrieved for NEB DH5α genome. Moreover, we found more phage genomic sequences in the former than in the latter strain (Supplementary Table 7).

4. Discussion

Several studies have revealed the modest capability of *E. coli* strains to acquire exogenous DNA by natural transformation and researchers have recently underlined the existence of a few peculiar DNA uptake mechanisms of natural transformation in this species (Sun et al., 2006; Guo et al., 2015; Hasegawa et al., 2018). *E. coli* laboratory strains, known for their high artificial transformation efficiency, demonstrated to undergo to natural transformation

in experiments mimicking natural conditions e.g. using freshwater or food extracts (Baur et al., 1996; Woegerbauer et al., 2002; Maeda et al., 2003; Maeda et al., 2004), whereas a limited number of publications verified natural competence in *E. coli* strains isolated from human and warm-blooded animals (Woegerbauer et al., 2002; Tsen et al., 2002; Matsumoto et al., 2016). Environmental *E. coli* strains, to our knowledge, were never tested for natural competence. In this study, we investigated the ability of the environmental *E. coli* strain ED1, isolated form the crustacean *Daphnia* sp., to acquire exogenous DNA, comparing the results with the ones showed by the laboratory *E. coli* strain DH5α in relation to the cell growth phase, amount of transforming DNA and in environmental-mimicking conditions, i.e. exposed to lake water and WWTP effluents.

We ascertained a higher transformation frequency (10^{-8}-10^{-9}) for the environmental strain than for the laboratory one (10^{-10}), observing a higher number of transformation events when high quantities of plasmidic DNA were used, up to a saturation level (Baur et al., 1996). Values retrieved for ED1 strain underlined the modest capability of transformation in *E. coli* strains, especially if compared with other bacterial strains known to be naturally competent, such as *Acinetobacter baylyi* BD413 (Lorenz et al., 1992) and *Bacillus subtilis* 168 (Hauser and Kanamata, 1994). As reported by Baur et al. (1996), our results showed higher transformation frequencies for ED1strain with cells grown at early exponential growth phase (0.4-0.6 OD_{600nm}) rather than at the stationary one. Log-phase cells were also used by Woegerbauer et al. (2002) who compared the transformation frequency and efficiency of laboratory and clinical isolates, revealing higher transformation rates for the former. In case of DH5α strain we recovered low values of transformation frequencies: we retrieved only two transformants in all the replicates in which Milli-Q water and 2 µg of DNA were applied. Nevertheless, other studies reported in case of DH5α strain higher numbers of transformants or transformation frequencies than the ones we obtained, likely due to differences of the adopted experimental protocols, which included, among the

others, variations of the bacterial growth condition and growth phase (Sun et al., 2006; Woegerbauer et al., 2002).

The protocol we adopted in our experiments was conceived to mimic conditions feasible in the environment. To this aim, strains were subjected to a few manipulation procedures before incubation on selective agar plates and were exposed to different kinds of waters considered as representative of a few habitats (i.e. ALW and treated wastewater). Moreover, temperatures of 20-23°C, closer to environmental values than the ones usually used in laboratory procedures, were maintained during the transformation protocol (not for the incubation), differently from what reported in literature i.e. 37°C (Sun et al., 2006), 10°C or temperature shifts (Baur et al., 1996). Although it was reported that disinfection by-products in the WWTP effluents can enhance the rate of bacterial transformation, promoting the spread of extracellular antibiotic resistance genes (Lu et al., 2020; Mantilla-Calderon et al., 2019; Augsburger et al., 2019; Jin et al., 2020), ED1 strain showed higher transformation efficiency in presence of ALW than treated wastewater. This could be due, on one hand, to a water composition of ALW that was more similar to that of the original habitat of the bacterium; on the other, lower transformation frequencies detected for ED1 strain in presence of treated water than ALW could be related to the peculiar chemical composition of the sampled water (Papageorgiou et al., 2016; Pereira et al., 2015). Thus, we cannot rule out that experiments performed with water collected in different moments could bring the same results. Certain natural and anthropic environments could supply optimal conditions for natural transformation. An example are biofilms in which cell density is very high and cells can be exposed to high concentrations of free DNA (even higher than the ones routinely used in laboratory procedures) derived from the dead neighboring cells (Baur et al., 1996; Hasegawa et al., 2018); this condition can result in ARGs acquisition and spread in the bacterial communities, as characterized in several studies (Petrovich et al., 2018). Moreover, clinically relevant ARGs enter freshwater systems through the outflow of WWTPs (Zhang et al., 2018).

Gram-positive and Gram-negative bacteria that are known to be naturally transformable usually share a similar DNA uptake machinery linked to the Type IV pili and Type II secretion systems (Claverys and Martin, 2003) and both ED1 and K12 NEB DH5α showed the presence of these genes in their genomes. Taking into account the peculiar *E. coli* DNA uptake machineries (Sun, 2018), we found the presence of the genes encoding the transcriptional regulator RpoS that regulates *E. coli* natural transformation (Zhang et al., 2012), as well as the RpoS-regulated genes *ydcV* and *ydcS*, which are involved in the DNA internalization into the inner membrane (Sun, 2016). Although we retrieved in both *E. coli* strain genomes the presence of the above-mentioned genes, we demonstrated that ED1 transformation frequency was higher than DH5α one. Even though we observed an overall genomic function-based similarity between the strains (using the RAST function-based comparison tool), we cannot exclude the existence of some signaling-dependent or regulatory mechanisms that can favor natural transformation in ED1 rather than in DH5α strain. Natural transformation is known to be a very complex mechanism activated differently among species and strains (Blokesch, 2016; Lorenz and Wackernagel, 1994). For instance, in *Haemophilus influenzae* natural competence was demonstrated to be triggered by a lack of phosphotransferase system (PTS) sugars and purine precursors (Mell and Redfield, 2014). Furthermore, since only a DNA-based analysis has been performed in our study, we do not have information about the effective production of the proteins corresponding to the natural transformation-related genes.

Genomic analysis allowed to identify a larger number of genes encoding for metabolic pathways in ED1 genome rather than in the one of K12 NEB DH5α strain, e.g. we found in ED1 genome the propanediol utilization pathway, which allows *E. coli* to grow in anaerobic conditions using rhamnose as carbon source (Liu et al., 2007) and the genes of methilcytrate cycle, which allows microorganisms to use propionate as a carbon/energy sources, being especially useful in the propionate-rich environments such as the

gastrointestinal tract (Upton and McKinney, 2007). Furthermore, we found several genes that may help ED1 to thrive in different habitats i.e. genes encoding for proteins related to the osmotic stress (involved in the synthesis and uptake of compatible solutes; Sim et al., 2014); genes involved in cell to cell aggregation and biofilm production, such as RTX that seems to be responsible for cell-surface adhesions, cells' aggregation and production of biofilm (Tchagang et al., 2018); CFA/I pili-related genes implicated in the bacterial adhesion through the production of fimbriae; and genes encoding the type III secretion injectosome (Zheng et al., 2019; Diepold et al., 2011; Table 1). Therefore, strain ED1 has different traits that may help it to thrive in the environment and that might be related to a high transformation rate success. Moreover, the higher total amount of the mobile genetic elements found in ED1 than in K12 NEB DH5α could be due to the fact that these elements are commonly found in bacteria exposed to a "horizontal gene pool", which can be easily found in several environments (Dobrindt et al., 2004). HGT is, indeed, known to contribute to bacterial adaptation to different habitats and, in the long term, to bacterial evolution (Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005; Vandecraen et al., 2017). This result is also in agreement with the data available on *Vibrio* species, the transformation proficiency of which appears to be more common in environmental strains than in clinical ones (Bernardy et al., 2016).

The environments where *E. coli* is known to survive include soil, water and manure besides several micro-habitats associated to plants, given the ability of some *E. coli* strains to colonize roots, leaf surfaces and endosphere (Van Elsas et al., 2011; Eissenberger et al., 2020; Wright et al., 2017). The capacity of an environmental and naturally transformable *E. coli* strain like ED1 to survive in soil and colonize the plant rhizosphere has relevant implications in the light of the antibiotic cycle and the One-health vision. The plant rhizosphere is indeed a well characterized, substrate-rich, hot spot for bacterial activity and abundance (Zhu et al., 2018), where naturally competent cells can find higher concentrations of free DNA and could, moreover, reach

the growth phase in which transformation occurs with high frequency (Mølbak et al., 2003; Sørensen and Jensen, 1998; Zhu et al., 2018; Ling et al., 2018). Relevant concentrations of ARGs can reach the plant rhizosphere e.g. through soil amended with manure, sewage sludge and treated wastewater (Chen et al., 2017; Wu et al., 2020; Riva et al. 2020). We selected lettuce as a model plant for the root system colonization experiments, as representative of raw-consumed vegetables of high economic importance in the ready-to-eat food industry. Our results showed that ED1 strain colonized efficiently the lettuce rhizosphere both in sterile and non-sterile soils and indicated that the rhizosphere colonization was stable over a period of 14 days. The ability of ED1 strain to acquire exogenous DNA in environmental mimicking conditions and to efficiently colonize the plant rhizosphere might represent a possible route of ARGs spread in the plant microbiome, potentially representing a risk for health through the consumption of raw vegetables (Nüesch-Inderbinen et al., 2015). In this perspective we analyzed the ED1 strain genome for the presence of virulence factors, revealing a higher number of virulence factors in this environmental and naturally competent strain than in the laboratory strain K12 NEB DH5α. Although further analyses are required to unveil any possible relation with human pathogenic *E. coli* strains, these data allow us to hypothesize a low and not relevant virulence for *E. coli* strain ED1 (Supplementary Table 6).

5. Conclusions

We demonstrated the ability of an environmental *E. coli* strain to acquire exogenous DNA by natural competence with relatively high frequency in exponential growth phase in environmental-like conditions, together with its capability, when applied to soil, to thrive in lettuce rhizosphere. These results confirm the importance to further investigate the possible spread of antibiotic resistant determinants through HGT in the environment and, particularly, in the rhizosphere of those plant species consumed as raw vegetables, to

elucidate the related food and human safety risks. Further studies on environmental *E. coli* strains could allow to strengthen our results and to understand the spread of this phenomenon.

6. Supplementary Materials

Supplementary materials are available at the following link:
https://www.frontiersin.org/articles/10.3389/fmicb.2020.574301/full

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Chapter IV

Natural transformation is impaired by Daphnia zooplankton in freshwater microcosms

Abstract

The spread of antibiotic resistance genes (ARGs) by natural transformation is usually underestimated in aquatic environments, although they represent one of the largest ARGs reservoirs. Considering that zooplankton plays a crucial role in aquatic ecosystems and is closely linked to bacteria, this study aims to evaluate the impact of zooplankton on the frequency of bacterial natural transformation. The interaction was studied setting up different microcosms in which the model bacterium *Acinetobacter baylyi* BD413 was subjected to the addition of a plasmid carrying a kanamycin resistance gene, as exogenous DNA, with and without the presence of *Daphnia obtusa*, as model animal of zooplankton. The presence of zooplankton resulted in a decrease of transformation frequency due to plasmid degradation, as supported by PCR and proteomic analyses. This study gives a clue on to the possible role which zooplankton and its resident bacteria might have in the diffusion of antibiotic resistance.

1. Introduction

Horizontal gene transfer (HGT) is defined as the exchange of genetic material across genomes of biological organisms, and it has been reported to occur in bacteria, fungi, and eukaryotic organisms (Tiwari et al., 2020). The three main mechanisms of HGT are conjugation (i.e. DNA transfer mediated by cell-to-cell contact), transduction (i.e. acquisition of DNA by viruses), and natural transformation (i.e. cellular uptake of exogenous DNA) (Hall et al., 2020). Natural competence is indicated as the specific physiological state in which bacterial cells are able to acquire free DNA (Blokesh et al., 2016). Moreover, several naturally competent bacteria develop active DNA-scavenging approaches to optimize the capture and acquisition of extracellular DNA (eDNA), even if it is damaged or is just a small fragment (Blokesh et al., 2016). Usually, the concentration of eDNA in aquatic environment is higher in the sediments than in water columns (Zarey-Baigi and Smith 2021). Once the eDNA is released by cells lysis, it could be present as free-eDNA, which is more easily transported into competent cells but also easier to be degraded within several days, or it could be adsorbed to sediments and humic substances, preventing its degradation by extracellular nucleases present in the environment (Sivalingam et al., 2020). Antibiotic resistant genes (ARGs) could be present as eDNA and could be transferred through natural transformation in different environments (Zarey-Baigi and Smith 2021). Interestingly water environments represent one of the hugest reservoirs of ARGs in which clinical and environmental bacterial can thrive (Abe et al., 2021; Zarey-Baigi and Smith., 2021). Moreover, eARGs, i.e. ARGs present in eDNA, are the dominant ARG fraction in receiving water environments. Several studies underlined that the presence of some pollutants derived from anthropogenic activity (i.e. disinfectants such as chlorine, triclosan and bromoacetic acid) can upregulate transformation and enhance its frequency (Winter et al., 2021). Natural transformation occurs in several complex microenvironments, including some host species: its magnitude in the

spreading of ARGs is underestimated because of the impossibility to track (Winter et al., 2021), facilitating the exchange of ARGs between unrelated species. Acquisition of ARGs through HGT mechanism, including natural transformation, needs to be understood to mitigate the spread of antibiotic resistant determinants derived from the environment among human pathogens (Winter et al., 2021).

Zooplankton plays a crucial role in aquatic ecosystem linking phytoplankton and bacteria to higher trophic levels such as fish (Xiong et al., 2020). Bacteria and zooplankton are closely linked to one another in several ecological functions. Zooplankton can be used as i) refuge: the body of zooplankton can be used as protection against environmental stress; ii) dispersal: migration of zooplankton speeds up the diffusion of bacteria through huge distances or iii) bioreactor: zooplankton bodies enhance growth of the inhabitant and transient bacterial community due to the physical and chemical characteristics (Tang et al., 2010). Since it is known that *Daphnia* unselectively filters water including free organic compounds continuously (Eckert & Pernthaler, 2014), we hypothesized that free DNA would be enriched in the animals and that this could lead to influence natural transformation of bacteria in the gut. We hypothesized that the zooplankton could also have a role on the availability of free DNA, including eARGs, thus impairing their acquisition by freshwater bacteria through natural competence. Due to these reasons the aim of this work was to evaluate the influence of zooplankton on natural transformation. To this aim we used two different model organisms, i.e. *Daphnia obtusa* as the zooplankton model (Tzkazyk et al., 2021) and *Acinetobacter baylyi* BD413 as the bacterial model of natural transformation (Borin et al., 2008; Rizzi et al., 2008; Seinz and Blokesh 2016).

2. Materials and Methods

2.1 *Daphnia obtusa* rearing

D. obtusa derived from a culture maintained in a small garden pond at the CNR-ISE in Verbania. Daphniids were sampled from the pond washed in artificial lake water, ALW (Zotina et al., 2003) and fed with *Kirchneriella* sp. and adapted to laboratory conditions over 48h (Eckert et al., 2016). Before the experiment they were kept without feeding for 12h and washed with ALW.

2.2 Natural transformation of *A. baylyi* BD413 with and without *D. obtusa*.

The study of natural transformation was carried on with *Acinetobacter baylyi* BD413 which was cultured on Luria Bertani (LB) broth (Sigma-Aldrich). Transformation was verified by using, as exogenous DNA, the plasmid pZR80(gfp); plasmid can by selected by its kanamycin resistance gene (Borin et al., 2008). Overnight *A. baylyi* BD413 preculture was inoculated (2:100) in LB medium and incubated at 30°C until cells reached an optical density (OD) at 600 nm between 0.4 and 0.5. Fifty µl of culture were added in a 1.5 ml tube containing 550 µl of artificial lake water (ALW) (Riva et al., 2020) with the addition of i) 200 µl of ALW as control; ii) 4 *D. obtusa* individuals together with 200 µl of ALW in which the daphniids were previously placed for 2h (in 2 ml of ALW volume); and iii) 200 µl of ALW in which 4 *D. obtusa* specimens were previously grown, namely Water surrounding *Daphnia*, WSD. A first evaluation of *A. baylyi* transformation frequencies was performed with different quantities of plasmid pZR80(gfp) to assess the working conditions of our experimental setup (Supplementary material paragraph 1). Two different amounts of plasmid pZR80(gfp), i.e. 0.98 (~ 1) and 1.98 (~ 2) ng) were then selected and added to the tubes and mixed gently (without pipetting to preserve *Daphnia* health). After one hour of incubation in the dark at room temperature, ALW plus *A. baylyi* BD413 was plated on selective plates of LB added with kanamycin (100 µg/ml). An aliquot of each replicate was serially diluted and used to evaluate the total cells' count on LB without selection. Aliquots of negative control (no plasmid) were plated on LB added with kanamycin (100

µg/ml). All plates were incubated at 30 °C for two days. Transformation was confirmed by antibiotic selection and the visualization of green fluorescent cells through fluorescence microscopy. Transformation frequency was calculated as the ratio between the number of transformants and the total number of culturable cells.

2.3 DNA degradation in presence of *D. obtusa*

DNA degradation was studied using the plasmid pCR™II-TOPO® (Invitrogen, Milan, Italy): 3 µg of plasmid (corresponding to 4.54×10^5 copy number/ml) were added into ALW tubes with a growing number of *D. obtusa* (from 0 to 10 for each tube), in order to observe any influence of their presence on the plasmid copy numbers. Microcosms reached a final volume of 1 ml. Following one hour of incubation, plasmid copy numbers were quantified by qPCR (CFX Connect, Bio-rad, Milan, Italy) using SsoAdvanced Universal SYBR Green Supermix (Bio-rad, Milan, Italy) with M13 Forward (−20) (5´-GTA AAA CGA CGG CCA G-3´) and M13 Reverse (5´-CAG GAA ACA GCT ATG AC-3´) primers at 200 nM (annealing temperature 55°C). Moreover, the fate of free DNA (plasmid pCR™II-TOPO®) exposed to the ALW, with a gradient of *Daphnia* density (from 0 to 10 for each 2 ml tube), was evaluated: after one hour from the addition of free DNA in ALW-containing tubes, its amount was measured through qPCR analyses as reported above. The experiment was also performed using a DNA fragment, amplified from pCR™II-TOPO® plasmid with primers M13Forward (-20) and M13Reverse (fragment of 250 bps) by PCR, instead of the plasmid. Standards for qPCR were measured by fluorometric quantification (Qubit, Invitrogen) of the plasmid or amplicon and by sequential dilution of the plasmid or amplicon, respectively.

2.4 Protein isolation and analysis by nano-liquid chromatography high resolution mass spectrometry (nLC-HRMS)

Two samples of 1 ml of ALW in which approximately 100 *D. obtusa* were reared for 4h at room temperature were collected. Samples were first freeze-dried and then resuspended in 20 µl of 50 mM ammonium bicarbonate.

Samples were prepared for proteomics and analyzed at UNITECH OMICs (University of Milano, Italy) with Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with nano electrospray ion source (Supplementary material paragraph 2). The MS data were searched using Thermo Scientific Proteome Discoverer software, version 2.5 (Thermo Scientific, Waltham, MA, USA). The detailed parameters for peptide search and quantification are described in Supplementary material paragraph 3. The database used for protein identification was a custom Uniprot database comprising *Daphnia* sp. proteins and *D. obtusa*-related bacteria (Eckert et al., 2016). For each protein, Gene Ontology Biological Process and Molecular Function terms were retrieved from Uniprot and Quick GO (https://www.ebi.ac.uk/QuickGO/). GO Terms were then clustered by similarity using ReViGo (Supek et al. 2011).

2.5 Statistical Analyses

Statistical analyses were conducted with R 4.1.1 through RStudio (RStudio Team, 2015) and with Calc Statistical Function of Microsoft R Office Excel. To evaluate differences between different treatments for Transformation frequency protocol, when 1 ng of plasmid was added, a Welch Anova and Tukey post hoc analyses were conducted, while an Anova analysis was performed for the data linked to the transformation frequency when 2 ng of plasmid were added (followed by Tukey post hoc test). Possible relation (monotonic function) between concentration of eDNA and Water surrounding *Daphnia* (WSD) and the number of *Daphnia* after qPCR analyses were studied by Spearman's rank correlation coefficient and Pearson correlation coefficient.

3. Results

We first assessed the quantity of DNA to be used in our experimental conditions by exposing *A. baylyi* to increasing DNA quantity (1, 2.5, 5, 10, and

50 ng). This allowed us to specifically select two DNA quantities, the variation of which affected the transformation frequency of the bacterium, i.e. 1 and 2 ng. Indeed, above the DNA quantity threshold of 5 ng we retrieved transformation frequencies of about 10^{-3}, independently of the DNA quantity used and thus making not possible to individuate any variation of transformation frequency related to a possible DNA degradation by the presence of zooplankton (Supplementary Fig. S1). Experiments were then carried out in ALW microcosms, where *A. baylyi* was exposed to plasmidic DNA (1 or 2 ng) with or without *D. obtusa* or with the addition, instead of the daphniids, of a drop of ALW in which four *D. obtusa* individuals were previously reared (namely Water surrounding *Daphnia*, WSD). As shown in Fig. 1A, using an amount of exogenous DNA equal to 1 ng, there were statistically significant differences among all the three different treatments (Welch ANOVA analysis $F_{138,85}$ = 0.001988; Tukey Test is reported in Supplementary Tab. S2): the lowest transformation frequency of *A. baylyi* was found when *Daphnia* individuals were added to the microcosms ($4.41 \times 10^{-6} \pm 1.98 \times 10^{-6}$), whereas in the treatment without the zooplankton the transformation frequency was higher, i.e. $3.63 \times 10^{-4} \pm 3.98 \times 10^{-5}$. The highest concentration frequency was however detected in the microcosms in which WSD was added ($6.76 \times 10^{-4} \pm 1.15 \times 10^{-5}$). The experiment was then performed using a higher quantity of exogenous DNA (2 ng) (Fig. 1B): as when 1 ng of DNA was used, a significant lower transformation frequency was observed in the microcosms with *Daphnia* specimens (Anova analysis $F_{22,591}$ = 0.001611; Tukey test is reported in Supplementary Tab. S3) than the ones without *Daphnia*. Differences in the transformation frequency between the microcosm without *Daphnia* and microcosms added with WSD were not detected in this case (Supplementary Tab. S4).

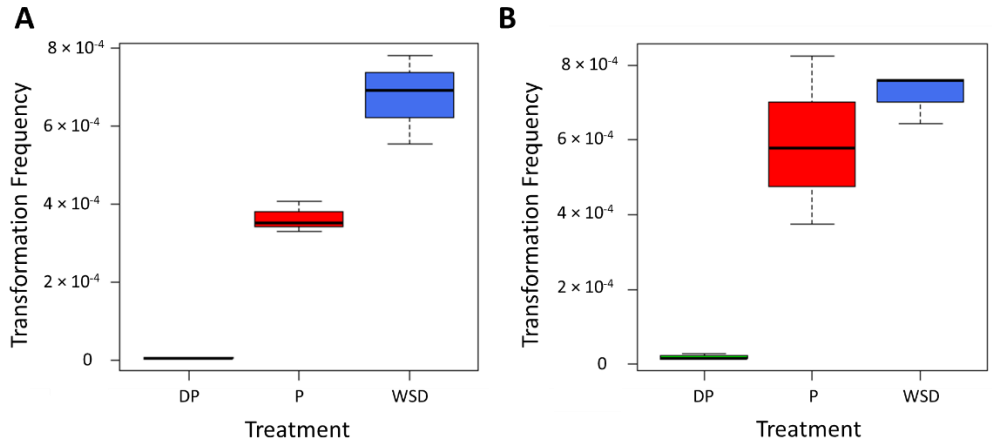

Figure 1. Transformation frequencies of *A. baylyi* BD431 with or without the presence of *Daphnia*. (**A**) Values found adding 1 ng of plasmidic DNA. (**B**) Values found adding 2 ng of plasmidic DNA. DP represents microcosms in which *D. obtusa* was added; P represents microcosms in which *D. obtusa* was not added; WSD represents microcosms in which WSD was added.

Since we retrieved low transformation frequencies when *Daphnia* was added to the microcosms, we evaluated the influence related to its presence in terms of DNA degradation, quantifying the concentration of plasmid pCR™II-TOPO® when exposed to increasing numbers of *Daphnia*. As shown in Fig. 2A, plasmid concentration showed a decreasing trend with a negative correlation (Spearman correlation, r= -0.8481) between the number of *Daphnia* individuals used during the experiments and DNA concentration in the microcosms: the addition of 6 or more daphniids drastically reduced the plasmid concentration in microcosms if compared to the plasmid concentration when no daphniid was added (Fig. 2A). On the other hand, we observed that, when microcosms were amended with WSD, in which increasing amounts of *Daphnia* were previously grown, a trend with a positive correlation (Spearman correlation, r=0.8057) was observed between transformation and animal density (Fig. 2B).

Degradation experiments were also carried out with DNA amplicon fragments, instead of the plasmid, showing a negative correlation with *Daphnia* density

for both gradient of *Daphnia* individuals (Pearson correlation, r=-0.842089574) (Fig. 2C) and WSD (Pearson correlation, r= -0,494727) (Fig. 2D).

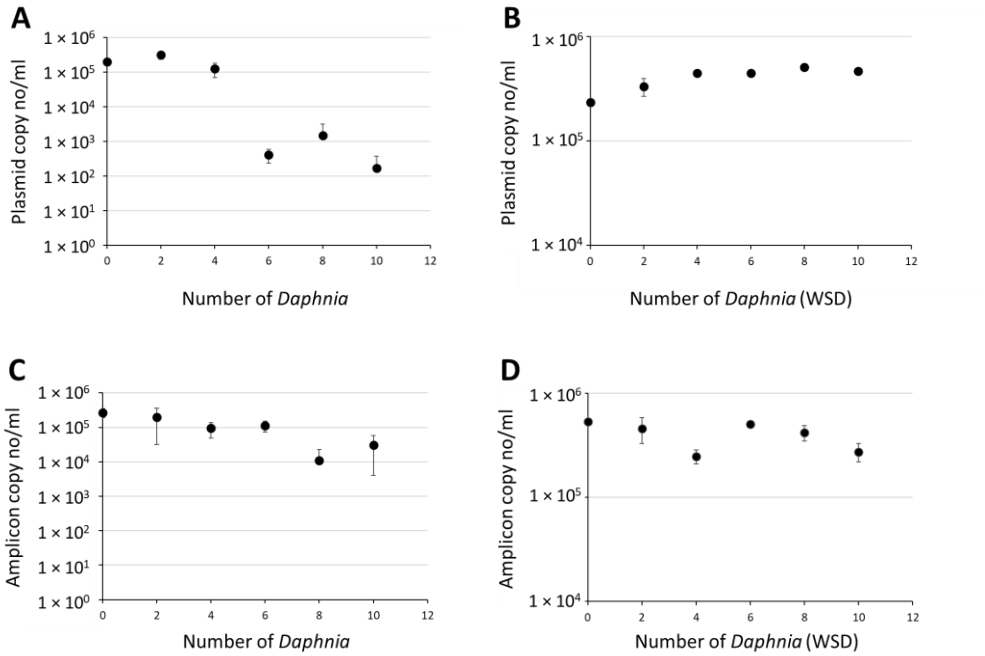

Figure 2. Plasmid DNA concentration (copy numbers/ml) at increasing numbers of *Daphnia* specimens by qPCR. Plasmid concentration in microcosms added (**A**) with increasing number of daphniids and (**B**) with water in which increasing number of daphniids were added. Amplicon concentration in microcosms added (**C**) with increasing number of daphniids and (**D**) with water in which increasing number of daphniids were added.

In order to identify proteins responsible of the decreasing of plasmid concentration when *Daphnia* individuals were added, we isolated and identified the proteins released by the zooplankton in ALW microcosms. By means of nLC-HRMS, 48 *Daphnia* proteins were identified (Supplementary Tab. S5), although 19 proteins are still uncharacterized. Interestingly, we could notice the presence of proteins related to DNA dephosphorylation and DNA degradation: alkaline phosphatase (E9HCS6) and DNA topoisomerase 2 (A0A165ADD4). In addition, we found proteins able to bind DNA: Histone

H3 (E9G2V5) and THAP-type domain-containing protein (E9H1C5). The analysis of Gene Ontology Molecular Functions Term associated to identified protein showed that there were two clusters related to nucleic acid interaction: enzymatic activity on DNA (GO:0003916, GO:0003755, GO:0004386), and nucleic acids binding (GO:0043565, GO:0000166, GO:0003723, GO:0003676, and GO:0003677). Other interesting functions were proteolysis and protein phosphorylation. We also identified 173 proteins of bacteria which have been previously indicated as inhabitants of *D. obtusa* (Eckert et al., 2016; Supplementary Tab. S6). Among these, we could observe the presence of proteins related to DNA degradation: endonuclease from Flavobacteria (A0A1G0AMD6), restriction endonuclease subunit S (Fragment; A0A367MDU0), DNA topoisomerase 1 from *Pseudomonas* (Q26B64), and DNA topoisomerase from Flavobacteria (A0A1W9KR15). Finally, it is noteworthy that there were proteins involved in reactions that could bring to a pH variation of the medium. For instance, among the proteins released by bacteria inhabiting *Daphnia* we found an amidohydrolase (A0A6M3V8B2), an amidase family protein (A0A2R3IW46), and an esterase (A0A315AWB6). The gene ontology analysis of bacterial proteins also showed the presence of proteins involved in acetate metabolism and transport of small molecules, in particular ions.

4. Discussion

The presence of eDNA in freshwater ecosystems is well known and is also due to anthropogenic pollution e.g. the release of treated wastewater in freshwater bodies (Zarey-Baygi et al., 2021).

Since *Daphnia* filters large quantities of water containing eDNA (Eckert et al., 2021), we tested whether a potential association with the animal might influence bacterial transformation to understand its possible role on ARGs spread. Indeed, we observed a difference in transformation frequency of *A. baylyi* BD413, which is well-known for its capability to acquire eDNA by natural

competence (Sainz and Blokesh 2012), in microcosms with or without *D. obtusa*. When *A. baylyi* BD413 was not in presence of *D. obtusa*, it showed a transformation frequency of about 10^{-4}, consistent with results obtained in other studies (Borin et al., 2008). On the other hand, when *D. obtusa* was added to the microcosms the transformation frequency of the bacterium was significantly lower. Acquisition of eDNA by natural transformation usually depends on different conditions, among which there are species specific factors e.g. it is induced by chitin in *Vibrio cholerae* or by starvation in *Bacillus subtilis* and *Haemophilus influenzae* (Zeaiter et al., 2018). Considering that *A. baylyi* BD413 is known to acquire eDNA constitutively (Seinz and Blokesh 2012), we assumed that in this system the limiting factor, which resulted in a lower transformation frequency in microcosms with *D. obtusa*, is represented by the amount of free DNA acquirable by the bacterium. This is also supported by qPCR results: a negative correlation between the concentration of pCR™II-TOPO® and number of *D. obtusa* individuals were found, indicating that the presence of daphniids led to a reduction of DNA available for natural transformation. As reported by Zhu (2016), who observed plasmid degradation in environmental waters, we detected a decrease of plasmid and amplicon concentrations in microcosms added with *D. obtusa*. Since similar total cell counts (on plates without selection) were obtained in all the three treatments (DP, P and WSD, Fig. 1), we excluded any interference due to the daphniid grazing activity on the bacteria.

The hypothesis of DNA degradation related to the presence of zooplankton (and associated bacteria) is further supported by proteomic results. Indeed, we documented the expression of different *Daphnia* sp. proteins, which might be linked to the decrease of eDNA concentration: DNA topoisomerase type II, which are known to generate transient breaks on double stranded DNA (McCledon and Osheroff, 2007) and alkaline phosphatase, which catalyzes the hydrolysis of phosphate monoester at basic pH values (Sharma et al., 2014). Broadening the research also to the bacterial inhabitants of *Daphnia obtusa* (Eckert et al., 2016), proteins related to DNA cleavage and degradation

were identified. Interestingly, we found that the protein with the highest spectral count was recognized as an endonuclease of Flavobacteria affiliated bacteria. The activity of extracellular endonucleases produced by Gram negative bacteria is known to be able to degrade DNA (Vafina et al., 2018) and this enzyme could be a major actor in reducing transformation frequency. Moreover, we identified several proteins i.e. amidases, amidohydrolases and esterases, which could catalyze such reactions releasing by-products that could act acidifying the surroundings at microscale level (Kim and Miura 2004; Wu et al., 2020; Rafeeq et al., 2021), enhancing free DNA degradation (Seymour et al., 2018). The identification of proteins involved in DNA degradation, together with data related to the differences in transformation frequencies in presence or absence of daphniids and qPCR results, allowed us to speculate that *Daphnia* and its inhabitant bacterial community together create an environment in which natural transformation could be inhibited due to enzymatical eDNA degradation. Further studies are needed to deeply characterize the secretome (i.e. protein secreted in water) of *Daphnia*, in order to identify other possible enzyme able to degrade DNA, and clarify the role of *Daphnia*-related bacteria in this process.

Natural transformation in WSD microcosms showed results comparable or higher with the ones obtained for microcosms without the addition of *Daphnia*. This might suggest that *Daphnia* and its related microbiota have a prominent role in DNA degradation, because their presence maybe enhanced, through the passage in the zooplankton gut, a closer interaction among the host, the DNA, the bacteria inhabitants, and released proteins. The difference observed in WSD with the addition of 1 or 2 ng of eDNA (considering that the same quantity of WSD was added in both experiments) could be due to some changes in the topological form of the plasmid (Demanéche et al., 2002; Borin et al., 2008), as suggested by qPCR results which showed a slight increase of the amplified fragment concentration in WSD microcosms added with plasmid, probably caused by a first enzymatic or chemical activity on DNA (which then should lead to degradation), which resulted in an easier

amplification of the plasmid. The increase of the amplified DNA concentration, found by qPCR analyses, in WSD microcosms added with plasmid in respect to the ones added with DNA amplicons could be due to some PCR biases related to DNA conformation (Lin et al., 2011): supercoiled DNA plasmid conformation promotes an overestimation of DNA in qPCR analyses (Hou et al., 2010). If we consider these biases, the data suggest that in WSD microcosms proteins released by daphniids are too diluted to be responsible for DNA degradation or that only a limited amount of DNA is degraded in these microcosms. Nonetheless, results observed in WSD microcosms should be further investigated to take into account all these aspects, from the synergism between *Daphnia* and its microbiota, to the topological isoform of the plasmid and to the use of higher DNA concentrations (or number of daphniids).

5. Conclusions

We showed that *D. obtusa*, together with its inhabitant community, impaired the natural transformation frequency of the constitutively competent bacterium *A. baylyi* BD413 by degrading the exogenous DNA present in the microcosm. Considering that *Daphnia* was indicated as a very promising organism to evaluate the persistence of specific gene (Eckert et al., 2016; Miner et al., 2012), this study helps to unveil the possible role of zooplankton in DNA exchange, also under the perspective to study ARGs diffusion in aquatic environments related to the agri-food system.

Moreover, we show that the presence of *Daphnia* decreases free DNA from the surrounding water, which is often considered an important contaminant of natural water bodies.

6. Supplementary Materials

1. Estimation of transformation frequencies of *Acinetobacter baylyi* BD413 with increasing quantity of DNA

Acinetobacter baylyi BD413 was cultured in Luria Bertani (LB) broth until the reaching of an optical density (OD) at 600 nm between 0.4 and 0.5. Fifty 50 µl of *A. baylyi* BD413 cells were then added to 550 µl of ALW and exposed to a varying amount of pZR80(gfp) i.e. 1, 2.5, 5, 10, and 50 ng. Following one hour of incubation, the mix was plated on LB added with kanamycin 100 µg/ml to count the transformants, whereas serial dilutions were plated on LB without selection to evaluate the total cells' count. All plates were incubated at 30 °C for two days. Transformation was confirmed by antibiotic selection and the visualization of green fluorescent cells through fluorescence microscopy. Transformation frequency was calculated as the ratio between the number of transformants and the total number of culturable cells.

2. Protein samples preparation

Protein concentration was determined by Nanodrop (Thermo Fisher, Italy) at the absorbance of 280 nm, considering a reference standard curve (0.125 - 1.000 µg/µl). Following determination of concentration, each protein sample resuspended in 20 µl of 50 mM ammonium bicarbonate was added of 1 µl of 100 mM DTT (dithiothreitol), incubated 55°C per 30 minutes, and then added of 2 µl of 150 mM IAA (iodoacetamide solution). Samples were then incubated in the dark for 20 minutes. Then, 5 µl of tripsine solution 0.2 µg/µL in 50 mM ammonium bicarbonate were added to the samples and incubated at 37°C overnight. Finally, 1 µL of 50% TFA (trifluoroacetic acid) was added. All samples were subjected to a purification procedure using 5 µg C18 zip-tip according to the following scheme: zip-tip according to procedure on the whole digested sample, concentration of the eluate (speedvac) and recovery of the eluate with 10 µL of 0.1% FA (formic acid).

3. Protein analysis by nano-liquid chromatography high resolution mass spectrometry (nLC-HRMS)

Four µL of each sample were then injected into nLC-HRMS. All samples were analyzed at using Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with nano electrospray ion source. Peptide mixtures were pre-concentrated onto an Acclaim PepMap 100 – 100 µm x 2cm C18 (Thermo Scientific) and separated on EASY-Spray column ES800A, 15 cm x 75 µm ID packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 µm, 100 Å using mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile 20/80, v/v) at a flow rate of 0.300 µL/min. The elution program is summarized in Supplementary Table S1. The temperature was set to 35°C and the sample were injected in duplicates. One blank was run between samples to prevent sample carryover. MS spectra were collected over an m/z range of 375 – 1500 Da at 120,000 resolutions, operating in the data dependent mode, cycle time 3 sec between masters scans. HCD was performed with collision energy set at 35 eV. Polarity: positive.

For data analysis, the MS data were searched using the Sequest HT search engine contained in the Thermo Scientific Proteome Discoverer software, version 2.5 (Thermo Scientific, Waltham, MA, USA). All data generated were searched using the Sequest HT search engine contained in the Proteome Discoverer software, version 2.5. The following criteria were used for the identification of peptide sequences and related proteins: trypsin as enzyme, three missed cleavages per peptide, mass tolerances of ± 20 ppm for precursor ions and ± 0.05 Da for fragment ions. Fixed Value PSM Validator node was used with a target-decoy strategy to give a final false discovery rates (FDR) at Peptide Spectrum Match (PSM) level of 0.01 (strict), considering maximum deltaCN of 0.05. Only peptides with high confidence, minimum peptide length of six amino acids, and rank 1 were considered. Protein grouping and strict parsimony principle were applied.

The experimental MS/MS spectra were correlated to tryptic peptide sequences by comparison with the theoretical mass spectra obtained by in

silico digestion of a custom Uniprot database downloaded in May 2021 (www.uniprot.org) comprising *Daphnia* sp. proteins and *Daphnia obtusa*-related bacteria: *Albidiferax* sp., *Deefgea* sp., *Flavobacteria* sp., *Limnohabitans* sp., *Methylophilus* sp., *Pseudomonas* sp., *Rhodoferax* sp., *Rubrivivax* sp., and *Vogesella* sp. (Eckert et al., 2016) with a total of 223275 entries.

For the quantification of the identified proteins, we used a label-free approach, based on the Peptide spectrum Matches (PSMs), also called spectral count. The discussed results are based on an arithmetic average of the spectral count for each protein in all replicates.

N.	**Time**	**Flow (µL/min)**	**%B**
1	0.000	*Run*	
2	0.000	0.300	4.0
3	3.000	0.300	4.0
4	103.000	0.300	28.0
5	113.000	0.300	40.0
6	114.000	0.300	95.0
7	117.000	0.300	95.0
8	120.000	0.300	4.0
9	123.000	0.300	4.0
10	126.000	0.300	95.0
11	129.000	0.300	95.0
12	132.000	0.300	4.0
13	135.000	0.300	4.0
14	138.000	0.300	95.0
15	141.000	0.300	95.0
16	144.000	0.300	4.0
17	New Row		
18	150.000	*Stop run*	

Table S1. Eluition program of High Resolution Mass Spectrometry analysis (nLC-HRMS)

Comparison between microcosms	p-value
DP-WSD	0.0000573
P-WSD	0.00378555
P-DP	0.0018761

DP: microcosm with the addition of *D. obtusa*; P: microcosm without the addition of *D. obtusa*; WSD: microcosm with the addition of Water surrounding *Daphnia*.

Table S2. Tukey test results obtained between transformation frequency when 1 ng of plasmid pZR80(gfp) was added to the microcosms.

Comparisons between microcosms	p-value
DP-WSD	0.001789
P-WSD	0.5161297
P-DP	0.0050614

DP: microcosm with the addition of *D. obtusa*; P: microcosm without the addition of *D. obtusa*; WSD: microcosm with the addition of Water surrounding *Daphnia*.

Table S3. Tukey test results obtained between transformation frequency when 2 ng of plasmid was added to the microcosms.

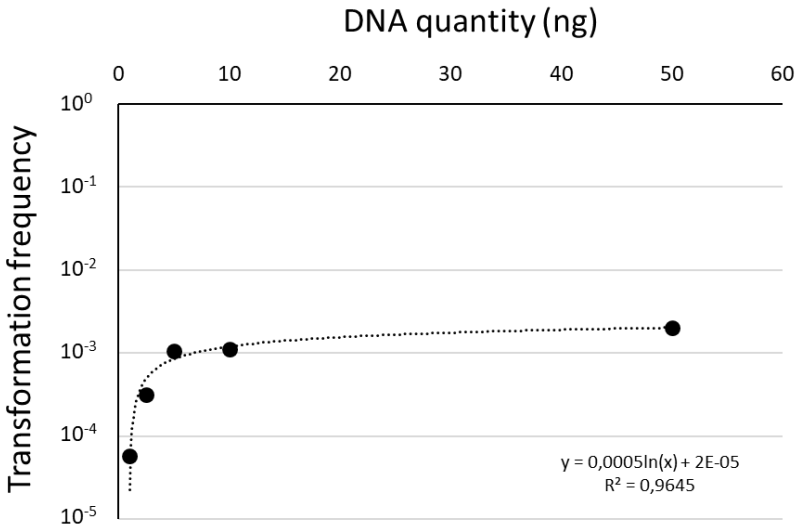

Figure S1: Transformation frequency obtained exposing *A. baylyi* BD413 at different amounts of exogenous DNA (1, 2.5, 5, 10, and 50 ng)

Accession Number	Description	Species	Genus	Gene name	Hits avg A	Hits avg B	Hits avg A+B
E9H8Q4	Uncharacterized protein VTG4	*Daphnia pulex*	*Daphnia*	VTG4	3	1	2
A0A164UQD2	Uncharacterized protein	*Daphnia magna*	*Daphnia*	APZ42_023719	3,5	0	1,75
E9FZS9	Myosin heavy chain isoform 1	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_347733	0	3	1,5
E9I0X9	Uncharacterized protein (Fragment)	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_229994	3	0	1,5
A0A0P5E676	6-phosphogluconolactonase	*Daphnia magna*	*Daphnia*		1	0	0,5
E9HCS6	Alkaline phosphatase	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_328304	1	0	0,5
A0A482DHQ2	ATP-binding cassette sub-family H-like protein 14	*Daphnia magna*	*Daphnia*		1	0	0,5
E9GGL5	DUF4806 domain-containing protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_317655	1	0	0,5
A0A4Y7M515	EOG090X04VI	*Daphnia longispina*	*Daphnia*	EOG090X04VI	0	1	0,5
A0A164S3G3	RNA helicase	*Daphnia magna*	*Daphnia*	APZ42_026680	0	1	0,5
A0A6C0T3C0	Serine protease chymortrypsin 448 (Fragment)	*Daphnia magna*	*Daphnia*		1	0	0,5
J9QSE1	Trypsin 208 (Fragment)	*Daphnia magna*	*Daphnia*		0,5	0,5	0,5
A0A0N8ERA1	Uncharacterized protein	*Daphnia magna*	*Daphnia*		1	0	0,5
E9H6H7	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_200882	1	0	0,5
A0A162BQP5	Uncharacterized protein (Fragment)	*Daphnia magna*	*Daphnia*	APZ42_009530	1	0	0,5
E9HEQ1	WD_REPEATS_REGION domain-containing protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_300684	1	0	0,5
A0A0P6F6K8	Alpha1,3fucosyltransferase	*Daphnia magna*	*Daphnia*		0,5	0	0,25
A0A0P5D7K5	Centrosomal protein of 290 kDa	*Daphnia magna*	*Daphnia*		0,5	0	0,25
A0A0P5V9B4	Chymotrypsin BI	*Daphnia magna*	*Daphnia*	APZ42_019737	0	0,5	0,25
A0A0N8CAN5	Coiled-coil domain-containing protein	*Daphnia magna*	*Daphnia*		0,5	0	0,25
A0A0P5BUI6	Cuticular protein analogous to peritrophins 1-F	*Daphnia magna*	*Daphnia*		0	0,5	0,25
A0A165ADD4	DNA topoisomerase 2	*Daphnia magna*	*Daphnia*	APZ42_016347	0,5	0	0,25
A0A4Y7MP53	EOG090X00FC (Fragment)	*Daphnia magna*	*Daphnia*	EOG090X00FC	0,5	0	0,25
A0A0P5IU70	Fibrillin-1	*Daphnia magna*	*Daphnia*		0	0,5	0,25
E9H1C5	Histone H3	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_231307	0,5	0	0,25

E9H1B1	Methionine aminopeptidase 2	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_57172	0	0,5	0,25
A0A0N8BYN0	Non-specific serine/threonine protein kinase	*Daphnia magna*	*Daphnia*		0,5	0	0,25
A0A4Y7N5E2	Nucleoporin GLE1	*Daphnia similis*	*Daphnia*	EOG090X0755	0	0,5	0,25
E9HNV4	Peptidase_M14 domain-containing protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_130340	0,5	0	0,25
A0A164ZGM4	Phosphatidylinositol 3-kinase catalytic subunit type 3	*Daphnia magna*	*Daphnia*	APZ42_017964	0,5	0	0,25
A0A164QSY7	Probable cytosolic iron-sulfur protein assembly protein Ciao1	*Daphnia magna*	*Daphnia*	Ciao1	0,5	0	0,25
A0A162QI64	Putative Serum response factor binding protein	*Daphnia magna*	*Daphnia*	APZ42_013755	0,5	0	0,25
E9GG91	Serine/threonine-protein phosphatase 2A activator	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_303405	0,5	0	0,25
E9GDV9	Sulfotransfer_1 domain-containing protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_49070	0,5	0	0,25
E9G2V5	THAP-type domain-containing protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_222106	0,5	0	0,25
E9HNZ1	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_262942	0,5	0	0,25
E9H9U9	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_308845	0,5	0	0,25
E9H2S0	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_252473	0	0,5	0,25
E9FYZ0	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_235424	0,5	0	0,25
E9H8N4	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_308685	0	0,5	0,25
E9GL29	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_304363	0	0,5	0,25
A0A0P5F6S4	Uncharacterized protein	*Daphnia magna*	*Daphnia*		0	0,5	0,25
E9FTL9	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_233248	0,5	0	0,25
E9HQX5	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_332773	0	0,5	0,25
A0A164MCM0	Uncharacterized protein	*Daphnia magna*	*Daphnia*	APZ42_031892	0,5	0	0,25
E9FRC9	Uncharacterized protein	*Daphnia pulex*	*Daphnia*	DAPPUDRAFT_232636	0	0,5	0,25
A0A164IDX9	Uncharacterized protein (Fragment)	*Daphnia magna*	*Daphnia*	APZ42_002266	0	0,5	0,25
A0A164HRP6	Uncharacterized protein (Fragment)	*Daphnia magna*	*Daphnia*	APZ42_003175	0	0,5	0,25

Supplemtary Table S6: Daphnia's proteins identified by nano-liquid chromatography high solution mass spectrometry (nLC-HRMS).

Accession Number	Description	*Species*	*Genus*	Gene name	Hits avg A	Hits avg B	Hits avg A+B
A0A1G0AMD6	Endonuclease	*Flavobacteria bacterium GWA2_35_26*	*Flavobacteria*	A2X21_03590	3,5	1	2,25
A0A6L9KPV4	Alpha/beta fold hydrolase	*Rhodoferax sp.*	*Rhodoferax*	GZ093_14035	2,5	1,5	2
A0A315E364	Porin_4 domain-containing protein	*Limnohabitans sp. 2KL-1*	*Limnohabitans*	B9Z47_02940	3	1	2
A0A1G0A8K6	Thiol peroxidase	*Flavobacteria bacterium GWF1_32_7*	*Flavobacteria*	Tpx	2,5	0,5	1,5
A0A2S5IJY3	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	HV87_23835	1,5	1,5	1,5
A0A2S7J781	Deaminase	*Rhodoferax sp. TS-BS-61-7*	*Rhodoferax*	C5F53_16860	1,5	1	1,25
A0A1Q3ZLK0	Chromosome partitioning protein ParB	*Flavobacteriia bacterium 40-80*	*Flavobacteriia*	BGO87_04145	0	2	1
A0A1G0BUV9	Glutamate-1-semialdehyde 2,1-aminomutase	*Flavobacteria bacterium RIFCSPLOWO2_12_FULL_31_7*	*Flavobacteria*	hemL	1	1	1
A0A519EHN8	Pseudouridine synthase	*Rubrivivax sp.*	*Rubrivivax*	EOP37_01175	1	1	1
A0A367MDU0	Restriction endonuclease subunit S (Fragment)	*Pseudomonas aeruginosa*	*Pseudomonas*	DT376_08190	2	0	1
A0A315EV10	Uncharacterized protein	*Limnohabitans curvus*	*Limnohabitans*	B9Z44_09520	1	1	1
A0A520B6G4	ABC transporter ATP-binding protein (Fragment)	*Rubrivivax sp.*	*Rubrivivax*	EOP35_26015	0	1,5	0,75
A0A509JEU5	DUF1329 domain-containing protein	*Pseudomonas aeruginosa*	*Pseudomonas*	CGU42_22245	1,5	0	0,75
A0A2S5IDJ2	Flagellin	*Pseudomonas aeruginosa*	*Pseudomonas*	HV87_15165	0	1,5	0,75
A0A1W9KTF9	Histidine kinase	*Rhodoferax ferrireducens*	*Rhodoferax*	BWK72_12565	1,5	0	0,75
A0A2S5IJB2	Peptidoglycan-associated protein	*Pseudomonas aeruginosa*	*Pseudomonas*	Pal	1	0,5	0,75
A0A437JZY7	4-hydroxythreonine-4-phosphate dehydrogenase PdxA	*Rubrivivax albus*	*Rubrivivax*	pdxA	1	0	0,5
A0A315B7Z2	Aldo/keto reductase	*Limnohabitans sp. MMS-10A-178*	*Limnohabitans*	B9Z32_07955	0,5	0,5	0,5
A0A2S7JYC6	Cation acetate symporter	*Limnohabitans sp. TS-CS-82*	*Limnohabitans*	actP	0,5	0,5	0,5
A0A2S5IE45	Chaperone protein HtpG	*Pseudomonas aeruginosa*	*Pseudomonas*	htpG	0,5	0,5	0,5
A0A7M2ZLN9	Class I SAM-dependent methyltransferase	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC1295_30720	1	0	0,5

Q26B64	DNA topoisomerase 1	*Flavobacteria bacterium (strain BBFL7)*	*Flavobacteria*	topA	0	1	0,5
A0A1G0ACN0	DUF4920 domain-containing protein	*Flavobacteria bacterium GWF1_32_7*	*Flavobacteria*	A2X07_04135	1	0	0,5
A0A5C7XBP9	Efflux RND transporter permease subunit	*Methylophilus sp.*	*Methylophilus*	E6Q52_04415	1	0	0,5
A0A1T1AXP5	Efflux transporter periplasmic adaptor subunit	*Rhodoferax fermentans*	*Rhodoferax*	RF819_04715	0,5	0,5	0,5
A0A1G3HWY5	Globin	*Rhodoferax sp. RIFCSPLOWO2_12_FULL_60_11*	*Rhodoferax*	A3H24_06855	0	1	0,5
A0A0P0MIM7	Glutamine synthetase	*Limnohabitans sp. 103DPR2*	*Limnohabitans*	glnA_2	1	0	0,5
A0A520BGU6	GNAT family N-acetyltransferase	*Rubrivivax sp.*	*Rubrivivax*	EOP35_20525	1	0	0,5
A0A1T1ARS7	Magnesium transporter CorA	*Rhodoferax fermentans*	*Rhodoferax*	RF819_08535	1	0	0,5
A0A080VNC4	Methyl-accepting chemotaxis protein	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC116_08200	0	1	0,5
A0A315CJK7	Molybdopterin converting factor subunit 1	*Limnohabitans sp. Jir61*	*Limnohabitans*	B9Z35_04025	1	0	0,5
A0A315EBK2	Phosphate acyltransferase	*Limnohabitans parvus II-B4*	*Limnohabitans*	plsX	0	1	0,5
A0A2S5I8K2	P-II family nitrogen regulator	*Pseudomonas aeruginosa*	*Pseudomonas*	HV87_05570	1	0	0,5
A0A1E4Q0V5	Poly(A) polymerase I	*Rubrivivax sp. SCN 70-15*	*Rubrivivax*	pcnB	1	0	0,5
A0A432IKH1	Protease (Fragment)	*Flavobacteriia bacterium*	*Flavobacteriia*	DSY82_04730	0,5	0,5	0,5
A0A315DFQ3	Ribosome maturation factor RimP	*Limnohabitans sp. Hippo3*	*Limnohabitans*	rimP	0	1	0,5
A0A315CS30	Transcriptional repressor NrdR	*Limnohabitans sp. Hippo4*	*Limnohabitans*	nrdR	0	1	0,5
A0A2T7T2J3	Translation initiation factor IF-2	*Limnohabitans sp. Rim28*	*Limnohabitans*	infB	1	0	0,5
A0A1P8K9X0	Type IV pili twitching motility protein PilT	*Rhodoferax saidenbachensis*	*Rhodoferax*	RS694_09690	1	0	0,5
A0A315B892	Uncharacterized protein	*Limnohabitans sp. WS1*	*Limnohabitans*	B9Z48_08860	1	0	0,5
A0A2S5I7C3	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	HV87_03150	0	1	0,5
A0A1P8K2A5	Uncharacterized protein	*Rhodoferax koreense*	*Rhodoferax*	RD110_25480	1	0	0,5
A0A1P8JQV4	Uncharacterized protein	*Rhodoferax koreense*	*Rhodoferax*	RD110_02060	0,5	0,5	0,5
A0A1G0A9E9	Uroporphyrinogen decarboxylase	*Flavobacteria bacterium GWF1_32_7*	*Flavobacteria*	hemE	1	0	0,5
A0A6M8SMB9	Rha family transcriptional regulator	*Deefgea sp. D17*	*Deefgea*	HQN60_00140	0,5	0	0,25

A0A315CB17	(2Fe-2S)-binding protein	*Limnohabitans sp. Jir61*	*Limnohabitans*	B9Z35_12370	0,5	0	0,25
A0A315D0R7	1-deoxy-D-xylulose-5-phosphate synthase	*Limnohabitans sp. Hippo4*	*Limnohabitans*	Dxs	0,5	0	0,25
A0A315B9R8	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	*Limnohabitans sp. MMS-10A-178*	*Limnohabitans*	B9Z32_03120	0,5	0	0,25
A0A315D4E8	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	*Limnohabitans sp. MMS-10A-160*	*Limnohabitans*	B9Z38_14865	0,5	0	0,25
A0A432ICX2	4-hydroxybutyrate CoA-transferase	*Flavobacteriia bacterium*	*Flavobacteriia*	DSY82_07495	0	0,5	0,25
A0A1Q8YIS9	50S ribosomal protein L23	*Rhodoferax antarcticus ANT.BR*	*Rhodoferax*	rplW	0	0,5	0,25
A0A315CRK3	ABC transporter ATP-binding protein	*Limnohabitans sp. Hippo4*	*Limnohabitans*	B9Z46_06180	0,5	0	0,25
A0A519G827	ABC transporter ATP-binding protein	*Rubrivivax sp.*	*Rubrivivax*	EOP38_10225	0,5	0	0,25
A0A315AQX8	ABC transporter permease	*Limnohabitans sp. T6-5*	*Limnohabitans*	B9Z51_09635	0	0,5	0,25
A0A2S7JRH1	ABC transporter substrate-binding protein	*Limnohabitans sp. TS-CS-82*	*Limnohabitans*	C5F52_12080	0	0,5	0,25
A0A3E1RJ34	ABC transporter substrate-binding protein	*Rhodoferax sp. IMCC26218*	*Rhodoferax*	DIC66_01560	0,5	0	0,25
A0A1C6LY13	ABC-type amino acid transport substrate-binding protein	*Vogesella sp. LIG4*	*Vogesella*	PSELUDRAFT_0970	0	0,5	0,25
A0A6N0BRT9	Acyl-CoA dehydrogenase	*Rhodoferax sp. BAB1*	*Rhodoferax*	HTY51_10155	0	0,5	0,25
A0A2R3IW46	Amidase family protein	*Pseudomonas aeruginosa*	*Pseudomonas*	CSB93_1441	0,5	0	0,25
A0A6M3V8B2	Amidohydrolase	*Pseudomonas aeruginosa*	*Pseudomonas*	ytcJ2	0	0,5	0,25
A0A1P8K4B5	Anti-anti-sigma factor	*Rhodoferax koreense*	*Rhodoferax*	RD110_20595	0	0,5	0,25
A0A515EMC3	Aspartate-semialdehyde dehydrogenase	*Rhodoferax sediminis*	*Rhodoferax*	Asd	0,5	0	0,25
A0A0P0MHQ4	ATP-dependent RNA helicase RhlE	*Limnohabitans sp. 103DPR2*	*Limnohabitans*	rhlE_4	0	0,5	0,25
C0BL61	Beta-lactamase	*Flavobacteria bacterium MS024-3C*	*Flavobacteria*	Flav3CDRAFT_1309	0,5	0	0,25
A0A3D5VKF6	Bifunctional rhamnulose-1-phosphate aldolase/short-chain dehydrogenase	*Rhodoferax sp.*	*Rhodoferax*	DHV01_01200	0	0,5	0,25
A0A3S3VIC1	Carboxymuconolactone decarboxylase family protein	*Pseudomonas aeruginosa*	*Pseudomonas*	PA52Ts2_6076	0	0,5	0,25

A0A6N0BTL9	Class II aldolase/adducin family protein	*Rhodoferax sp. BAB1*	*Rhodoferax*	HTY51_16300	0,5	0	0,25
A0A1P8K647	Conjugal transfer protein TraR	*Rhodoferax saidenbachensis*	*Rhodoferax*	RS694_02070	0,5	0	0,25
A0A1P8KDC1	C-type cytochrome biogenesis protein CcsB	*Rhodoferax saidenbachensis*	*Rhodoferax*	RS694_16395	0	0,5	0,25
A0A6N0BQ42	Cysteine--tRNA ligase	*Rhodoferax sp. BAB1*	*Rhodoferax*	cysS	0,5	0	0,25
A0A2S7JKK5	Cytochrome c-type biogenesis protein	*Limnohabitans sp. TS-CS-82*	*Limnohabitans*	C5F52_23445	0	0,5	0,25
A0A3S2UAK9	D-alanine--D-alanine ligase	*Rubrivivax albus*	*Rubrivivax*	Ddl	0,5	0	0,25
A0A5B8CRC7	Dephospho-CoA kinase	*Methylophilus medardicus*	*Methylophilus*	coaE	0,5	0	0,25
A0A3E1R7A8	Dihydroorotase	*Rhodoferax sp. IMCC26218*	*Rhodoferax*	DIC66_20150	0	0,5	0,25
A0A1Q3ZL86	DNA alkylation repair protein	*Flavobacteriia bacterium 40-80*	*Flavobacteriia*	BGO87_03420	0	0,5	0,25
A0A515EMB3	DNA mismatch repair protein MutS	*Rhodoferax sediminis*	*Rhodoferax*	EXZ61_06215	0	0,5	0,25
A0A1W9KR15	DNA topoisomerase	*Rhodoferax ferrireducens*	*Rhodoferax*	BWK72_18050	0,5	0	0,25
A0A519ILW4	DUF3619 family protein	*Rubrivivax sp.*	*Rubrivivax*	EOP39_00750	0,5	0	0,25
A0A2T7UDQ8	DUF490 domain-containing protein	*Limnohabitans planktonicus II-D5*	*Limnohabitans*	H663_010615	0,5	0	0,25
A0A5C7T6D4	EAL domain-containing protein	*Rhodoferax sp.*	*Rhodoferax*	E6Q78_01930	0	0,5	0,25
A0A519HFD5	Efflux RND transporter periplasmic adaptor subunit (Fragment)	*Rubrivivax sp.*	*Rubrivivax*	EOP39_25760	0,5	0	0,25
A0A520C374	Elongation factor P	*Rubrivivax sp.*	*Rubrivivax*	Efp	0	0,5	0,25
A0A315AWB6	Esterase	*Limnohabitans sp. MMS-10A-178*	*Limnohabitans*	B9Z32_11730	0,5	0	0,25
A0A1W9KVR7	Exopolyphosphatase	*Rhodoferax ferrireducens*	*Rhodoferax*	BWK72_06685	0	0,5	0,25
A0A2V3GFS3	ExsD (Fragment)	*Pseudomonas aeruginosa*	*Pseudomonas*	C0044_12825	0,5	0	0,25
A0A519YZA5	Fasciclin domain-containing protein	*Rubrivivax sp.*	*Rubrivivax*	EOP36_14285	0,5	0	0,25
A0A315EHB4	Flagellin	*Limnohabitans sp. Rim8*	*Limnohabitans*	B9Z36_09835	0	0,5	0,25
A0A5C7YVA9	FtsX-like permease family protein	*Limnohabitans sp.*	*Limnohabitans*	E6Q48_03640	0	0,5	0,25
A0A1P8JUK9	Glutamyl-tRNA amidotransferase	*Rhodoferax koreense*	*Rhodoferax*	RD110_09880	0	0,5	0,25
A0A2K4Y2T9	Helicase ATP-binding domain-containing protein	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC669_33595	0	0,5	0,25
A0A519G1H7	Histidine kinase	*Rubrivivax sp.*	*Rubrivivax*	EOP38_17910	0	0,5	0,25

A0A519ECD6	Histidine kinase	*Rubrivivax sp.*	*Rubrivivax*	EOP37_08330	0	0,5	0,25
F3LVN5	Histidine kinase	*Rubrivivax benzoatilyticus JA2 = ATCC BAA-35*	*Rubrivivax*	RBXJA2T_18824	0	0,5	0,25
I0HX06	Histidine kinase	*Rubrivivax gelatinosus (strain NBRC 100245 / IL144)*	*Rubrivivax*	pill	0,5	0	0,25
A0A495B3P2	Histidine kinase	*Vogesella indigofera*	*Vogesella*	C8E02_2974	0,5	0	0,25
A0A495AVY1	Histidine kinase	*Vogesella indigofera*	*Vogesella*	C8E02_3339	0,5	0	0,25
A0A519I7U4	Histidine kinase (Fragment)	*Rubrivivax sp.*	*Rubrivivax*	EOP39_13430	0	0,5	0,25
A0A519ZIJ7	Histidine kinase (Fragment)	*Rubrivivax sp.*	*Rubrivivax*	EOP40_13355	0,5	0	0,25
A0A315D7L3	Hydroxyacid dehydrogenase	*Limnohabitans sp. Bal53*	*Limnohabitans*	B9Z50_08860	0,5	0	0,25
A0A1E4PSB2	Hydroxyacid dehydrogenase	*Rubrivivax sp. SCN 70-15*	*Rubrivivax*	ABT20_06455	0	0,5	0,25
A0A6N0TMD0	L-aspartate oxidase	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC1505_22515	0,5	0	0,25
I0HQZ5	Lipoprotein	*Rubrivivax gelatinosus (strain NBRC 100245 / IL144)*	*Rubrivivax*	RGE_20910	0,5	0	0,25
A0A1P8KEZ0	LPS-assembly protein LptD	*Rhodoferax saidenbachensis*	*Rhodoferax*	lptD	0	0,5	0,25
A0A1C6N5R3	L-threonine dehydratase	*Vogesella sp. LIG4*	*Vogesella*	ilvA	0,5	0	0,25
A0A2M6VH49	Maltoporin	*Limnohabitans sp. B9-3*	*Limnohabitans*	B9Z42_04910	0,5	0	0,25
A0A2S5I8I1	Methionine ABC transporter substrate-binding protein	*Pseudomonas aeruginosa*	*Pseudomonas*	HV87_05220	0,5	0	0,25
A0A485I135	Methyltransferase	*Pseudomonas aeruginosa*	*Pseudomonas*	NCTC13621_06095	0	0,5	0,25
A0A5B8CTK0	MoxR family ATPase	*Methylophilus medardicus*	*Methylophilus*	FIU01_09055	0,5	0	0,25
A0A367M9U7	NAD(P)/FAD-dependent oxidoreductase (Fragment)	*Pseudomonas aeruginosa*	*Pseudomonas*	DT376_13880	0,5	0	0,25
A0A1G3IJ38	NADH-quinone oxidoreductase subunit I	*Rhodoferax sp. RIFCSPLOWO2_12_FULL_60_11*	*Rhodoferax*	nuoI	0	0,5	0,25
A0A515EUY3	NADPH-dependent 2,4-dienoyl-CoA reductase	*Rhodoferax sediminis*	*Rhodoferax*	EXZ61_21355	0	0,5	0,25
A0A515DES8	NADPH-dependent 7-cyano-7-deazaguanine reductase	*Rhodoferax sediminis*	*Rhodoferax*	queF	0,5	0	0,25
A0A515EKY4	NodB homology domain-containing protein	*Rhodoferax sediminis*	*Rhodoferax*	EXZ61_03550	0,5	0	0,25

A0A520BHM2	Non-specific serine/threonine protein kinase (Fragment)	*Rubrivivax sp.*	*Rubrivivax*	EOP35_20000	0,5	0	0,25
A0A2S5IC52	Nucleoside diphosphate kinase	*Pseudomonas aeruginosa*	*Pseudomonas*	Ndk	0	0,5	0,25
A0A315CW04	NUDIX hydrolase	*Limnohabitans sp. Hippo4*	*Limnohabitans*	B9Z46_06970	0	0,5	0,25
A0A315AXZ6	Oligopeptidase A	*Limnohabitans sp. MMS-10A-178*	*Limnohabitans*	B9Z32_09690	0,5	0	0,25
A0A519YYC7	Oxoglutarate dehydrogenase (succinyl-transferring)	*Rubrivivax sp.*	*Rubrivivax*	EOP36_11960	0	0,5	0,25
A0A519HTZ8	PAS domain-containing protein (Fragment)	*Rubrivivax sp.*	*Rubrivivax*	EOP39_21295	0	0,5	0,25
A0A515D6M1	Patatin	*Rhodoferax sediminis*	*Rhodoferax*	EUB48_00995	0	0,5	0,25
A0A3S2UPM3	PEP-CTERM sorting domain-containing protein	*Rubrivivax albus*	*Rubrivivax*	ENE75_14830	0,5	0	0,25
A0A1E4PTU2	Peptidase S41	*Rubrivivax sp. SCN 70-15*	*Rubrivivax*	ABT20_05450	0,5	0	0,25
A0A3E1MTT2	Peptidase S8 and S53 subtilisin kexin sedolisin (Fragment)	*Pseudomonas aeruginosa*	*Pseudomonas*	DZ962_33275	0,5	0	0,25
A0A1G0B0W7	Peptidase_M23 domain-containing protein	*Flavobacteria bacterium RIFCSPLOWO2_12_FULL_35_11*	*Flavobacteria*	A3F91_13305	0	0,5	0,25
A0A0P0M7J7	Peripla_BP_6 domain-containing protein	*Limnohabitans sp. 63ED37-2*	*Limnohabitans*	L63ED372_00781	0,5	0	0,25
A0A2S7JRC7	Phage virion morphogenesis protein	*Limnohabitans sp. TS-CS-82*	*Limnohabitans*	C5F52_14475	0	0,5	0,25
A0A2S7JH21	Phosphatase	*Rhodoferax sp. TS-BS-61-7*	*Rhodoferax*	C5F53_04425	0,5	0	0,25
A0A1T1ARH3	Phosphoenolpyruvate--protein phosphotransferase	*Rhodoferax fermentans*	*Rhodoferax*	RF819_07950	0	0,5	0,25
A0A515ET29	PLP-dependent aminotransferase family protein	*Rhodoferax sediminis*	*Rhodoferax*	EXZ61_17520	0,5	0	0,25
A0A2T7UBV5	Polyphosphate kinase	*Limnohabitans planktonicus II-D5*	*Limnohabitans*	Ppk	0	0,5	0,25
F3LM63	ProQ domain-containing protein	*Rubrivivax benzoatilyticus JA2 = ATCC BAA-35*	*Rubrivivax*	RBXJA2T_03848	0,5	0	0,25
A0A3S2TT44	Pseudouridine synthase	*Rubrivivax albus*	*Rubrivivax*	ENE75_04735	0,5	0	0,25
A0A2U0YBE9	Putative Zn-dependent protease	*Rhodoferax sp. YR267*	*Rhodoferax*	C7516_110117	0,5	0	0,25
A0A519E1M5	Pyruvate kinase	*Rubrivivax sp.*	*Rubrivivax*	Pyk	0	0,5	0,25

A0A0P0MER5	RecBCD enzyme subunit RecC	*Limnohabitans sp. 103DPR2*	*Limnohabitans*	recC	0	0,5	0,25
A0A0P0MHK1	Riboflavin biosynthesis protein	*Limnohabitans sp. 103DPR2*	*Limnohabitans*	ribF	0,5	0	0,25
A0A1G0AJS3	Ribonuclease R	*Flavobacteria bacterium GWA2_35_26*	*Flavobacteria*	Rnr	0,5	0	0,25
A0A515EUR9	Sensor domain-containing diguanylate cyclase	*Rhodoferax sediminis*	*Rhodoferax*	EXZ61_20940	0	0,5	0,25
A0A315B0L8	Short-chain dehydrogenase	*Limnohabitans sp. MMS-10A-178*	*Limnohabitans*	B9Z32_09670	0	0,5	0,25
A0A1P8K3M7	Short-chain dehydrogenase	*Rhodoferax koreense*	*Rhodoferax*	RD110_10385	0	0,5	0,25
A0A7M3ATV8	Sigma-54-dependent Fis family transcriptional regulator	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC737_25665	0	0,5	0,25
A0A7M3F4J6	TolC family protein	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC151_01255	0	0,5	0,25
A0A1G0ANJ3	TonB-dependent receptor	*Flavobacteria bacterium GWA2_35_26*	*Flavobacteria*	A2X21_01810	0	0,5	0,25
A0A315AX81	TPR_REGION domain-containing protein	*Limnohabitans sp. WS1*	*Limnohabitans*	B9Z48_15300	0,5	0	0,25
A0A1E4IGJ6	TPR_REGION domain-containing protein	*Rubrivivax sp. SCN 71-131*	*Rubrivivax*	ABS84_12765	0,5	0	0,25
A0A315BBN9	Transcriptional regulator	*Limnohabitans sp. MMS-10A-178*	*Limnohabitans*	B9Z32_04425	0,5	0	0,25
A0A2T7SYG7	Transcriptional regulator	*Limnohabitans sp. Rim28*	*Limnohabitans*	B472_05870	0,5	0	0,25
A0A1E4PVV2	Transcriptional regulator	*Rubrivivax sp. SCN 70-15*	*Rubrivivax*	ABT20_03990	0,5	0	0,25
A0A1Q8YDS9	Transcriptional regulator, LysR family	*Rhodoferax antarcticus ANT.BR*	*Rhodoferax*	BLL52_2439	0	0,5	0,25
A0A432IR67	Triosephosphate isomerase	*Flavobacteriia bacterium*	*Flavobacteriia*	tpiA	0,5	0	0,25
A0A3D5VSG8	tRNA pseudouridine synthase A	*Rhodoferax sp.*	*Rhodoferax*	truA	0,5	0	0,25
A0A5C7Z7I5	Type I secretion system permease/ATPase	*Limnohabitans sp.*	*Limnohabitans*	E6Q49_12550	0	0,5	0,25
A0A1Q3ZEX6	Uncharacterized protein	*Flavobacteriia bacterium 40-80*	*Flavobacteriia*	BGO87_09320	0	0,5	0,25
A0A1Q3ZHM4	Uncharacterized protein	*Flavobacteriia bacterium 40-80*	*Flavobacteriia*	BGO87_12575	0	0,5	0,25
A0A2M6VS40	Uncharacterized protein	*Limnohabitans sp. 15K*	*Limnohabitans*	B9Z40_14460	0,5	0	0,25
A0A315C918	Uncharacterized protein	*Limnohabitans sp. JirII-29*	*Limnohabitans*	B9Z39_09175	0	0,5	0,25
A0A315BHS6	Uncharacterized protein	*Limnohabitans sp. WS1*	*Limnohabitans*	B9Z48_07200	0	0,5	0,25

A0A315E6I4	Uncharacterized protein	*Limnohabitans sp. 2KL-51*	*Limnohabitans*	B9Z49_01790	0	0,5	0,25
A0A0C6EU03	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	DT376_17850	0,5	0	0,25
B3G221	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC1323_31795	0,5	0	0,25
A0A2K4XHI8	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	RW109_RW109_00118	0,5	0	0,25
A0A5F1BJU4	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	ECC04_025620	0,5	0	0,25
A0A241XSN9	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	CAZ10_11105	0,5	0	0,25
A0A2S5IG85	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	HV87_20230	0	0,5	0,25
A0A7M3B4T6	Uncharacterized protein	*Pseudomonas aeruginosa*	*Pseudomonas*	IPC737_01695	0	0,5	0,25
A0A1P8JZ07	Uncharacterized protein	*Rhodoferax koreense*	*Rhodoferax*	RD110_18700	0,5	0	0,25
A0A1T1AW00	Uncharacterized protein	*Rhodoferax fermentans*	*Rhodoferax*	RF819_17650	0	0,5	0,25
A0A6L9KWP7	Uncharacterized protein	*Rhodoferax sp.*	*Rhodoferax*	GZ093_16075	0	0,5	0,25
A0A437JUZ4	Uncharacterized protein	*Rubrivivax albus*	*Rubrivivax*	ENE75_14910	0	0,5	0,25
A0A519EDW6	Uncharacterized protein	*Rubrivivax sp.*	*Rubrivivax*	EOP37_06815	0	0,5	0,25
A0A519FZF3	XRE family transcriptional regulator	*Rubrivivax sp.*	*Rubrivivax*	EOP38_20490	0,5	0	0,25
A0A519HA57	YkgJ family cysteine cluster protein	*Rubrivivax sp.*	*Rubrivivax*	EOP39_28260	0,5	0	0,25
A0A1H0W6E5	Phosphoglycolate phosphatase	*Albidiferax sp. OV413*	*Albidiferax*	SAMN05216303_10873	0,5	0	0,25

Supplemtary Table S6: proteins of bacteria which have been previously indicated as inhabitants of *D. obtusa* identified by nano-liquid chromatography high solution mass spectrometry (nLC-HRMS).

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Chapter V

Construction of an environmental donor strain to evaluate the conjugal plasmid transfer in lettuce rhizosphere

Abstract

The diffusion of antibiotic resistance in the agri-food system is a public issue for food safety and global health. The reuse of treated wastewater for irrigation purpose is a common practice, but the presence of antibiotic resistance genes (ARGs) could contribute to the spread of antibiotic resistance, through horizontal gene transfer (HGT), posing a risk on food safety e.g. by eating raw vegetables. The study of AR determinants' spread through conjugation in the agri-food system, especially in plant's environments, is mandatory to understand its magnitude. Aim of the study was to construct a donor strain isolated from treated wastewater to study conjugal transfers to the bacterial communities inhabiting different plant's niches (i.e. rhizosphere), to simulate the possible spread of ARGs due to irrigation. Particularly, the strain of *Klebsiella variicola* subsp. *variicola* EEF15 was prepared taking advantage of the insertion on the chromosome of a cassette with a constitutively expressed red fluorescent protein (mCherry), and of the insertion of a mobilizable plasmid carrying a green fluorescent protein (Gfp), under the control of a repressible promoter, to track the conjugal events. Moreover, the strain ability to survive in lettuce rhizosphere, used as model for raw-eaten vegetables, was verified, making the strain *Klebsiella variicola* subsp. *variicola* EEF15::lacI^{q}-pLppmCherry-Gent^{R} with the plasmid pKJK5::gfp the perfect candidate to study ARGs spread through conjugation in the agri-food system.

1. Introduction

The diffusion of antibiotic resistant determinants in several environments linked to the agri-food system is a public concern for food safety and global health. Horizontal gene transfer (HGT) could have an important role in the spread of antibiotic resistance genes (ARGs) (Smalla et al., 2018), as it can be enhanced in specific hot spots such as mycosphere, residuesphere, and wastewater treatment plants (WWTPs) (Riva et al., 2020a). For instance, it has been found that treated wastewater form WWTPs can promote the stabilization of a resistome in freshwater bodies (Corno et al., 2019; Di Cesare et al., 2016). Water reuse is becoming a common practice to combat the water crisis caused by global warming (Riva et al., 2020a). The reuse of treated wastewater for irrigation purposes could contribute to the diffusion of ARGs which could enter into the agri-food system and could potentially be acquired by pathogenic strains (Riva et al., 2020b). The presence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) belonging to potential pathogenic bacteria in fresh products and farm environment have been found (Perera et al., 2020; Araújo et al., 2017; Yang et al., 2020; Nüesch-Inderbinen et al. 2015; Schierstaedt et al., 2019; Cerqueira et al., 2019). Moreover, the ability of several ARB and ARGs to spread and survive in several plant's microenvironments have been demonstrated (Xu et al. 2021; Cernava et al., 2019; Zhang et al., 2020).

Antibiotic resistance genes are present both as intracellular (iARGs) and extracellular ARGs (eARGs). Usually, iARGs are more abundant in rich environments such as manure, sewage sludge or sediment and they are usually spread by conjugation and transduction; moreover, the persistence of host range plasmids in soil and their diffusion in the inhabitant community have been demonstrated (Fan et al., 2019; Klumper et al., 2015; Zarey-Baigi et al., 2021). Conjugation occurs in several environments and depends on several factors including. i) genetic background of donor and recipients, ii) environmental characteristics, such as temperature, cell to cell contact

opportunity and nutrient availability, iii) plasmid characteristics (Zarei-Baigi et al., 2021), iv) selection pressure, such as the presence of emerging micropollutants (Feng et al., 2021). Conjugation without antibiotic selection between a laboratory-derived donor strain and a complex community or in complex environments have been already investigated taking advantage of fluorescent proteins that allowed to recognize donors from the transconjugants (Klumper et al., 2015; Berthold et al., 2016; Musovic et al., 2010; Fan et al., 2019).

Rhizosphere is defined as the small portion of soil attached to the roots of the plants. Looking at this plant's microenvironment in a wider way, it could be defined as the soil influenced by the root: plants give to the soil the basic resources and there is an exchange between organic and inorganic pool mediated by microbes, with implications for climatic change, food security, gas emission, soil fertilization and carbon sequestration (York et al., 2016). Plant could be defined as an holobiont with its bacterial community, especially with rhizosphere: plant associated microbes could be considered as a second genome of plants, contributing to respond to nutrient acquisition, to pathogen suppression and environmental stress factors such as drought (Xun et a., 2021). The high content of nutrients, together with water fluxes and bacterial mobility make rhizosphere a hot spot of HGT (Van Elsas et al., 2003). Due to all these evidences i.e. i) the presence of ARGs and ARB in soil and treated wastewater which could be used for crops irrigation, and ii) the high exchange of genetic material in the rhizosphere, the aim of this study was to construct a bacterial donor strain of environmental origin (isolated from treated wastewater) belonging to genus *Klebsiella*, to study conjugal transfer with the inhabitant community of the rhizosphere. Specifically, the preparation of a strain derived from treated wastewater was done to simulate the spread of ARGs from an environmental *Enterobacteriaceae* member to the rhizospheric bacterial community of lettuce, used as model of raw-eaten vegetables.

Klebsiella genus was chosen because it is a member of the family of *Enterobacteriaceae* and it is considered an emerging pathogen for humans.

Moreover *K. variicola* has been isolated form several different sources such as plants microenvironments e.g. rhizosphere, humans, insects and animals and several evidences of kingdom-crossing from plants to humans have been found. Moreover, together with its pathogenicity and its wide distribution, it is intrinsically resistant to ampicillin (Barrios-Camacho et al., 2019; Rodriguez-Medina et al., 2019).

2. Materials and Methods

2.1 Bacteria isolation and identification

1.1 L of water collected from the effluent from the largest municipal WWTP of Milan municipality (Milano-Nosedo, Northern Italy) were filtered through a 0.22 µm membrane filter GSWP04700 by vacuum pomp. Bacteria on the filter were resuspended in saline solution (0.9% NaCl), serially diluted, plated on Violet Red Bile Lactose Agar medium added with 0.1g/L of cycloheximide and incubated at 30°C for 48h. Single colonies were randomly picked up and spread three times on the same medium in order to obtain pure cultures. Pure cultures were stored at -80°C in 20% glycerol. Genomic DNA was extracted by boiling cell lysis (Ferjani et al., 2015) and identified by 16S rRNA gene sequencing analyses through BLAST alignment on the NCBI database (Riva et al., 2021).

2.2 Lettuce plants and agricultural soils

Experiments were conducted with two different set-ups. Survival experiments in lettuce's rhizosphere were carried out with already grown plant of *Lactuca sativa* transplanted in agricultural soil collected in Triuggio (Monza and Brianza, Italy), while filter mating conjugation experiments between the donor strain and the inhabitant community of lettuce rhizosphere were carried out with already grown *Lactuca sativa* transplanted in agricultural soil collected from CRUCIAL (Closing the Rural Urban Nutrient Cycle) agricultural field site (Taastrup, Denmark) from a plot fertilized by NPK. All the experiments were performed at least one week after the transplantation of lettuce plants in the

agricultural soil. Lettuce plants were grown as described by Riva et al. (2020b).

2.3 Root survival by *Klebsiella variicola* subsp. *variicola* EEF15

The rifampicin-resistant mutant, RIF-R *Klebisella variicola* subsp. *variicola* EEF15, was obtained as described in Riva et al. (2020b). RIF-R *Klebisella variicola* subs *variicola* EEF15 was used to verify its ability to survive in lettuce rhizosphere. Plants were bacterized with 1×10^8 and 1×10^9 cells of EEF15 RIF-R per gram of soils; indicatively 300 grams of soil were added per pot. After one week rhizosphere was collected (Marasco et al., 2018) and the presence of EEF15 RIF-R was assessed by plating (Riva et al., 2020b).

2.4 Donor strain construction

Among the strains isolated from the WWTP effluent, the strain *Klebsiella variicola* subsp. *variicola* named EEF15 was selected to be used as donor strain for conjugation studies. Following the evaluation of its sensitivity to kanamycin (100 µL/ml) and gentamycin (100 µL/ml) (Klumper et al., 2015), EEF15 was i) chromosomally tagged with a cassette containing the gene encoding for a mCherry, a constitutive expressed $LacI^q$ gene and a gentamycin resistance gene; ii) supplemented with a host broad range plasmid namely pKJK5::gfp::Kan^R, carrying a green fluorescent protein gene under the control of $lacI^q$ repressible promoter, resulting in a no gfp expression in the donor, which would be only expressed in a putative acceptor. The plasmid and the gene cassette were inserted by electroporation at 1800 V (Fournet-Fayard et al., 1995). Transformed cells were selected on LB agar plates added, respectively, with kanamycin (100 µg/ml) and gentamicin (100 µg/ml). Insertion of the gene cassette and the loss of the plasmid was carried out as per McKenzie and Craig., (2006) and verified by fluorescence microscopy and PCR analyses (Lambertsen et al., 2007). Primers used to verify the loss of the plasmid used to insert the gene cassette were i) Backbone pGRG36Fw 1 5'-TAG AGC GTC GCT ATT GGC AG-3'; Backbone pGRG36Rv 1 5'-TGC TCA ACG AGT TCG CTT CT-3'; and ii) Backbone

pGRG36Fw 8 5'-TGC TAG AGG CAT TAC GCT CG-3'; Backbone pGRG36Rv 8 5'-GCA AAG CGG GCA AAT ACC AA-3'. Primers were designed using Primer designing tool-NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR amplification of 16S rRNA gene was performed as described by Mapelli et al. (2013), modifying the annealing temperature at 58°C

Finally, the acquisition of the plasmid carrying the Gfp was verified by fluorescence microscopy (Gfp expression) adding IPTG in the growth media. A schematic view of the donor strains is summarized in Figure 1.

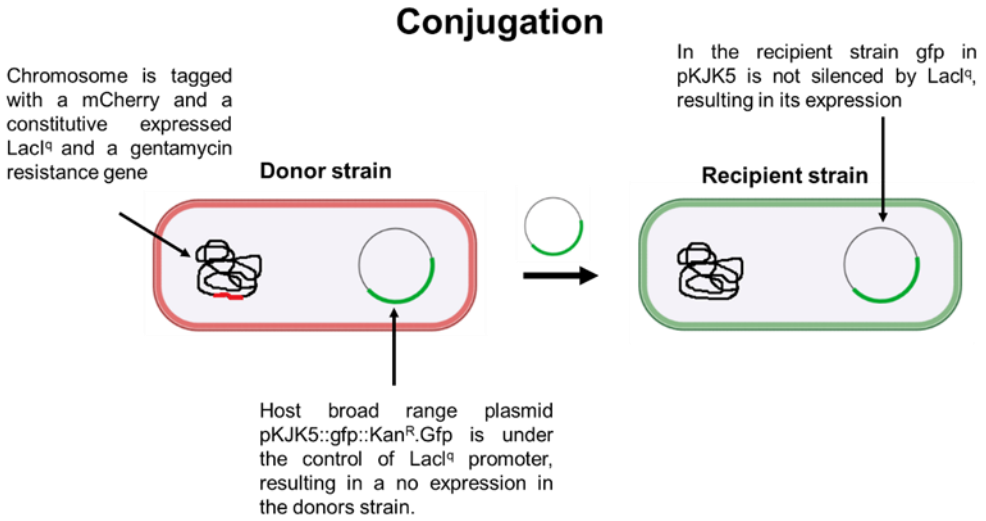

Figure 1: Scheme resuming the characteristics of the constructed donor strain. Chromosome of donor is tagged with a cassette encoding for a mCherry, a constitutive expressed $LacI^q$ and a gentamycin resistance gene. Broad host range plasmid pKJK5::gfp::Kan^R. Gfp is under the control of $LacI^q$ promoter, resulting in a no expression in the donor strain, which is red due to the mCherry gene expression. In the recipient strain Gfp in pKJK5 is not silenced by $LacI^q$, resulting in its expression.

2.5 Assessment of the ability of strain EEF15::$lacI^q$-pLppmCherry-Gm^R with plasmid pKJK5::gfp to donate DNA

To verify the ability of *Klebsiella variicola* subsp. *variicola* EEF15::$lacI^q$-pLppmCherry-$Gent^R$ with pKJK5::gfp to donate the DNA by conjugation,

conjugative experiments by filter mating assays (Schlecter et al., 2019), using as recipient *Pseudomonas putida* KT2440 were performed. Transconjugants were selected plating the conjugation mix on cetrimide agar (Millipore) added with kanamycin (100 µg/ml): donor growth was inhibited on cetrimide agar, while the growth of *P. putida* KT2440 without the conjugative plasmid was inhibited by the presence of kanamycin. The insertion of the plasmid in the recipient cells was verified by fluorescent microscopy (monitoring the Gfp expression) and by PCR amplification by ITS-PCR (Mapelli et al., 2013).

2.6 Conjugation between EEF15 ::lacI^{q}-pLppmCherry-Gent^{R} pKJK5::gfp and the inhabitant community of lettuce's rhizosphere

Rhizosphere was collected from lettuce plants as described by Marasco et al. (2018). Basically, conjugation between strain EEF15::lacIq-pLppmCherry-Gent^{R} pKJK5::gfp and the inhabitant community of lettuce rhizosphere was conducted as already performed by Klumper et al. (2014), changing two steps of the protocol i.e. i) the microbiome of the rhizosphere was extracted as described by Fan et al. (2019); ii) a cell density of 3.38×10^{8} cells/ml, instead of 3.38×10^{7} cells/ml, for the donor strain and the recipients were used.

3. Results

3.1 Donor strain selection

In order to select only Enterobacteriaceae, the selective medium Violet Red Bile Lactose Agar was used during isolation procedures. A total number of 15 strains were isolated and identified by PCR analyses on 16S rRNA gene (Table 1). Specifically, a total of number of 14 strains, of which 10 *Klebsiella*, 2 *Enterobacter*, 1 *Citrobacter*, 1 *Kluveira* and 1 *Raoultella* were isolated. After antibiotic resistance evaluation, *Klebsiella variicola subsp. Variicola* EEF15 was used for conjugation donor construction because it was sensitive for both kanamycin (100 µg/ml) and gentamycin (100 µg/ml).

Strain	Specie
EEF1	*Enterobacter sichuanensis*
EEF3	*Kluyvera georgiana*
EEF4	*Klebsiella michiganensis*
EEF5	*Citrobacter pasteurii*
EEF6	*Klebsiella michiganensis*
EEF7	*Klebsiella michiganensis*
EEF8	*Klebsiella grimontii*
EEF9	*Klebsiella huaxenensis*
EEF10	*Klebsiella michiganensis*
EEF11	*Klebsiella grimontii*
EEF12	*Klebsiella michiganensis*
EEF13	*Enterobacter hormaechei* subsp*. hoffmanii*
EEF14	*Raoultella ornithinolytica*
EEF15	*Klebsiella variicola* subsp*. variiicola*

Table 1: List of strains isolated from the effluent of the WWTP of Nosedo (Milan, Italy)

3.2 EEF15 ability to donate pKJK5::gfp

To verify the ability of *K. variicola* subsp. *variicola* EEF15::lacI^{q}-pLppmCherry-Gent^{R} to donate the plasmid, the strain of *Pseudomonas putida* KT2440, already known as able to acquire DNA by conjugation (Berthold et al., 2016), was used as acceptor. Filter mating experiments, based on Schlecter et al. (2019), were performed using a ratio between the donor strain and the recipient equal to 1:1 (10^9 cell of donor strain: 10^9 cells of recipient strain). After incubation, several colonies were found on the selective plates where dilutions of the conjugation mix were plated. The identity of the transconjugants was verified by ITS-PCR profiling, showing to be the same of the recipient *P. putida* KT2440: plasmid insertion was instead verified by fluorescence microscopy and antibiotic selection.

3.3 Rhizosphere colonization by strain EEF15

The ability of EEF15 to colonize the lettuce rhizosphere was verified using EEF15 RIF-R strain. Seven days after the bacterization of lettuce's rhizosphere, we found that $2.62 \times 10^7 \pm 1.21 \times 10^7$ CFU/gram of soil of rifampicin resistant bacteria were able to colonize the rhizosphere, when 1×10^8 EEF15 RIF-R cells/gram of soil were applied. Conversely, when 1×10^9 EEF15 RIF-R cells/gram of soil were used, $2.87 \times 10^8 \pm 1.53 \times 10^7$ CFU/gram of soil of rifampicin resistant bacteria were isolated from bacterized plant rhizosphere. No rifampicin-resistant colony forming units (cfu) were detected in the negative control. ITS-PCR profiles of all the colonies that we obtained confirmed the identity of the applied strain.

3.4 Conjugation with the inhabitant community of lettuce rhizosphere

The ability of the donor strain to transfer pKJK5::gfp plasmid to rhizosphere's microbiome, measured as transfer frequency was evaluated. After the extraction of the inhabitant community of lettuce's rhizosphere, a conjugation mix with a ratio 1:1 donors:recipients was prepared and incubated at 25 °C on soil extract medium for a period of 48 hours. Following incubation, filters were analysed by fluorescence stereomicroscopy (Figure 2).

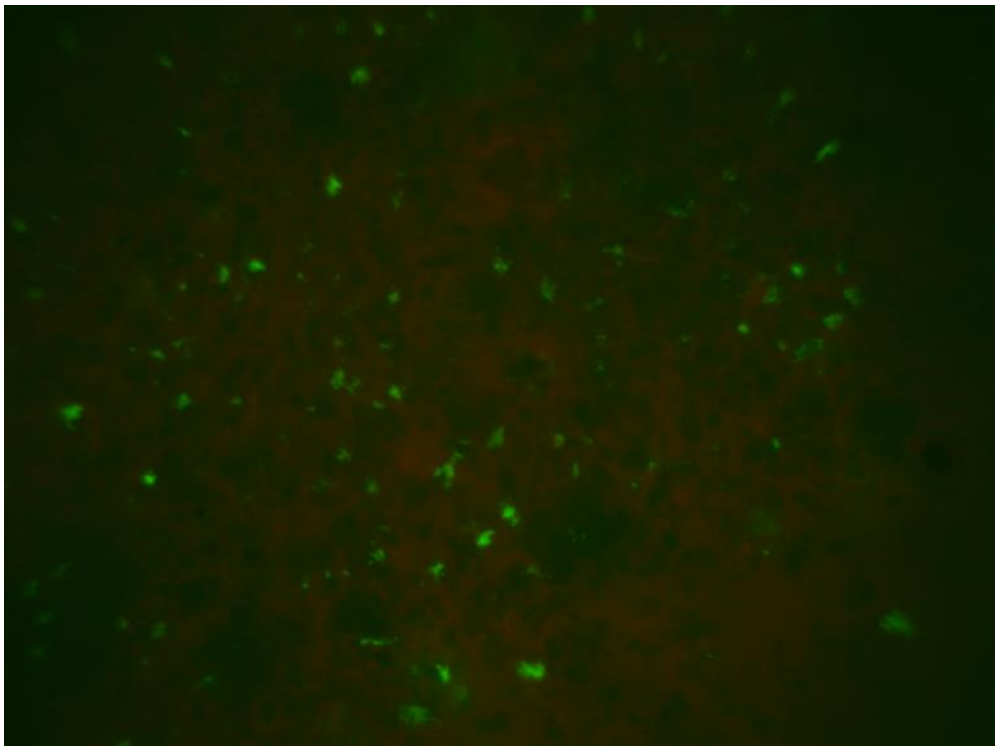

Figure 2: Filter used for the filter mating assay between EEF15 pKJK5::gfp and the lettuce rhizosphere community, visualized by fluorescence stereomicroscopy (picture area 0.6 μm^2). Green spots are the transconjugants who received pKJK5::gfp plasmid in which there is the expression of the Gfp, while the donor strain is observable as red spots due to the expression of the mCherry.

Transfer frequency of EEF15 (Table 2) was equal to $2.42 \times 10^{-3} \pm 5.71 \times 10^{-4}$. No green events (presence of transconjugants) were detected when only the donor strain or the recipient were applied to the filters.

Filter	Transfer frequency
Filter n° 1	2.48×10^{-3}
Filter n° 2	2.43×10^{-3}
Filter n° 3	1.66×10^{-3}
Filter n° 4	3.05×10^{-3}

Table 2: Transfer frequency of plasmid pKJK5::gfp between EEF15 and the inhabitant community of lettuce rhizosphere calculated per each filter. Transfer frequency is calculated as:

(Transconjugants per pictures × filter area)/(picture area (μm^2) × recipients introduced originally).

4. Discussion

Conjugation is a wide studied HGT mechanism mediated by pili or adhesines and pores. It could happen between the same bacterial species or between taxonomically distant bacteria (Zarey-Baigi et al., 2021) and it have been studied in several different environments e.g. plant's microenvironments, sediment, and sewage sludge (Van Elsas et al., 2003). As already mentioned, conjugation transfer is influenced by physical factors e.g. temperature, nutrient ability and cell to cell contact (Zarey-Baigi. 2021) and by anthropogenic factors e.g. presence of emerging pollutants which have been described to enhance the spread of ARGs (Feng et al., 2021). The description of conjugation-based DNA transfer in different environments is thus mandatory to understand the magnitude of ARGs diffusion in relation to different conditions which could be found (Smalla et al., 2018). In this study we hence constructed a donor strain of environmental origin to study conjugative transfer events in complex bacterial communities, such as the ones associated to plant rhizosphere, without the use of a selection e.g. an antibiotic or a selective medium to identify transconjugants (Klumper et al., 2015; Berthold et al., 2016; Fan et al., 2019; Xu et al., 2021). Our construct was based on the one(s) prepared by Musovic et al. (2010), which have been widely used to assess conjugative transfer to complex communities where, however, the donor strain was represented by laboratory model ones. The use of laboratory strains could indeed influence the evaluation of the phenomenon: in some cases, laboratory strains are optimized for some functions (Zebec and Scrutton, 2018). However environmental strains could have some traits which may help them to thrive in the environment improving also their HGT proficiency (Riva et al., 2020b; Bernardy et al., 2016).

As described by Klumper et al. (2014), EEF15::lacI^q-pLppmCherry-Gent^R strain was able to donate pKJK5::gfp plasmid to a complex bacterial

community as confirmed by fluorescence stereomicroscopy. *Klebsiella variicola* is known to survive in several habitats linked to the agri-food system. Indeed, it is a bacterial member of the plant microbiome e.g. rhizosphere and endosphere, and some strains have been described as plant growth promoters (Duran-Bedolla et al., 2021). EEF15 was selected to be engineered as donor strain of conjugation also because it belongs to Enterobacteriaceae family, which members are known to be possible pathogens, or commensals, being able to survive in several environments linked to the agri-food system e.g. soil, plant microenvironments, freshwater bodies, human and animal gut (Touchon et al., 2020; Riva et al., 2020b; Leimbach et al., 2013; Nüesch-Inderbinen et al., 2015; Wang et al., 2020; Shikora et al., 2012). Specifically, *Klebisella variicola*, which belong to the *Klebisella pneumoniae* complex, is considered an emerging pathogen already isolated from humans (Barrios-Camacho et al., 2019): however, its presence in the effluent of WWTPs is still not required in some countries such as Italy (PCFC 2005), underlining the necessity to improve the wastewater management, considering also AR determinants (Corno et al., 2019).

The ability to donate the plasmid, together with its belonging to Enterobacteriaceae family, and the ability to colonize the rhizosphere, a plant's microenvironment rich of nutrients, where bacterial activity is enhanced (Zhu et al., 2018), make EEF15 strain a perfect candidate to study conjugation events in the rhizosphere, as well as in other plant microhabitats. Moreover, the ability of EEF15 to spread a plasmid with ARGs to the complex lettuce rhizospheric community highlights the importance to investigate the dimension of ARGs spread in environments linked to the agri-food system, specifically to raw-eaten vegetables, to underline the possible risks for human health.

5. Conclusion

In this study we constructed and tested a donor strain, isolated from treated wastewater, to evaluate gene transfer to the rhizospheric microbiome, to mimic ARGs spread by conjugation. The ability of EEF15 strain to donate the plasmid to the microbiome of rhizosphere without any selective pressure, and its ability to colonize the plant rhizosphere highlight the importance to study the magnitude of the diffusion of AR determinants in the agri-food system, through a one health approach, to understand the possible risk related to human health (Smalla et la., 2018; Larsson et al., 2018).

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Chapter VI

Microbial assisted phytodepuration for water reclamation: environmental benefits and threats

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Abstract

Climate changes push for water reuse as a priority to counteract water scarcity and minimize water footprint especially in agriculture, one of the highest water consuming human activities. Phytodepuration is indicated as a promising technology for water reclamation, also in the light of its economic and ecological sustainability, and the use of specific bacterial inocula for microbial assisted phytodepuration has been proposed as a further advance for its implementation. Here we provided an overview on the selection and use of plant growth promoting bacteria in Constructed Wetland (CW) systems, showing their advantages in terms of plant growth support and pollutant degradation abilities. Moreover, CWs are also proposed for the removal of emerging organic pollutants like antibiotics from urban wastewaters. We focused on this issue, still debated in the literature, revealing the necessity to deepen the knowledge on the antibiotic resistance spread into the environment in relation to treated wastewater release and reuse. In addition, given the presence in the plant system of microhabitats (e.g. rhizosphere) that are hot spot for Horizontal Gene Transfer, we highlighted the importance of gene exchange to understand if these events can promote the diffusion of antibiotic resistance genes and antibiotic resistant bacteria, possibly entering in the food production chain when treated wastewater is used for irrigation. Ideally, this new knowledge will lead to improve the design of phytodepuration systems to maximize the quality and safety of the treated effluents in compliance with the 'One Health' concept.

1. Introduction

Water scarcity is a highly relevant and still unresolved global issue. In 2018, the World Economic Forum ranked the water crisis among the top 3 global risks for the third consecutive year (WEF, 2018) and, given the continuous world population growth and the impact of climate change, global water demand is predicted to increase of 55% by 2050 (UN, 2015; Gain et al., 2016; Ercin et al., 2014). Among anthropogenic activities, agriculture is the most water-consuming one, using for irrigation purposes about 75% of the world's freshwater (FAO, 2015; Sato et al., 2013; Falkenmark, 2013). The consequences of water scarcity on agriculture are particularly relevant in countries currently facing an intense growth of population and economy and characterized by harsh environmental conditions, such as those located in the Middle East and North Africa (MENA) region that is among the most arid areas of the world. Here, the economic growth is forecast to determine an increase in water demand of 47% by 2035 (Frascari et al., 2018), intensifying the competition among different water-consuming sectors. This may negatively affect the allocation of freshwater to agriculture and, eventually, threaten food security. Furthermore, climate changes have already intensified phenomena like inconstant rainfall distribution and drought in Europe (Lehner et al., 2006; NOAA, 2018), exacerbating their consequences on agriculture and promoting the development of alternative solutions also on those territories once considered safe in terms of water availability (Rolli et al., 2015).

In this context, pursuing an improved water reuse is a priority to fight water crisis and minimize water footprint in agriculture (Van der Hoek et al., 2002) and, accordingly, the reuse of treated wastewaters is a priority for the European water management policy (SCHEER, 2017). In fact, non-conventional water resources (e.g. wastewaters) can offer complementary supplies to alleviate water shortage in areas where renewable water sources are particularly limited (Qadir et al., 2007) and it is estimated that at least twenty million hectares of cultivated lands worldwide are irrigated with

wastewaters (Bouaroudj et al., 2019). Unfortunately, this approach frequently implies the release of untreated wastewaters in agriculture fields, and eventually in the food chain (Garner et al., 2016), representing an issue particularly in the least developed countries (LDCs) and in the MENA region.

Among wastewater treatment technologies, constructed wetlands (CWs) are engineering systems based on the purifying processes that occur in natural wetlands, i.e. the ability to remove pollutants and nutrients present in the water that flows through, thus enhancing water quality (Carvalho et al., 2017). Compared to the conventional wastewater treatment processes, CW systems need low building and maintenance costs, require less external energy and can be implemented at both small and large scales: these features make them highly suitable for wastewater treatment in the LDCs (Mahmood et al. 2013). CWs can be used as unique treatment to cleanup wastewaters produced by small communities, being effective in decreasing chemical and biochemical oxygen demand (COD and BOD) and removing total suspended solids (TSS) and nutrients (i.e. ammonia and phosphorus) (Vymazal, 2010; Wang et al., 2018). Moreover, CWs can be used as tertiary treatment for the final cleaning step in conventional wastewater treatment plants, which are not specifically designed for the removal of micropollutants (e.g. emerging organic contaminants – EOCs- like pharmaceuticals and personal care products) (Verlicchi and Zambello, 2014; Li et al., 2014). The CW system is indeed suitable for the improvement of water quality of different types of wastewaters including domestic (Wang et al., 2018) and industrial (Wu et al., 2015) ones.

As in natural wetlands, in CW systems several physicochemical and biological processes take place spontaneously at the same time, allowing the removal of organic and inorganic compounds by soil/sediment sorption and sedimentation, phytodegradation and plant uptake. In addition, the soil/sediment matrices and the plant root apparatus provide a niche for the development of microbial communities that are pivotal for the nutrient and pollutant removal from wastewaters and can at the same time sustain plant growth (Faulwetter et al., 2009) (Fig. 1). Recently, different studies have

demonstrated that the addition of specific bacteria to the plant species used in CWs can be a promising strategy to boost the phytodepuration performances (Prum et al., 2018; Rehman et al., 2018; Syranidou et al., 2016), given their capacity to promote plant growth and remove different pollutants (see chapter 2 and 3 of this review).

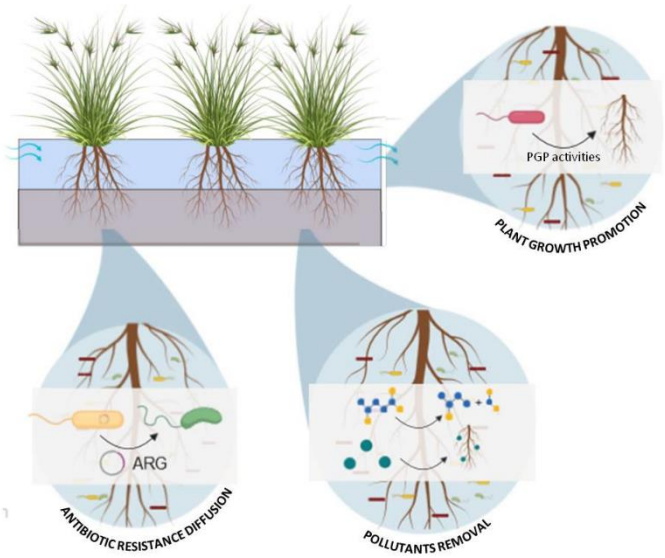

Figure 1: Bacteria mediated processes in phytodepuration system. Bacterial populations can play a beneficial effect in phytodepuration systems acting as Plant Growth Promoting strains and contributing to the degradation of micropollutants of emerging concerns (e.g. pharmaceuticals). On the other side, bacteria are involved in the phenomenon of antibiotic resistance diffusion into the environment through Horizontal Gene Transfer events, which allow the spread of antibiotic resistance genes.

CWs have been proposed as a solution to reduce contaminants of emerging concern like antibiotics, antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), typically present in wastewaters containing human and animal stools (Chen and Zhang, 2013). Nevertheless, contrasting results are reported in the literature on this topic, as shown in the chapter 4 of this review. The possible occurrence of horizontal gene transfer (HGT), the mechanism supporting the spread of ARGs (Jechalke et al., 2014) is an issue in phytodepuration technology (Fig. 1), considering that it can be enhanced in

the bacterial community inhabiting plant rhizosphere (Chen et al., 2019). This critical aspect urgently needs to be addressed, since the spread of antibiotic resistance through treated wastewater used in agriculture could increase the presence of environmental ARB that can potentially be recruited by the plant and eventually enter the food production chain.

In this review, we propose some considerations on the advantages and the possible risks correlated with the use of selected bacterial inocula in CWs for microbial assisted phytodepuration to improve nutrient and micropollutant removal, focusing specifically on the threat of antibiotic resistance diffusion into the environment amplified by HGT events.

2. PGP bacteria support to plant growth under adverse conditions

When growing in adverse environments plants face a number of growth-limiting factors, such as water scarcity, high salinity, reduced nutrients bioavailability and toxicity of polluted water and soil (Soussi et al., 2016). These conditions determine oxidative stress, reduced photosynthetic rate and plant growth, thereby affecting negatively plant productivity and related ecosystem services, including soil and water depuration (Khan et al., 2015). Plant growth-promoting (PGP) bacteria are known to establish an intimate association with the plant rhizosphere and endosphere, where they can sustain plant growth and development by counteracting the effects of stress conditions through a variety of mechanisms that have been extensively reviewed and can be categorized as direct and indirect (Abilash et al., 2016; de Bashan et al., 2012; Riva et al., 2019). PGP bacteria can directly promote plant growth acting as biofertilizers by increasing the bioavailability of key nutrients including nitrogen, phosphorus and iron. Moreover, PGP bacteria sustain the growth of associated plants by mechanisms that interfere with the production and regulation of phytohormones, such as auxins and ethylene, orchestrating root development and plant biotic and abiotic stress response (Backer et al., 2018).

2.1 Mechanisms relevant for plant growth promotion in CWs

The adverse conditions encountered by plants growing in constructed wetland (CW) ecosystems seem to be mostly related to the toxicity of the pollutants contaminating the wastewaters, including pharmaceuticals, azo-dyes and metals in high concentrations. These compounds are often slightly hydrophobic and can easily enter the roots and be translocated through the xylem to the plant tissues, where they determine an increase of the reactive oxygen species (ROS) and the activation of the plant stress response supported by an increase of the respiration rate (Ferreira et al., 2014; Christou et al., 2016; Mesa-Marín et al., 2018). Such physiological changes alter the carbon balance against plant biomass production, eventually inducing a reduction of the depuration rate in the CW system (Ferreira et al., 2016). PGP bacteria ability to counteract abiotic stress and to enhance the development of the root system represents thereby a valid biotechnological resource to sustain plant development and enhance the detoxification process (Vergani et al., 2017). The ability of indole-3-acetic acid (IAA) producing bacteria to promote the development of the root apparatus represents a significant advantage for the plant that can explore an increased volume of soil for water and nutrient uptake (Backer et al., 2018), but can be also exploited to increase the phytodepuration rate of wastewaters. Plant associated bacteria also interfere with the production of stress and senescence related hormone, i.e. ethylene, through the expression of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Nascimento et al., 2018). This enzyme hydrolyses the immediate precursor of ethylene in plants, thereby lowering the concentration of endogenous ethylene related to stress response in polluted waters and indirectly promoting plant growth. However, the mechanisms underlying microbial-assisted phytoremediation remain widely unclear and variable, depending on the bacteria and plant species and on the type of contamination. Different studies report that plant stress tolerance and phytoremediation performances benefit from the enhancement of antioxidant

enzymes activity (e.g. catalase, superoxide dismutase, glutathione reductase) triggered by the inoculated bacteria (Rajkumar et al., 2012; Backer et al., 2018). This type of mechanism assumes particular interest when the target pollutants in the wastewaters are hydrophilic organic contaminants and metals that can be taken up by the roots, translocated and eventually metabolized within the plant tissues. Alternatively, PGP bacteria can exert a protective effect towards the plant, decreasing roots respiration rate and reducing the energy consumption for antioxidant enzymes related to the stress response, hence leading to higher root biomass production and increased phytoremediation potential (Mesa Marin et al., 2018).

3. PGP bacteria application in phytodepuration

As previously mentioned, in phytodepuration systems like CWs the pollutants present in wastewaters can impair plant growth and performance. PGP bacteria have the potential to improve the efficiency of CW plants by i) decreasing stress response and in turn increasing plant growth and services and ii) degrading phytotoxic compounds (Faulwetter et al., 2009). The chemical pollutants occurring in wastewaters are indeed hardly degradable compounds and the supplementation in CWs of microbes owing specific metabolic pathways able to degrade chemical compounds and to promote plant growth has been indicated as a strategy to accelerate and enhance wastewaters depuration (Shehzadi et al., 2014; Wu et al., 2015).

3.1 Methods for PGP bacteria selection, application in CWs and testing

The successful exploitation of PGP bacteria in CW systems foresees different research steps, including the isolation of the proper bacterial strains, their taxonomic identification in order to omit potential pathogens, the functional characterization to select the most promising ones. The whole procedure must also include the assessment of bacterial effects on the mesocosm-scale CW system and the quality of the treated wastewater effluent. A survey of the peer-reviewed articles published in the last 10 years on this topic showed that different approaches can be adopted in the pipeline that leads from bacteria

selection (Table 1) to bacteria inoculum (Table 2) and performance validation (Table 3).

Different authors performed the PGP bacteria selection establishing a collection of strains from the endosphere or rhizosphere of wetland plant species commonly used in phytodepuration systems (Kabra et al., 2013; Saleem et al., 2018; Salgado et al., 2018. Table 1). PGP bacteria were isolated from the same plant species used in the CW to be bioaugmented (Ijaz et al., 2015) or from a different plant species grown in CWs treating the wastewater type of interest (Syranidou et al., 2016). Alternatively, PGP bacteria used in CWs were isolated from a site polluted with the target contaminant that had to be removed from the wastewater (Lingua et al., 2015; Prum et al., 2018; Rehman et al., 2018). The selection of the most promising strains suitable for assisted phytodepuration was then performed by analyzing the isolates for the potential capacity to tolerate/degrade the target contaminants and for the plant growth promotion ability both *in vitro* and *in vivo* (Table 1).

Once the best candidates were selected, pilot scale experiments were implemented to validate their possible application in CWs. As summarized in Table 2, most of the studies applied a consortium of two or more bacteria rather than single strain inocula to CW system, and the bioaugmentation was usually performed once, at the beginning of the experiment. The bacteria inoculation was performed by adding a cell suspension of determined concentration directly into the wastewater (Salgado et al., 2018; Shehzadi et al., 2014) or supplied around the plant root apparatus (Lingua et al., 2015; et al., Syranidou et al., 2016). As an alternative, before placing the plant in the CWs, it was possible to dip the root apparatus in the bacterial cells suspension as proposed by Prum et al. (2018).

Different chemical parameters were measured to verify the efficacy of the PGP bacteria inoculation in enhancing the CW depuration performance (Table 3). Obviously, the pollutant removal in the effluent must be evaluated, comparing wastewaters treated with inoculated and non-inoculated plants in

CWs. Chemical and biological oxygen demands (COD and BOD) were routinely measured as general parameters that provide an indication of effluent quality. Moreover, the target pollutants in CWs, varying according to the wastewater origin, were quantified. For instance, heavy metals and emerging organic contaminants (EOCs) such as pharmaceuticals and personal care products were monitored in industrial and domestic wastewater effluents (Carvalho et al., 2014; Syranidou et al., 2016; Zhang et al., 2018). In addition, the quality of the treated wastewater was assessed through ecotoxicological tests using model microbes, plants or fishes (Table 3).

To complete the analysis of the bacterial inocula effect on a CW system, plant growth promotion was also evaluated, generally measuring plant dry biomass, root and shoot lengths and ROS concentration (Table 3). Furthermore, a key aspect for the validation of the PGP bacteria to be used in assisted phytodepuration was the persistence of the inoculum into the system and its capacity to establish a tight bacteria-plant interaction. Such analyses can be performed using different molecular methods as reviewed by Rilling and coworkers (2018) and showed in Table 3.

Table 1 (next page): Overview of the scientific literature reporting *in vivo* bioaugmentation experiments in Constructed Wetlands (CWs). The table includes CW type (FTW = floating treatment wetland; VF = Vertical flow; SF = subsurface flow), wastewater type, plant species and the criteria applied for the selection bacteria in each study. ACC = 1-Aminocyclopropane-1-Carboxylate deaminase activity, P sol. = phosphate solubilization, sid. = siderophore production, IAA = Indole-3-acetic acid production

References	CW type	Wastewater type	Plant species	Bacteria applied for bioaugmentation in CW		
				Bacterial isolation site	Characterization of pollutant tolerance/degradation	Characterization of PGP potential
Ijaz et al. (2015)	microcosm (FTW)	sewage effluent	*Brachiaria mutica*	*Brachiaria mutica* shoot	COD/BOD removal	ACC, P sol., sid.
Kabra et al. (2013)	mesocosm	textile effluent	*Glandularia pulchella*	*Glandularia pulchella* root	dye decolorization	-
Lingua et al. (2015)	mesocosm	nitrate polluted water	*Phragmites australis*	forest soil	-	IAA, sid., P sol., *in vivo P. australis* promotion
Prum et al. (2018)	mesocosm (VF)	arsenic polluted water	*Echinodorus cordifolius*	arsenic contaminated soil	arsenic tolerance	-
Rehman et al. (2018)	microcosm (FTW)	oil field wastewater	*Brachiara mutica/Phragmites australis*	crude oil-contaminated site	hydrocarbon degradation	IAA, ACC, sid., P sol.
Saleem et al. (2018)	microcosm (FTW)	phenol polluted water	*Typha domingensis*	Plant rhizosphere and shoot	phenol degradation	ACC
Salgado et al. (2018)	mesocosm	domestic wastewater	*Typha latifolia*	*Typha domingensis* plant	COD/nitrogen/phosphorus removal	-
Shezhadi et al. (2014)	mesocosm (VF)	textile effluent	*Typha domingensis*	wetland plants roots	textile effluent degradation	ACC
Syranidou et al. (2016)	microcosm (static)	EOCs and metals polluted water	*Juncus acutus*	*Juncus acutus* endosphere	BPA/antibiotic/metals tolerance	IAA, ACC, P sol., sid., organic acid production
Watharkar et al. (2015)	mesocosm (static)	textile effluent	*Pogonatherum crinitum*	*Petunia grandiflora* rhizosphere	dye decolorization	-
Zhao et al. (2016)	mesocosm (SF)	sewage effluent	*Acorus calamus*	CW/commercial bacteria	nitrogen removal	-

Table 2: List of the different methods applied in the literature for the inoculation of bacteria in a CW system. The table reports for each reference the type of inoculum used, the way and the frequency of the microbe addition to CW.

References	Bacteria inoculation methods in CW		
	Inoculum type	**Inoculation method**	**Frequency of inoculation**
Ijaz et al. (2015)	Consortium	wastewater inoculation	once
Kabra et al. (2013)	single strain	soil inoculation	daily (for 15 days)
Lingua et al. (2015)	single strain	bacterial suspension around root apparatus	once
Prum et al. (2018)	Consortium	root dipping	once
Rehman et al. (2018)	Consortium	wastewater inoculation	once
Saleem et al. (2018)	Consortium	wastewater inoculation	once
Salgado et al. (2018)	Consortium	wastewater inoculation	once
Shehzadi et al. (2014)	Consortium	wastewater inoculation	once
Syranidou et al. (2016)	Consortium	bacterial suspension around plant base	once
Watharkar et al. (2015)	single strain	cell immobilization	once
Zhao et al. (2016)	Consortium	bacterial suspension around plant base	thrice

Table 3: List of the parameters measured in the literature to assess the effect of bacteria inoculation in CW systems. The table refers to different bacteria-mediated effects possibly enhancing CW performance (i.e. pollutant removal, effluent toxicity, plant growth promotion) besides inoculum persistence.

References	Bacterial effect assessment			
	Pollutant removal	**Effluent toxicity**	**Plant growth promotion**	**Inoculum persistence**
Ijaz et al. (2015)	BOC, COD, TS, TDS, TSS, DO, oil and grass, SO4, Cl, total N, PO4, Na, K, metals	Fish toxicity test	-	Plating + RFLP analyses
Kabra et al. (2013)	dyes, COD, BOD, TOC	Plant toxicity test	-	-
Lingua et al. (2015)	nitrogen, ammonium	-	N° of stems/sprouts/leaves; stem length; shoot and root fresh/dry biomass and weights	Plating
Prum et al. (2018)	Arsenic	-	ROS and IAA levels	-
Rehman et al. (2018)	COD, BOD, Na, K, metals, residual oil	Fish toxicity test	fresh and dry biomass, root and shoot length	Plating + RFLP analyses
Saleem et al. (2018)	phenol, COD, BOD, TOC	-	fresh and dry biomass	Plating + RFLP analyses
Salgado et al. (2018)	COD, NH4, PO4	-	-	-
Shehzadi et al. (2014)	dyes, COD, BOD, TOC, TDS, TSS	Ames test	fresh and dry biomass	Plating + RFLP analyses
Syranidou et al. (2016)	metals, BPA, antibiotics	-	dry biomass	ARISA
Watharkar et al. (2015)	dyes, BOD, COD	Plant and fish toxicity test	-	-
Zhao et al. (2016)	nitrogen, COD, BOD	-	-	high-throughput sequencing

3.2 PGP bacteria contribution to increase plant performances

PGP bacteria can improve the fitness of plants used in CW systems. As reported by Shehzadi and coworkers (2014), the inoculation of the wetland plant *Typha domingensis* with the endophytic strains *Microbacterium arborescens* TYSI04 and *Bacillus pumilus* PIRI30 in a vertical flow CW reactor promoted plant growth besides improving the depuration of the effluent. In the system, textile effluent negatively influenced the development of *T. domingensis,* however the endophytes inoculation reduced the toxic effects of textiles, restoring the plant biomass production up to the values of *T. domingensis* plants irrigated with clean tap water and used as control in the study. Likewise, the addition of a bacterial consortium to a floating treatment wetland enhanced *Brachiara mutica* and *Phragmites australis* growth that was normally inhibited by the presence of oil-contaminated water (Rehman et al., 2018). In the study, bacterial inoculation restored plant health and increased root (36-46%) and shoot (35-36%) biomass and root (15-29%) and shoot (4-21%) length, enabling these two plant species to cope with the oil-induced stress.

The influence of PGP bacteria on wetland plants can be also indirectly evaluated by measuring the decrease in plant content of antioxidative enzymatic activity, which is linked to the oxidative stress induced in plants (Prum et al., 2018; Syranidou et al., 2016). It is known that PGP bacteria stimulate enzymatic and non-enzymatic antioxidant responses that scavenge ROS compounds related to stress condition (Marasco et al., 2013; Wang et al., 2012). The monitoring of antioxidative enzymatic activity has been used by Syranidou et al. (2016) in a microcosm scale experiment designed for the depuration of water artificially contaminated with metals, bisphenol-A and antibiotics to simulate urban wastewaters. The authors showed a significant reduction of activity of several enzymes involved in anti-oxidant defense in *Juncus acutus* plants inoculated with a consortium of endophytic PGP bacteria.

3.3 PGP bacteria contribution to nutrient removal

CWs are often used as secondary treatment systems for municipal wastewater cleanup in rural communities where wastewater treatment facilities are not present due to the high cost of treatment processes (Ghrabi et al., 2011; Fountoulakis et al., 2017). Phytodepuration is also used as tertiary treatment when the common treatment plant is not efficient enough in the achievement of legal standards for organic matter and nutrient removal to allow the safe discharging of treated water in the environment (Ijaz et al., 2015). Wetland plants, such as P*hragmites* and *Typha* spp., are able to uptake nitrogen and phosphorus from wastewaters (Vymazal, 2010; Fountoulakis et al., 2017). However, the addition of bacteria in CWs can help plants in the removal of these contaminants and contribute to organic matter decrease by the indigenous microbial communities, improving the quality of treated wastewaters (Ijaz et al., 2015; Salgado et al., 2018).

Salgado and coworkers (2018) investigated the effects of the application of four indigenous rhizobacteria to *Typha latifolia* on the performance of a vertical flow CW system treating domestic wastewaters. The study showed that the system removed over 75% of the organic matter, ammonium and phosphate and it had better performance than i) *T. latifolia* CW mesocosms without bacteria inoculation and ii) CW mesocosms established using plant with sterilized root surface supplemented by the four selected rhizobacteria. This interesting experiment suggests that the inoculation of the selected indigenous rhizobacterial strains activated the overall microbiome associated to *T. latifolia* in the bioaugmented plants, resulting in a synergic effect that significantly improved the effluent quality. Another study analyzed the removal of nitrogen from nitrate polluted and urban wastewaters using pilot scale-CWs enriched with non-indigenous bacteria aiming to achieve the legal standards of nitrogen emission in surface waters and to prevent the risk of eutrophication in lakes and rivers (Zhao et al., 2016). The addition of the selected PGP

bacteria improved the performance of the system and ameliorated the efficiency of denitrification up to 10% during the wastewater phytodepuration process.

3.4 PGP bacteria contribution to micropollutant removal

Wastewaters contain several categories of micropollutants, including metals and organic contaminants of emerging concern (e.g. pharmaceuticals, personal care products, pesticides), which pose serious risks for human health if released into the environment (Li et al., 2017; Pruden et al., 2012; Tezel and Pavlostathis, 2015; Zuccato et al., 2000). In this review, we focused on EOCs that are the prevalent class of micropollutants occurring in urban wastewater and show a relationship with the antibiotic resistance diffusion in the environment, as illustrated in chapter 4. EOCs that are frequently present in wastewater at trace concentrations and are hardly removed by common wastewater treatment plants, which are not tailored for their elimination (Gorito et al., 2017). CWs are proposed as tertiary treatment for the removal of micropollutants from industrial and municipal effluents (Verlicchi and Zambello, 2014; Garcia-Rodríguez et al., 2014; Wu et al., 2015), basing on the capacity of certain plant species to accumulate organic and inorganic compounds from water and considering their potential association in the rhizosphere and endosphere with pollutant degrading bacteria (Borruso et al., 2017). Bacterial degradation has been demonstrated to be the main process involved in micropollutant removal in CWs treating urban wastewaters (Carvalho et al., 2014; Li et al., 2016; Zhang et al., 2018), hence through the addition of microbial inocula having a specific degrading pathway it is in principle possible to achieve treated effluents at reuse grade. In a recent study, the endophytic bacteria *Rhizobium radiobacter* and *Diaphonobacter nitroreducens*, isolated from reed plants and characterized for plant growth promotion and carbamazepine degradation capacities, were inoculated to hairy root cultures of *Armoracia rusticana*. The inoculated hairy root cultures showed higher carbamazepine removal rate (21% and 10% for the cultures

inoculated with *Rhizobium radiobacter* and *Diaphonobacter nitroreducens* respectively) compared to the non-inoculated one (Sauvêtre et al., 2018) and, even though the plant-endophyte synergy needs to be further investigated, these data clearly indicated that plant associated microbes could be successfully exploited for municipal wastewater treatment in phytodepuration system. A CW mesocosm experiment for the depuration of urban wastewaters was recently described by Saleem and coworkers (2018): in a floating treatment wetland, *T. domingensis* was able to remove a small amount of phenol from the polluted water, however the addition of three phenol-degrading bacterial strains to the system significantly improved the phenol removal, which increased from 0,146 g/m^2/day to 0,166 g/m^2/day. Other studies have demonstrated that the addition of bacteria inocula to plants can be also effective for the treatment of textile wastewaters, contaminated by toxic dyes. In two different pilot scale experiments, Kabra et al. (2013) and Watharkar et al. (2015) inoculated respectively plants of *Glandularia pulchella* and *Pogonatherum crinitum* with two isolates belonging to the species *Pseudomonas monteilii* and *Bacillus pumilus*. The results highlighted a synergic effect of the plant and bacteria components, showing that inoculated plants were more efficient in dye removal and BOD and COD decrease compared to i) non-inoculated plants and ii) reactor systems with the presence of the sole bacterium without the plant.

An additional advantage of assisted phytodepuration is that the combined use of plants and bacteria is useful to deal with mixed pollution. Syranidou et al. (2016) investigated the potential of endophytic bacteria to sustain the removal of metals and emerging organic pollutants, such as bisphenol-A and antibiotics, by *Juncus acutus* plants in CWs. The results showed that beneficial effect of the bacteria in phytodepuration was especially evident when high concentration of contaminants where used, significantly improving the removal of organics and metals in shorter time by inoculated plants compared to the non-inoculated ones.

4. Antibiotic resistance dimension in phytodepuration

The majority of antibiotics are not completely metabolized and absorbed by animal and human's bodies, thus they are discharged in municipal and hospital wastes in their original or transformed chemical species (Huang et al., 2017; Berglund et al., 2014). As other EOCs, antibiotics escape wastewater treatment systems, which are not specifically designed to remove these compounds, thus representing micropollutants in treated effluents (Luo et al., 2014). Antibiotics are considered "pseudo-persistent" contaminants because of their constant addition through municipal wastewaters at low concentrations into the environment (Gorito et al., 2017) and can reach biologically active amounts due to accumulation as reported for the waters and sediments of Italian rivers where they have been measured at ppm and ppb concentrations respectively (Zuccato et al., 2000). The principal concern for their release is related to the selection and spread of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) (Rizzo et al., 2013; Liang et al., 2018; Yan et al., 2019), as already hypothesized by Sir Alexander Fleming, the discoverer of the first antibiotic, during his Nobel Lecture in 1945. Indeed, antibiotic resistance is nowadays one major concern threatening human and animal health due to the emergence of multi-drug resistant pathogens, which cannot be eliminated by pharmacological therapy with serious consequences for the overall society. The genetic determinants of antibiotic resistance (i.e. ARGs) are typically present in mobile genetic elements (MGE) and are considered micropollutants themselves since, if acquired by a receiving sensitive cell, can produce a new antibiotic resistant mutant.

The study of the antibiotic resistance dimension in the environment has to include the processes occurring in wastewater treatment plants (WWTPs), identified as one of the main sources of ARB and ARGs (Amos et al. 2015; Czekalski et al. 2014). The inadequacy of WWTPs in antibiotic removal from wastewaters has been identified as a major cause for the ubiquitous occurrence of ARB and ARGs in different environmental compartments (Amos et al., 2015; Tang et al.; 2016; LaPara et al., 2011; Ju et al., 2019). For

instance, recent findings suggest that the use of struvite as crop fertilizer can increase the diversity and the concentration of ARGs in the microbiome inhabiting soil and plant microhabitats, i.e. roots and leaves (Chen et al., 2017). Moreover, WWTPs are a suitable environment for antibiotic resistance selection and spread due to the continuous contact between the bacteria involved in the biological treatment processes and the antibiotics present in the wastewaters at sub-inhibitory concentrations (Rizzo et al., 2013). The presence of sub-lethal concentrations of antibiotics in WWTPs and in their effluents is a peculiar ecological driver favoring the ARB selection and the outbreak of antibiotic resistance (Andersson and Hughes, 2014). Other EOCs (e.g. biocides) and metals are typical stressors in WWTPs that might exert selective pressure contributing to the enrichment of ARGs and ARB through selection or co-selection events (Berendonk et al., 2015; Di Cesare et al. 2016). Indeed, the numerous bacteria thriving in wastewater, WWTPs and related habitats (e.g. biofilm, sludge) can host a myriad of ARGs and a better wastewater treatment technology is required to limit the risk of antibiotic resistance spread.

4.1 ARB & ARG threat in CWs

Phytodepuration of wastewater has gained increasing attention as low-cost and eco-friendly system for water reclamation and reuse, producing effluent of high quality for irrigation purposes (Petroselli et al., 2015; Petroselli et al. 2017). Several works have highlighted the efficacy of CWs in limiting the diffusion of antibiotics, ARGs and ARB (Hijosa-Valsero et al., 2011; Chen et al., 2015) by removing antibiotics from wastewaters with different mechanisms such as biodegradation, plant uptake, substrate absorption, photolysis and volatilization (Liu et al., 2013; Chen et al., 2016a). Chen and Zhang (2013) demonstrated that CWs used as tertiary treatment of WWTP were more effective than biological filter and UV disinfection for ARG removal. Chen et al. (2016a) proved that different mesocosm-scale CWs treating raw domestic wastewaters reached a removal efficiencies of total antibiotics from the aqueous phase comprised between 75.8 and 98.6%, and those of total ARGs

varied between 63.9 and 84%. Berglund and coworkers (2014) assessed the effluent quality of surface-flow experimental wetlands exposed to antibiotics at concentrations commonly found in wastewaters, showing that the CW system did not promote the dispersal of ARGs and leading to hypothesize that the low ARG concentration detected was probably due to a background genetic resistance present in the wetlands.

The fate of ARGs in CWs and their effluents is extremely influenced by the operating conditions of the CW (e.g. plant species, substrate type, flow type and velocity, system configuration) and environmental factors (e.g. temperature) (Liu et al., 2013; Chen and Zhang 2013; Chen et al., 2016a; Fang et al., 2017; Huang et al., 2017). For instance, the relative abundance of tetracycline resistance genes (*tet*) seemed to be higher in the CW effluents of an up-flow system compared to down-flow treatment (Huang et al., 2017). Likewise, the use of different substrates can influence the removal of *tet* genes, as shown by Liu and coworkers (2013) in CWs established using volcanic (CW1) or zeolite (CW2) substrates. The results of the study indicate that the absolute abundance of *tet* and the universal bacterial 16S rRNA genes decreased in both CW1 and CW2 effluents, while the relative abundance of *tet* genes increased in CW1, pointing out an increase of the resistant populations over the total bacterial community.

During the summer, an increase of ARGs concentration was registered in wastewater effluent compared to the winter period (Fang et al., 2017). Indeed, temperature has a key role for the regulation of several microbial processes and metabolisms in CWs, eventually influencing the microbial community structure (Truu et al., 2009). High temperature and solar irradiation positively influenced the degradation of different pharmaceuticals such as diclofenac, ibuprofen, ketoprofen, naproxen, salicylic acid, triclosan, and carbamazepine (Li et al., 2014) and antibiotics possibly share the same fate. On the other side, higher concentrations of ARGs were reported in natural and constructed wetlands during the summer period compared to winter (Li et al., 2019). Nevertheless, the influence of temperature on ARG fate is under debate and

a different study revealed that low temperatures promote antibiotic resistance and horizontal gene transfer (HGT) of integron-associated ARGs (Miller et al., 2014).

A further issue on the efficacy of CWs in restricting ARGs and ARB diffusion in the environment has been overlooked until recently. The natural microbial community associated to CW plants could become itself a reservoir for specific ARGs due to the continuous exposure to antibiotics and ARB present in the raw or partially treated wastewater. In a ten years-experiment, Fang et al. (2017) showed that CW system could promote the increase of ARG level in the effluent mostly because of the accumulation of these genes into CW sediment. Similar results were obtained by Song and coworkers (2018) using synthetic wastewater, with negligible ARG abundance, as CW inflow. The effluent presented a lower abundance of ARGs compared to the CW sediments, however the ARG abundance was higher in the effluent compared to the inflow, unveiling the role of the natural bacterial community associated to *Oenanthe javanica* plants in the antibiotic resistance diffusion.

4.2 Role of horizontal gene transfer (HGT) in the ARG diffusion into the environment and within the plant microbiome

HGT is a crucial mechanism involved in evolution that consists in the movement of genetic material across different organisms. In bacteria it is generally divided in three main mechanisms, i.e. transformation, transduction and conjugation (Aminov 2001; Van Elsas et al., 2003; Baltrus 2013). In the last few years HGT has been increasingly investigated in relation to the spread of ARGs that are frequently linked to MGE (Petrovich et al., 2018) and could be finally internalized by pathogenic bacteria representing a risk for human health (Song et al., 2018; Headd and Bradford 2018).

HGT events have been examined in many different studies mimicking environmental conditions (Qiu et al., 2018; Baur et al., 1996; Hasegawa et al., 2018). For instance, Klümper and colleagues (2015) showed the possible spread of different plasmids in a soil bacterial community highlighting the

importance of HGT in this environment. In a recent study, MGEs have been discovered in metal contaminated soil amended with pig manure (Garbisu et al., 2018), in agreement with the previous knowledge that manure's utilization as fertilizer in crops may enhance the abundance of ARGs in the soil microbiota by HGT (Chen et al., 2016b; Gotz and Smalla 1997). The frequency of HGT events depends on many different factors, beside the specific molecular nature of the mobile element and donor/receiving strains. As a general factor, HGT could be enhanced by selective pressure due to anthropogenic activities that release into the environment different classes of pollutants, e.g. metal or antibiotics under the minimal inhibitory concentration (MIC) (Santos et al., 2018). Furthermore, HGT events are enhanced in environments with high cell density and metabolic activity (Ulrich et al., 2015). Conjugation, for example, may depend on the ratio of donor to recipient strains and on the dimension and the conformation of the plasmid (Zeaiter et al., 2018). The HGT promoting conditions can occur in specific hot spots in natural and engineered ecosystems, mostly characterized by the presence of dense aggregates of colonies where cell-to-cell contact is boosted. Notably, even low concentrations of antibiotics were proved to determine the aggregation of bacterial cells in aquatic ecosystems, thus creating conditions prone to cell-cell DNA transfer (Corno et al., 2014; Eckert et al., 2019).

Biofilm and mycosphere are two examples of environmental hot spots for HGT events. Qui and colleagues (2018) visualized ARGs transfer through conjugation in an activated sludge bacterial biofilm, and several studies underlined that conjugation and transformation events happen at a higher frequency when bacterial cultures are in biofilm rather than in the planktonic state (Madsen et al., 2012). Another HGT hot spot is the mycelia produced by Fungi that provide a high amount of nutrients and a wide surface to which bacterial cells can adhere, enhancing the frequency of the conjugation events (Berthold et al., 2016). High concentrations of MGEs have been detected in water ecosystems like WWTPs (Petrovich et al., 2018), where the frequency of HGT events can be increased due to the presence of a dense microbial

population and sublethal concentrations of antibiotics acting as selective pressure toward the resistance acquisition. WWTP effluents can influence the spread of ARGs through MGEs in aquatic sediments (Chu et al., 2018) and it has been demonstrated that the release of tertiary treated urban wastewater can increase ARG concentration in the surface layers of the receiving water bodies (LaPara et al., 2011). Moreover, Di Cesare and co-authors (2016) demonstrated that chemical disinfection of urban wastewaters, treated in different WWTPs of Northern Italy, could favour the survival of bacterial populations resistant to antibiotics, able to form cell aggregates and harbouring different ARGs.

Major HGT hot spots related to the plant niche are rhizosphere, phyllosphere and spermosphere (Aminov 2011; Chen et al., 2019; Van Elsas et al., 2003) and, accordingly, HGT events in the plant microbiome have been extensively reported. Conjugation between exogenous and indigenous bacteria have been detected in bacterial communities of poplar plants (Ulrich et al., 2015) while plasmid transfer from *Pseudomonas putida* to indigenous bacteria have been studied on the surface of alfalfa sprout (Mølbak et al., 2003). Likewise, Björklöf and colleagues (1995) demonstrated that conjugative plasmid transfer occur in the phyllosphere of bean plants. Microcosm scale experiments showed that conjugation events can occur in barley seedling sphermosphere, where the transfer of plasmid RP4 from a *Pseudomonas* strain donor to the indigenous bacteria has been detected (Sorensen and Jensen 1998).

In the frame of phytodepuration, the rhizosphere is the most crucial hot spot of HGT events. In phytodepuration systems such as CWs, the root apparatus and its associated microbial community are continuously exposed to substances (i.e. pharmaceutical, biocides, metals) promoting selection and co-selection events. Moreover, in this habitat bacterial populations are metabolically active due to the high nutrient content, live in high concentration and tight contact and harbour abundant MGEs (Chen et al., 2019). Plasmid transfer has been studied in barley and pea rhizosphere (Mølbak et al., 2003;

Sørensen and Jensen 1998) and a higher relative abundance of IncP-1 plasmid has been detected in lettuce rhizosphere in comparison with bulk soil (Jechalke et al., 2014). Accordingly, a recent study suggests that plasmid transfer in rhizosphere soil of maize seedlings occurs with higher abundance than in bulk soil (Zhu et al., 2018). The chemical composition of root exudates, including sugars, organic acids and amino acids, enhances HGT and plasmid transfer events in the rhizosphere (Zhu et al., 2018), as specifically shown for nodulation-inducing flavonoid (Ling et al., 2016). The investigation of HGT events in the rhizosphere of plants in phytodepuration systems is still neglected, revealing a gap of knowledge that should be taken into account for the overall evaluation of this wastewater treatment technology, especially in the frame of ARG spread into the environment.

Even if there is massive evidence on HGT occurrence and ARGs presence in the environment, limited information is still available on their relationship in both natural and human impacted ecosystems, although previous studies suggest that HGT events, such as conjugation, happen in nature at several orders of magnitude higher than *in vitro* studies (Davies and Davies 2010). According to the available literature, HGT events in plant microbiome are influenced by different abiotic factors such as pH, temperature, micro- and macro-nutrients, oxygen and moisture content, and by biotic agents such as the presence of antagonistic, syntrophic or competing organisms (Van Elsas et al., 2003). Unfortunately, the high variability of such parameters over time and space makes extremely difficult the comprehension of the spread and dynamics of antibiotic resistance in the environmental niches where HGT can take place, hampering the modelling of antibiotic resistance fate that could be useful for successful water reuse management strategies.

5. Concluding remarks and research perspectives

In this review, we presented the advantages related to the implementation of microbial assisted phytodepuration. Bacterial inocula have indeed the potential to contribute to the biological wastewater cleanup in phytodepuration

systems that nowadays are proposed as a low maintenance solution for water reclamation at suitable quality for water reuse in agriculture. Thanks to different PGP mechanisms, bacteria are able to support the growth of wetland plants improving their service in term of nutrient and pollutant removal, through the action of a more developed root apparatus and higher biomass. Microorganisms, enriched in plant rhizosphere, can also degrade recalcitrant micropollutants, such as pharmaceuticals, that are not targeted by common wastewater treatment plants, directly improving the effluent quality.

On the other side, we evidenced the possible peculiar risk of this technology in terms of antibiotic resistance diffusion in the environment. Designed wetlands seem to have a high efficiency in the removal of pollutants of emerging concerns, such as antibiotics, ARGs and ARB, although this is still debated given some contrasting results present in the literature. We highlighted that the pollutant removal processes in phytodepuration are performed in the rhizosphere, which is reported as a hot spot for HGT that could drive the diffusion of ARGs and the ARB. Although the root apparatus of plants used in phytodepuration of urban wastewaters is constantly exposed to ARB and low concentration of biocides and antibiotics, we point out that HGT of ARGs is still not sufficiently investigated in this type of wastewater treatment facilities. In particular, we recommend the need of a deep characterization of the antibiotic resistance profile and the related genetic determinants for the bacteria selected as promising inocula for assisted CW phytodepuration, characterization that is lacking in all the literature works discussed in this review. Some authors demonstrated a role of the natural community associated to plants in CWs in increasing the ARG concentration in the effluent, making important to clarify the dynamics of antibiotic resistance in response to the addition of bacterial inocula in such systems. The investigation of such aspects is pivotal in the frame of the ‘One-Health’ approach, especially when the treated wastewater is reused for irrigation purposes, entering the food production chain and eventually contributing to the diffusion of ARGs that could be acquired by pathogenic bacteria.

Even though further studies are necessary to deepen our knowledge on the above-mentioned phenomena, assisted phytodepuration can be considered a valuable approach able to enhance the quality of treated wastewater and it can be part of a wider strategy to boost water reuse worldwide.

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Chapter VII

Conclusion and future perspective

Global warming is enhancing water scarcity, but the demand of water is going to increase (WEF, 2019; UN, 2015). In order to optimize the use of water, the use of treated wastewater to irrigate crops has to be a priority for water management policy in several countries e.g. Europe (SCHEER, 2017). However, treated wastewater derived from wastewater treatment plants (WWTPs) promotes the stabilization of a resistome, i.e a cluster of microbial genes which encodes for proteins responsible for antibiotic resistance machineries in bacteria, in freshwater bodies (Kumar and Kumar, 2021; Corno et al., 2018), which are then used for irrigation.

Diffusion of antibiotic resistance (AR) determinants in environment is a pressing problem to be counteracted in 21st century (Smalla et al., 2018). Globalization has lowered barriers between humans, animals and different environments allowing bacteria to move among them. The exchange of bacteria among these three compartments could enhance the diffusion of antibiotic resistant genes (ARGs) derived from anthropogenic environment or which could be previously harboured in environmental harmless bacteria, among pathogenic bacteria derived from human and animal environments (Larsson et al., 2018). The connection between anthropogenic environments with the natural one(s) requires fighting AR determinants diffusion through a one-health approach in which all the connections established among the different environments are considered (Larsson et al.,2018; Smalla et al., 2018). There are a lot of evidence about the presence of AR determinants, divided in both antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), in the agri-food system (Summerlin III et al., 2021; Schierstaedt et al., 2019; Kampouris et al., 2021.). However, in order to understand the magnitude of the risks to which humans could be exposed, further studies linked to the characterization of the different sources of AR determinants, the routes of diffusion and possible strategies of mitigation have to be carried out (Larsson et al.,2018; Smalla et al., 2018).

The aim of the PhD project was to evaluate the possible routes of diffusion of AR determinants in the agri-food system, through horizontal gene transfer

(HGT) mechanisms, with a closer look to the possible spread of AR determinants considering their journey from WWTPs to freshwater bodies, until their possible acquisition by bacterial community of plants of agricultural interest, hence possibly posing a risk to food safety and human health. Specifically, when possible, I preferentially decided to use environmental strains during HGT experiments to mimic as possible conditions that could be encountered in the agri-food system especially because environmental strains could possess several traits, due to the continuous exposure to “horizontal gene pool”, which could help them to survive in environment (Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005; Vandecraen et al., 2017), acting also on the frequency of HGT, as already studied in *Vibrio* species (Bernardy et al., 2016).

One of the several problems linked to AR diffusion is the possibility that a pathogen, including fecal indicator bacteria (FIB), derived from an antrhopogenic environments has to acquire an ARGs from harmful bacteria which usually thrive in the environments (Larsson et al., 2018). FIB, which *E. coli* is a member, can enter in the agri-food system (freshwater bodies) through different ways e.g. WWTPS, sewage discharge and fecal direct deposition; indeed their presence have been detected in sediments of lakes and on fish (Korajkic, et al. 2019). However less attention have been devoted to the interaction of FIB with small invertebrates such as zooplankton. Considering the interaction between zooplankton and bacteria (Tang et al., 2010) and the constitutive presence of AR determinants in acquatic evironments (Corno et al., 2019) I verified the possible interaction between *E. coli* and Daphnia. I first evaluated the relationship established by different environmental strains of *E. coli* isolated from *Daphnia obtusa* with the host itself, highlighting the role of the latter in helping *E. coli* to adapt to the freshwater system, allowing also the interaction with other bacteria. Due to the positive interconnection founded between zooplankton and bacteria, natural transformation (Winter et al. 2020), of which magnitude is underestimated, was further investigated to understand implications that could have on AR

determinants. Moderate ability of natural transformation of *E. coli*, mimicking environmental conditions that could be found in food system, have been previously evaluated (Hasegawa et al., 2018). In this thesis, I however demonstrated the ability of an environmental *E. coli* strain to acquire DNA by natural transformation in conditions similar to the ones which could be found in freshwater bodies, detecting a higher frequency than the one measured for a laboratory strain. Moreover, the strain ability to colonize lettuce rhizosphere was verified, highlighting the possible spread of AR determinants in the agri-food system. The impact of zooplankton, represented by the model *Daphnia obtusa*, on natural transformation was also monitored by the use of the environmental bacterial strain *Acinetobacter baylyi* BD413. I decided to use A. *baylyi* BD413 because it is known to be constitutively competent to acquire exogenous DNA with a relatively high frequency (Seinz and Blokesh, 2013); and the aim of the study was to monitor the influence of zooplankton on transformation frequency, which is usually dependent on a plethora of specific factors (Blokesh, 2016) which could influence transformation frequency instead of zooplankton. This study has thus revealed that the zooplankton could decrease the transformation frequency through DNA degradation, highlighting its possible role in counteracting AR diffusion. Results obtained in chapters II, III and IV help to unveil the possible role that zooplankton could have in the spread of AR determinants, through natural transformation, in the agri-food system: on one hand, even if *Daphnia* reduces the abundance of *E. coli* in water (Ismail et al., 2019), It could help *E. coli* to survive and to adapt to freshwater environment thanks to a short-term association, which could be considered as a concret risk linked to AR diffusion do to the ability of *E. coli* to acquire eDNA in condition which could be encountered in freshwater bodies as discovered in chapter III and other studies (Hasegawa et al., 2018; Baur et al. 1997). On the other hand, as demonstrated in chapter IV, zooplankton could help to limit the diffusion of ARGs by degrading them and limiting their diffusion through natural transformation. Results obtained in these chapters devoted to the study of natural transformation linked to freshwater bodies and

zooplankton underline the complexity of AR diffusion in the environments and how it is important to further study it to completely understood the spread of AR in freshwater bodies.

The last part of the PhD project was devoted to the study of conjugation in the rhizosphere of the plants, one of the main hot spot of HGT linked to plants microenvironments (Riva, V et al., 2020). To simulate the spread of AR determinants which could happen through the use of treated wastewater used for irrigation purpose the aim of the fifth chapter was the construction of an environmental donor strain to study conjugation in a complex bacterial community: specifically, a *Klebsiella variicola* subsp. *variicola* isolated from treated wastewater derived from a WWTP was genetically manipulated as described by Musovic et al. (2010). Its abilities to donate a plasmid carrying an antibiotic resistance gene to the complex community of lettuce' rhizosphere, and to colonize the root apparatus were demonstrated making this donor strain an optimal candidate to study the possible route of diffusion of AR determinants through conjugation. Data collected about the possible AR diffusion routes, focused on the description of the reuse of treated wastewater for irrigation purpose, underline the complexity of AR diffusion in its linked environments, unveiling some cold and hot spots, corroborating that a one health approach is mandatory to counteract AR diffusion phenomenon. Moreover, data suggest that it could be easier to limit the possible diffusion of AR determinants nearby the effluent of WWTPs, in order to minimize the conditions which would have to be considered to counteract the possible spread of AR determinants once treated wastewater are released in the water bodies. Studies conducted in this PhD work help to understand the magnitude of AR diffusion, due to HGT mechanisms, in the agri-food system underlining some possible routes of diffusion of AR determinants and mapping different ARGs hot spots, to create a rank risk map and a risk assessment plan to counteract AR spread together with technologies able to reduce AR determinants concentration and a more responsible and informed behaviour to minimize the risk for human health.

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APPENDICES

Publications

Riva, F., Riva, V., Eckert, E. M., Colinas, N., Di Cesare, A., Borin, S., Mapelli, F., & Crotti, E. (2020). An Environmental *Escherichia coli* Strain Is Naturally Competent to Acquire Exogenous DNA. *Front. Microbio*., 11, 2131.

Riva, V., **Riva, F**., Vergani, L., Crotti, E., Borin, S., & Mapelli, F. (2020). Microbial assisted phytodepuration for water reclamation: Environmental benefits and threats. *Chemosphere*, 241, 124843.

National and international conferences

Oral presentation:

F. Riva, E. Crotti, "Possible routes of antibiotic resistance diffusion through horizontal gene transfer in environments linked to the agri-food system". "First Virtual Workshop on the developments in the Italian PhD research on food science, technology and biotechnology". Page 350, 14-15 September 2021. Title: page 350

Poster presentations:

F. Riva, V. Riva, E. M. Eckert, N. Colinas, A. Di Cesare, S. Borin, F. Mapelli and E. Crotti "Natural transformation of an environmental *Escherichia coli* in conditions which may be encountered in the agri-food system". "ISME Virtual Microbiology Ecology Summit #UnityinDiversity" 11-12 November 2020.

F. Riva, E. Crotti "The Dimension of Antibiotic Resistance in the Agri-Food System"XXIV Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology" Florence, 11-13 Sep 2019.

F. Riva, E. Eckert, F. Mapelli, S. Borin, E. Crotti “Natural transformation in environmental-like conditions of an *Escherichia coli* strain isolated from *Daphnia obtusa*” “Microbiology 2019. XXXII SIMGBM Congress” Florence, 19-22 June 2019.

Courses:

Physalia Courses “Metabarcoding for Microbial Communities” 23-27 November 2020.

Physalia Courses “Metagenomics, metatranscriptomics, and multi'omics for microbial community studies” 1-5 June 2020.