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Genomic and Functional Analysis of Members of the Gut Microbiota: Novel Insight on *Clostridium ramosum*, *Escherichia coli*, and Enterobacteriaceae

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XXXII CYCLE

Abstract (English)

Gut microbiota is a collection of bacteria, archaea, and eukarya that inhabits the mammalian gastrointestinal tract. The composition of this microbial community is host specific, evolves throughout lifetime, and can create an intricate and mutually beneficial relationship with the host (Backhed, 2005; Neish, 2009).

The first project of this thesis focused on the deep characterization of a collection of *E. coli* and non- *E. coli* Enterobacteriaceae (NECE) isolated from the fecal samples of 20 healthy volunteers, in order to determine whether the relationship between PFGE genotyping, phylogroups, genetic determinants, and functional features can be established among the isolates. Previous research mainly focused on the characterization of antibiotic resistance acquisition of virulent clinical *Escherichia coli* and other species of the family Enterobacteriaceae, whereas strains isolated from healthy subjects have been less studied and mostly investigated only in comparative studies as controls of clinical isolates (Johnson et al., 2000; Kudinha et al., 2013; Lee et al., 2019).

A total of 51 different strains of *E. coli* and 32 NECE belonging to the species *Klebsiella pneumoniae, K. oxytoca, Enterobacter cloacae, E. aerogenes, E. kobei, Citrobacter freudnii, C. amalonaticus, Hafnia alvei, Cronobacter, Morganella morganii,* and *Serratia liquefaciens* were collected. According to Clermont phylogrouping, the 27% of *E. coli* isolates belongs to B2 phylogroup, the 24% to B1, 14% to F, 14% to A, 10% to D, 8% to E and 4% to C. In *E. coli* isolates was registered a great variability of pathogenicity-associated features suggesting that the genetic determinants of virulence had a role in shaping their genome. Most of the *K. pneumoniae* strains were found positive for adhesin and siderophores genes. Several strains of *E. coli* had the potential to cause extraintestinal infections because of the presence of genes associated with adhesins, siderophores, and toxins. However, most of *E. coli* strains did non form biofilm and a total of 49 out of 51 strains were sensitive to all the tested antibiotics. On the other hand, several NECE were resistant to amoxicillin plus clavulanic acid, but sensitive to all the other tested antibiotics. Among *E. coli* and NECE strains isolates, strains that potentially act like pathogens and innocuously inhabited the gut of healthy subjects were identified on the basis of the presence of virulence genes, capacity of biofilm production, and genetic material transfer through conjugation. These strains may occasionally act as etiologic agents of extra-intestinal infections, albeit most *E. coli* and NECE strains were susceptible to a wide range of antibiotics and confirmed that the problem of antibiotic resistance is restricted to

frequent and inappropriate use the antibiotics, and to the hospital setting where antibiotic pressure is highest.

The second project of this thesis focused the study of *Clostridum ramosum*, a bacterium that seem involved in obesity development due to its role in the modulation of serotonin availability in the intestinal epithelium that cause an increased nutrient absorption (Mandić et al., 2018). The presence of *C. ramosum* was investigated in mice strains prone to obesity and in mice subjected to a high-fat diet in order to identify endogenous strains. From a total of 85 mice, it was possible to isolate only 3 different strains of endogenous *C. ramosum*. Isolation of *C. ramosum* from intestinal samples of a number of obese mice was quite rare, suggesting that this species probably is not a common inhabitant of the gut microbiota of obese mammals, in disagreement with previous evidences (Turnbaugh et al., 2009; Ferrer et al., 2013).

Abstract (Italiano)

Il microbiota intestinale è un insieme di batteri, archea ed eucarioti che risiedono nel tratto intestinale dei mammiferi. La composizione di questa varia comunità microbica è specifica per ogni individuo e crea una intricata e mutualistica relazione con esso evolvendosi durante l'arco della vita (Backhed, 2005; Neish, 2009).

Il primo progetto di questa tesi ha riguardato la caratterizzazione di ceppi di *E. coli* e di non-*E. coli* Enterobatteriacee (NECE) isolate dai campioni fecali di 20 soggetti sani, con lo scopo di comprendere se esistono delle relazioni tra di loro sulla base del genotipo attribuito dalla PFGE, del filogruppo, dei determinanti genici di virulenza e delle strutture funzionali caratterizzanti. Negli studi precedenti la ricerca su questi microrganismi si è focalizzata principalmente sulla caratterizzazione dell'antibiotico resistenza di isolati clinici virulenti di *Escherichia coli* e di altre specie appartenenti alla famiglia delle Enterobatteriacee, mentre questi ceppi isolati da soggetti sani sono stati poco studiati o solamente caratterizzati in studi comparativi impiegati come controllo per isolati clinici (Johnson et al., 2000; Kudinha et al., 2013; Lee et al., 2019).

Sono stati isolati un totale di 51 biotipi diversi di *E. coli* e 32 di NECE cappartenenti alle specie *Klebsiella pneumoniae, K. oxytoca, Enterobacter cloacae, E. aerogenes, E. kobei, Citrobacter freudnii, C. amalonaticus, Hafnia alvei, Cronobacter, Morganella morganii,* e

Serratia liquefaciens. L'assegnazione del filogruppo secondo Clermont ha rivelato che il 27% degli isolati di *E. coli* appartiene al filogruppo B2, il 24% al B1, il 14% all'F, il 14% all'A, il 10% al D, l'8% all'E e il 4% al C. Tra i ceppi di *E. coli* isolati è stato possibile osservare una grande variabilità i termini di determinanti genici di virulenza che suggeriscono come queste caratteristiche associate a patogenicità abbiano un grande impatto nel modellare il genoma degli isolati. Molti dei ceppi isolati di *E. coli* potrebbero causare infezioni extraintestinali dal momento che presentano geni associati ad adesine, siderofori e tossine. I ceppi di *E. coli* generalmente non formano biofilm, e 49 su 51 sono sensibili agli antibiotici comunemente usati per il trattamento delle infezioni da Gram-negativi. Al contrario, numerosi isolati NECE siano risultati resistenti all'amoxicillina e acido clavulanico ma sensibili agli altri antibiotici testati. Tra i ceppi di *E. coli* e di NECE che risiedono in modo asintomatico nel tratto gastrointestinale dei 20 soggetti sani, sono stati osservati ceppi che potenzialmente potrebbero comportarsi da agenti eziologici di infezioni extra-intestinali dal momento che presentano numerosi geni di virulenza, sono capaci di produrre biofilm, e di trasferire materiale genetico tramite coniugazione. Questi ceppi potrebbero occasionalmente essere agenti eziologici di infezioni extraintestinali, anche se comunque sono suscettibili ad un numero elevato di antibiotici, confermando come il problema dell'antibiotico resistenza sia ristretto ad un uso frequente ed inappropriatodi questi farmaci e alle strutture ospedaliere in cui ne viene fatto un impiego maggiore.

Il secondo progetto riguarda lo studio di *Clostridium ramosum*, un batterio che sembra avere un ruolo nell'insorgenza dell'obesità a causa della capacità di modulare la disponibilità di serotonina nell'epitelio intestinale, con conseguente aumento dei livelli di assorbimento dei nutrienti (Mandić et al., 2018). La presenza di *C. ramosum* è stata valutata in topi soggetti ad una dieta ricca di grassi o obesi, al fine di individuarne ceppi endogeni. Da un totale di 85 topi, è stato possibile isolare solo 3 ceppi endogeni di C. ramosoum.

L'isolamento di *C. ramosum* dai campioni intestinali di numerosi topi obesi è stato un evento piuttosto raro, suggerendo che questa specie non è un comune abitante del microbiota mammiferi obesi, in disaccordo con precedenti evidenze (Turnbaugh et al., 2009; Ferrer et al., 2013).

INDEX

1.0 INTRODUCTION

1.1 Gut microbiota

The collection of bacteria, archaea, viruses and eukarya that colonize the gastrointestinal tract is named ‘gut microbiota’, and it has co-evolved with the host over thousands of years creating an intricate and mutually beneficial relationship with it (Backhed, 2005; Neish, 2009). It has been estimated that the number of microorganisms that resides in the GI are more than 10^{13}, a number that is actually of the same order as the number of human body cells , and the amount of their genomic content is many times higher than the human genome, accounting for more than 1000 different species (Sender et al. 2016). Together, the collection of microorganisms and their respective genomes creates the “microbiome”.

The gut microbiota plays pivotal and essential roles in the gastrointestinal tract: it is involved in nutrition, as a modulator of metabolic responses, in the immune system development and response, and in the maintenance of host health (Ventura, et al., 2018). For these reasons the gut has been proposed to be regarded as an essential component of the human body: “the forgotten organ” (Kashyap et al., 2017). Nowadays it is widely accepted that gut microbiota affects many aspects of human health even if many information regarding mechanisms of action are still missing (Ventura et al., 2018). Nevertheless, several studies have confirmed the notion that the intestinal microbial communities play an important role in energy homeostasis and may also modulate weight gain or loss and obesity-associated disorders (Rosenbaum, et al., 2015). Moreover, the gut microbiota and their metabolites, have also been associated with blood pressure regulation, chronic kidney disease and cardiovascular disease. In addition, alterations in gut microbial composition or function have been associated with

changes in host immune status, inflammatory bowel disease, allergy, and asthma (Marques et al., 2018; Rooks and Garrett, 2016; Sircana et al., 2018; Carding et al., 2015).

Many studies have focused on how different external factors can modulate and shape the composition and function of the gut microbiota (Marchesi et al., 2016). One of the most important modifiers of the gut microbiota is the diet: macronutrients, micronutrients, as well as prebiotics, probiotics food additives, and other minor components including contaminants can exert a relevant effect in the composition modulation (Graf et al., 2015; Wang et al., 2015; Roca-Saavedra et al., 2018).

1.2 Fingerprinting techniques

Before the development of molecular biology techniques, microorganisms were usually characterized on the basis of their morphological and physiological characteristics. Several problems related to reproducibility, discriminatory power, or untypeability are often associated with the use of phenotypic methods. Such shortcomings have led to the development of genotypic methods. Most of them rely on visualization of DNA bands obtained from restriction digestion, hybridization, or PCR amplification. Some of the DNA fingerprinting techniques are based on the presence/absence of the restriction sites, while others are based on the homologies to short oligonucleotide primers. The outcomes of these methods are banding patterns or "DNA fingerprints" that are used to compare strains and obtain a hierarchical clustering.

1.2.1 RAPD-PCR

The Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) is a tool widely used since 1990's to assess intra-specific genetic variation (Welsh and McClellan, 1990). This technique is based on the arbitrary amplification of polymorphic DNA sequences. Amplification is carried out using a single, non-specific primer, no longer than 8-12 bp, whose sequences are random and not designed to be complementary to any particular site in the chromosome. Other commonly utilized single primers are the minisatellite derived from the core-sequence of the wildtype phage M13 (5'-GAGGGTGGCGGTTCT-3'), or the microsatellite sequence (5'-GACAGACAGACAGACA-3') (Meyer et al., 2001).

These primers bind in different and randomly distributed sequences on the denatured genomic DNA, under low stringency conditions. Since the primers do not anneal on any particular genetic locus, several priming events can result from variations in experimental conditions, making this PCR fingerprinting technique subjected to rigorous standardization laboratory methods. The amplification creates short amplicons that can be seen after gel electrophoresis and proper staining, the so called "DNA fingerprint". Different DNA-fingerprinting identifies diverse biotypes, allowing in some case to distinguish among strains belonging to the same species (Figure 1.1).

One of the main RAPD-PCR advantages is that no prior sequence information is required, and that the entire genomic sequence is explored. However, the major disadvantage is lack of inter-laboratory reproducibility because a little change in protocol, primers, polymerase or DNA extraction may give different results. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible (Fakruddin et al., 2013).

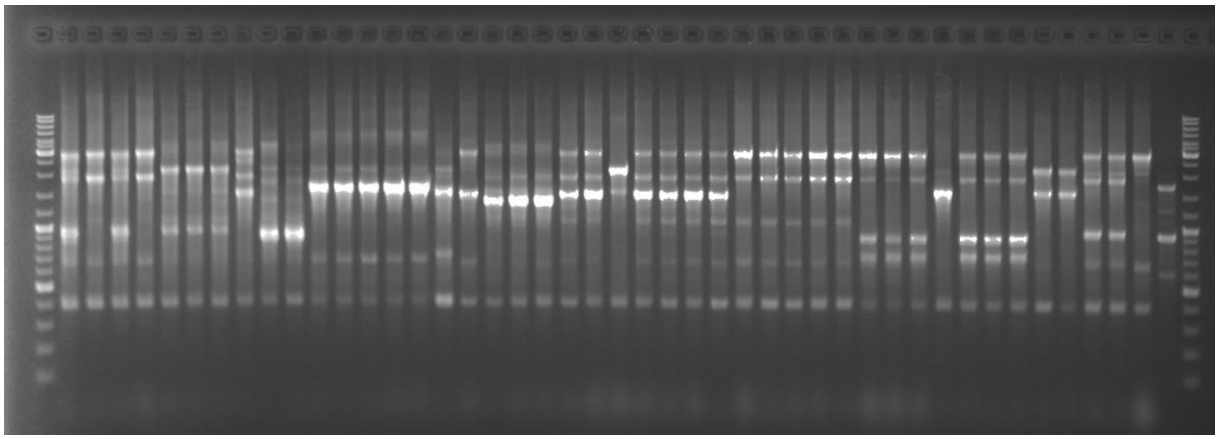

Figure 1.1 *RAPD-PCR fingerprinting of bacteria isolated from the same fecal sample, with similar and different profiles.*

1.2.2 Enterobacterial repetitive intergenic consensus

Enterobacterial repetitive intergenic consensus (ERIC) sequences are described as intergenic repetitive units first described in *Escherichia coli*, *Salmonella enterica* serovar *typhimurium*, and other members of the Enterobacteriaceae, as well as *Vibrio cholerae* (Sharples and Lloyd, 1990; Hulton et al., 1991). The ERIC sequence is an imperfect palindrome of 127 bp (Figure 1.2) found only in intergenic regions, apparently only within transcribed regions (Hulton et al., 1991). The number of copies of the ERIC sequence is different among

species: it was estimated by extrapolation that there are probably 30 copies in *E. coli* K-12 and maybe 150 in *S. enterica* typhimurium LT2 (Hulton et al., 1991). It is not clear the function of these copies (Duchaud et al., 2003).

These sequences are important for studying the evolution of enterobacteria. They are longer than others, more informative in comparative analyses, and present in a wider range of species among Enterobacteriaceae family. These sequences have been used as a template for primer design in order to discriminate closely related strains, or for the detection and differentiation of diverse bacteria in a sample of interest. ERIC-PCR can be used in investigation of pathogens epidemiology and to describe bacterial contamination in food (Ventura et al., 2003; Alippi et al., 2004; Yan et al., 2007; Ye et al., 2008).

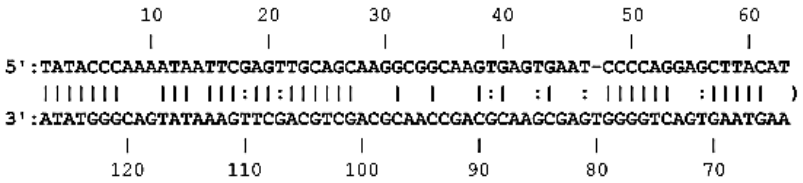

Figure 1.2 *The ERIC sequence: the 127 bp sequence is shown as a hairpin where lines connect bases in the two complementary DNA arms.*

ERIC-PCR succeeded in a wide range of different bacterial families other than Enterobacteriaceae and Vibrionaceae, suggesting that ERIC sequences may occur throughout the bacterial kingdom (Versalovic et al., 1991; Lupski and Weinstock 1992).

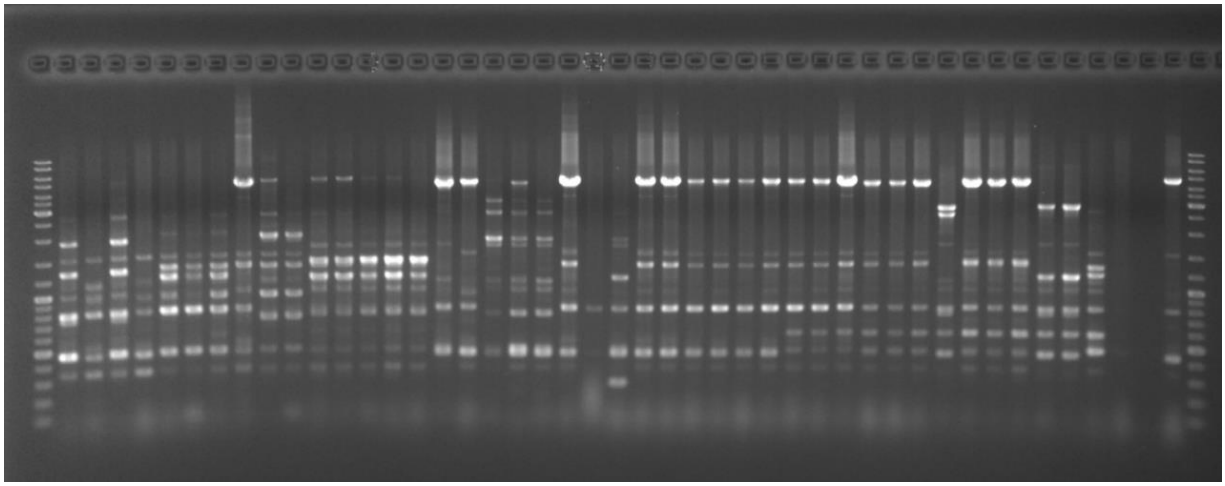

Figure 1.3 *ERIC-PCR fingerprinting of bacteria isolated from the same fecal sample, with similar and different profiles.*

1.2.3 Pulsed Field Gel Electrophoresis (PFGE)

In 1982, for the first time, DNA molecules larger than 50 kb were separated by using two alternating electric fields. Pulsed Field Gel Electrophoresis (PFGE) increased by two orders of magnitude the size of DNA molecules routinely separated and analysed (Schwartz et al., 1982). This allowed DNA investigations from bacteria and viruses to mammals (Smith et al., 1986a). PFGE showed excellent ability to separate small, natural linear chromosomal DNAs ranging in size from 50-kb of a parasite's microchromosomes to Mbp of yeast's chromosomes.

PFGE allows the construction of a physical map by previous digestion of DNA with restriction endonucleases. For PFGE it is advisable to use enzymes which have relatively few cut sites and give larger fragments from the target DNA and databases are available to find the most suitable enzyme for PFGE migration of the diverse species (Bhagwat, 1992). Analysis with rare-restriction enzymes provides information on genome size, and allows the genetic characterization of the strains, tracing the genetic evolution of a particular strain, or building up physical and genetic maps of bacterial chromosomes and studying chromosomal dynamics among bacteria (Grouthues et al., 1991; Kohara et al., 1987; Chang et al., 1990; Basim et al., 1998).

The gel where the digested samples run was placed into the chamber for horizontal electrophoresis. One field was homogeneously generated by two rows of dot electrodes. The other field was nonhomogeneous and generated by a number of dot electrodes as cathode and one dot electrode as anode. At the beginning, the electric field was switched at a 90 ° angle (Schwartz and Cantor, 1984). The authors called this system "pulsed field gradient electrophoresis" (PFGE).

Later in 1986, a new device for PFGE with a controlled homogeneous electric field (contour-clamped homogeneous electric field, CHEF) was developed (Chu et al., 1986). In this system, multiple dot electrodes are fixed around a hexagonal contour and clamped to potentials appropriate for a homogeneous field (Figure 1.4). The voltage is periodically switched in three directions; one that runs through the central axis of the gel and two that run at an angle of 60 ° either side. The switching pulse time is equal for each direction and this results in net forward migration of the DNA. The run takes longer than a normal gel electrophoresis because the size of the fragments is bigger, and the DNA doesn't move in a straight line through the gel.

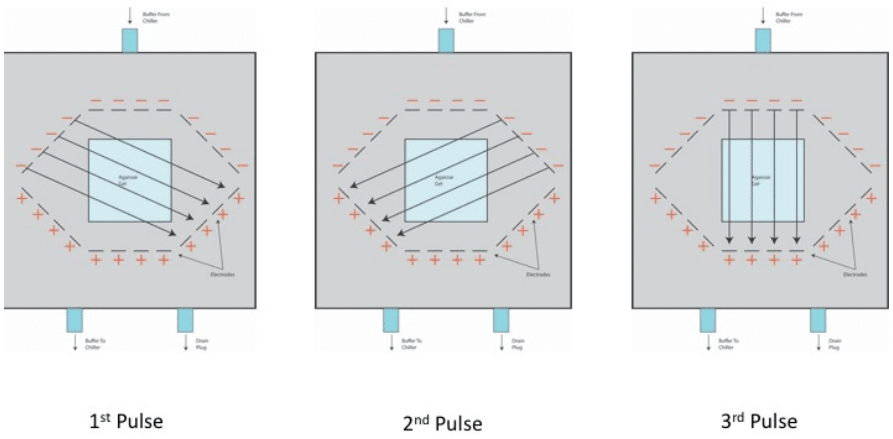

Figure 1.4 *Horizontal electrophoresis chamber of contour-clamped homogeneous electric field CHEF, with multiple dot electrodes fixed around a hexagonal contour. The voltage periodically switches in three directions: one that runs through the central axis of the gel and two that run at an angle of 60 ° at either sides.*

Nowadays, CHEF is the most widely used for PFGE, because it ensures the most effective fractionation of DNA molecules in a wide range of sizes. PFGE became the standard method used for bacteria typing. With the PFGE, the migration of DNA fragments obtained using different enzymes is a powerful tool for the quick resolution of bacterial genome into a small number of large fragments (Figure 1.5). In this way, it is possible to compare different migration patterns and establish a degree of relatedness among different strains of the same species. PFGE is a very useful technique in epidemiological studies, as well as population studies (Parizad et al., 2016).

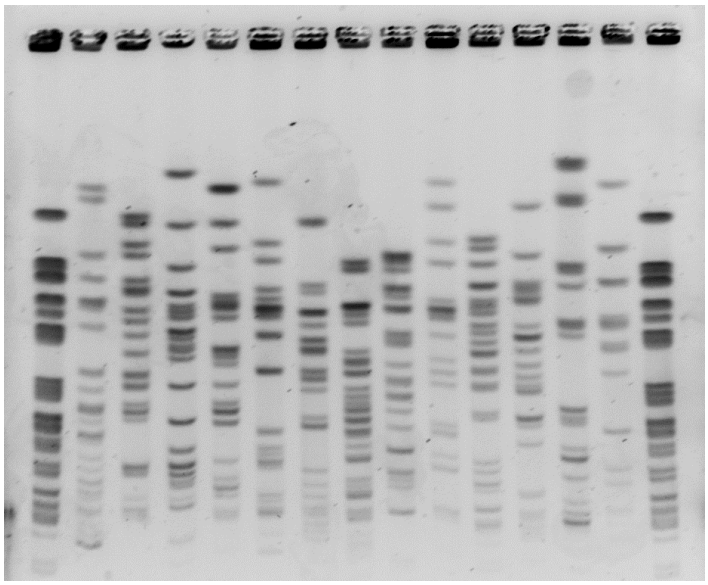

Figure 1.5 *PFGE fingerprinting of bacteria belonging to the same species, with similar and different profiles. In the first and in the last columns is loaded a reference strain as molecular marker.*

2.0 Isolation and molecular typization of *E. coli* and enterobacteriaceae from 20 healthy volunteers

2.1 INTRODUCTION

2.1.1 Enterobacteriaceae

The family Enterobacteriaceae includes Gram-negative, facultatively anaerobic non-spore-forming rods bacteria. It belongs to the γ-class of Proteobacteria and to the order Enterobacteriales. Enterobacteriaceae are ubiquitous bacteria that can be found worldwide in different ecological niches such as soil, water, vegetation, and animals (Brenner, 2006; Janda and Abbott, 2006; Janda, 2006). Certain species are common inhabitants of animal and human microbiota, among which many are frequently associated with diarrheal disease and severe extraintestinal infections (Janda and Abbott, 2006; Leimbach et al., 2013).

Yersinia pestis, the agent of the plague, is one of the most important pathogens in human history and it belongs to the family Enterobacteriaceae, like other bacteria such as *Salmonella enterica* serotype *typhi, Shigella,* and *Escherichia coli,* that currently represent a major public health concern due to virulence and antibiotic resistance. Enterobacteriaceae can cause both intestinal and extraintestinal diseases. The transmission route of intestinal infection is fecal-oral due to either consumption of contaminated food or water, or by direct contact with animals or the environment, or also by person-to-person. Virulent enterobacteriaceae can also translocate from the gut to blood, resulting in extraintestinal disease, often observed in immunocompromised hosts or persons with cirrhosis, or undergoing chemotherapy. Other enterobacteriaceae are important pathogens of plants and insect endosymbiont.

Generally, the bacteria of this family are motile, with the exception of the genera *Arsenophonus, Biostraticola, Klebsiella, Moellerella, Obesumbacterium, Raoultella, Shimwellia, Tatumella*, and of the endosymbionts *Buchnera, Sodalis*, and *Wigglesworthia*. They are catalase positive and oxidase negative, with the exception of *Plesiomonas*. They use

the Embden–Meyerhof pathway for sugar metabolism and acid production from glucose fermentation. Enterobacteracee can be distinct from other Gram-negative rod-shaped bacteria on the basis of cell geometry, flagellar arrangement, oxidase production, sodium requirement, as well as the presence of the Enterobacterial common antigen (ECA) (Francino et al. 2006).

Sequencing the 16S rRNA genes, it is possible to classify the members of this family in 52 genera and approximately 290 species (Hata et al., 2016). In Figure 2.1 is reported the phylogenetic relationships between the type strains of a few enterobacteriaceae species, based on the sequence comparison of this gene.

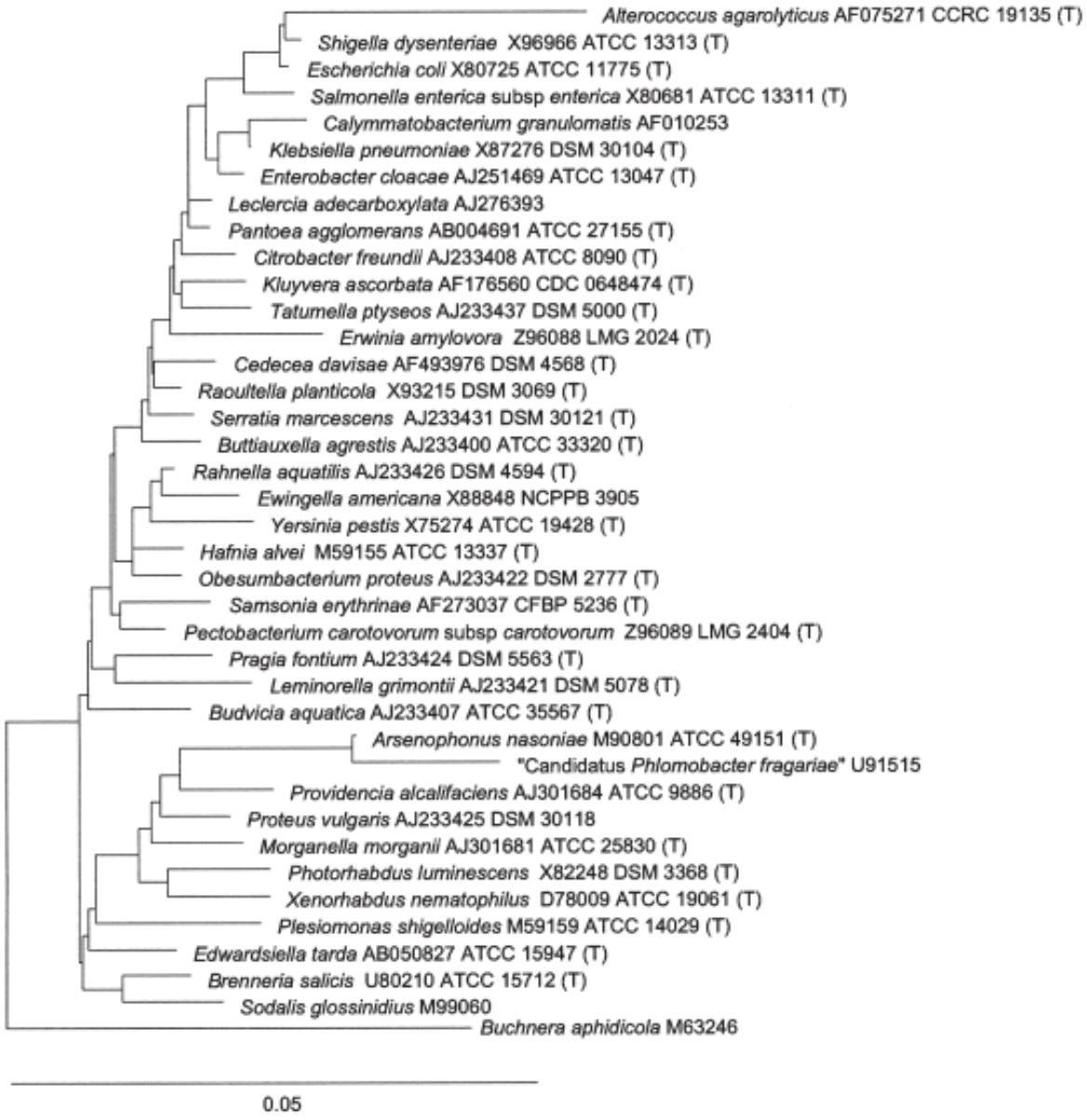

Figure 2.1 *Phylogenetic relationships between the type strains of Enterobacteriaceae*

2.1.2 Habitats

Enterobacteriaceae are ubiquitous, categorized into three groups on the basis of host that they can colonize: human and animals, plants, or insects associated endosymbionts. Few plants associated pathogens can cause infections also in humans like *Pantoea agglomerans*, a plant contaminant. Human pathogens such as *Salmonella* and *E. coli O157:H7* are capable of colonize or invade plants and infection outbreaks in humans have been associated with raw plant consumption. In this case bacteria colonization happens inside the plant tissue rather than on the surface (Tyler and Triplett, 2008).

Among the Enterobacteriaceae family the following genera can cause severe human infections: *Budvicia, Buttiauxella, Cedecea, Citrobacter, Cronobacter, Edwardsiella, Enterobacter, Escherichia, Ewingella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Moellerella, Morganella, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Salmonella, Serratia, Shigella, Tatumella, Trabulsiella, Yersinia,* and *Yokenella*.

2.1.3 *Escherichia coli*

Escherichia coli is a gram negative, facultative anaerobic, rod-shaped bacterium belonging to the γ-Proteobacteria class and Enterobacteriaceae family. It is a pervasive inhabitant of warm-blooded animals and reptile intestine (Kaper et al., 2004; Lukjancenko et al., 2010). Usually, it resides in the mucous layer of the caecum and colon and it can behave as commensal and harmless strain as well as potential pathogen. *E. coli* starts to colonize human gut during the first hours after birth and it became soon the most abundant facultative anaerobe of the infant's microbiota.

E. coli is able to survive in different ecological environments because it is a very versatile species, with metabolic and regulatory capabilities exploited to colonize different niches and to survive in critical situation for long periods. It is present in soil, water, and food. Some strains express many virulence genes, giving to *E. coli* the capacity of causing infections. Based on the body sites where *E. coli* can cause infections, two main groups of *E. coli* have been recognized: IPEC, intestinal pathogenic *E. coli* and ExPEC, extraintestinal pathogenic *E. coli*. Inside these two groups, it is possible to classify *E. coli* strains on the basis of the disease they cause.

Among IPEC strains, the recognized pathotypes are the enteroaggregative *E. coli* (EAEC), the enterohaemorrhagic *E. coli* (EHEC), the enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), the enterotoxigenic *E. coli* (ETEC), the diffusely adherent E. coli (DAEC), and the adherent invasive *E. coli* (AIEC) (Figure 2.1 and Figure 2.2) (Diaz et al., 2001; Kaper et al., 2004; Wirth et al., 2006; Hendrickson, 2009; Crossman et al., 2010). These pathotypes are characterized by different sets of the virulence factors (VFs).

Among ExPEC, uropathogenic *E. coli* (UPEC) are the most relevant strains, followed by septicemia-associated *E. coli* (SEPEC) and avian pathogenic *E. coli* (APEC) (Kaper et al., 2004; Crossman et al., 2010; Croxen and Finlay, 2010). Under poor hygiene conditions, EPECs are the main cause of diarrhoea in children (Kaper et al., 2004).

Pathotype (acronym)	Diseases	Symptoms	Virulence factors	Ref.
Enteric E.coli				
EnteroPathogenic *E. coli* (EPEC)	Diarrhoea in children	Watery diarrhoea and vomiting	Bfp, Intimin, LEE	[1]
EnteroHaemorrhagic *E. coli* (EHEC)	Haemorrhagic colitis, HUS	Bloody diarrhoea	Shiga toxins, Intimin, Bfp	[1,3]
EnteroToxigenic *E. coli* (ETEC)	Traveler's diarrhoea	Watery diarrhoea and vomiting	Heat-labile and sheat-stable toxins, CFAs	[4,5]
EnteroAggregative *E. coli* (EAEC)	Diarrhoea in children	Diarrhoea with mucus and vomiting	AAFs, cytotoxins	[6,7]
Diffusely Adherent *E. coli* (DAEC)	Acute diarrhoea in children	Watery diarrhoea, recurring UTI	Daa, AIDA	[8]
EnteroInvasive *E. coli* (EIEC)	Shigellosis-like	Watery diarrhoea; dysentery	Shiga toxin, hemolysin, Cellular invasion, Ipa	[1,7]
Adherent Invasive *E. coli* (AIEC)	Associated with Crohn disease	Persistent intestinal inflammation	Type 1 fimbriae, Cellular invasion	[9,10]
Extraintestinal E. coli (ExPEC)				
UroPathogenic *E. coli* (UPEC)	Lower UTI and systemic infections	Cystitis, pyelonephritis	Type 1 and P fimbriae; AAFs, hemolysin	[1,11]
Neonatal Meningitis *E. coli* (NMEC)	Neonatal meningitis	Acute meningitis, sepsi	S fimbrie; K1 capsule	[12,13]
Avian Pathogenic *E. coli* (APEC)	Probable source of food-borne disease	-	Type 1 and P fimbriae; K1 capsule	[14,15]

Bfp: Bundle-forming pili; LEE: Locus for enterocyte effacement; HUS: haemolytic-uraemic syndrome; CFA: colonization factor antigen; AAF: aggregative adherence fimbria; Daa: diffuse adhesin; AIDA: adhesin involved in diffuse adherence; Ipa: Invasion plasmid antigen.

Table 2.1 *E. coli pathogenic types (Allocati et al., 2013).*

EHEC strains are usually food-borne pathogens capable of causing haemorrhagic colitis or haemolytic-uremic syndrome. They can also produce toxins similar to those produced by *Shigella dysenteriae*, and for this reason they are called Shiga toxin producing *E. coli* (STEC)

(Bilinski et al., 2012). The most common pathogen causing travellers' diarrhoea are ETEC strains, responsible for an infection characterized by mild or severe outbreaks in humans.

EAEC strains are the second cause of travellers' diarrhoea worldwide, albeit they can be found also in the gut of asymptomatic humans. EAEC strains are also present in children from developing countries or in HIV-infected patients (Qadri, et al., 2005; Al-Abri et al., 2005).

Also DAEC and EIEC can cause diarrhoea in children (Servin, 2005). EIECs are closely related to *Shigella* spp. and can cause watery diarrhoea and occasionally dysentery in both children and adults (Kaper et al., 2004). AIECs have been recently identified as a pathotype always present in humans with Crohn's disease (Darfeuille-Michaud, 2002; Negroni et al., 2012).

APECs are pathotypes found in the intestine of healthy birds, that cause extraintestinal diseases in several avian species, and represent a possible cause of food-borne diseases (Rodriguez-Siek et al., 2005; Johnson et al., 2007).

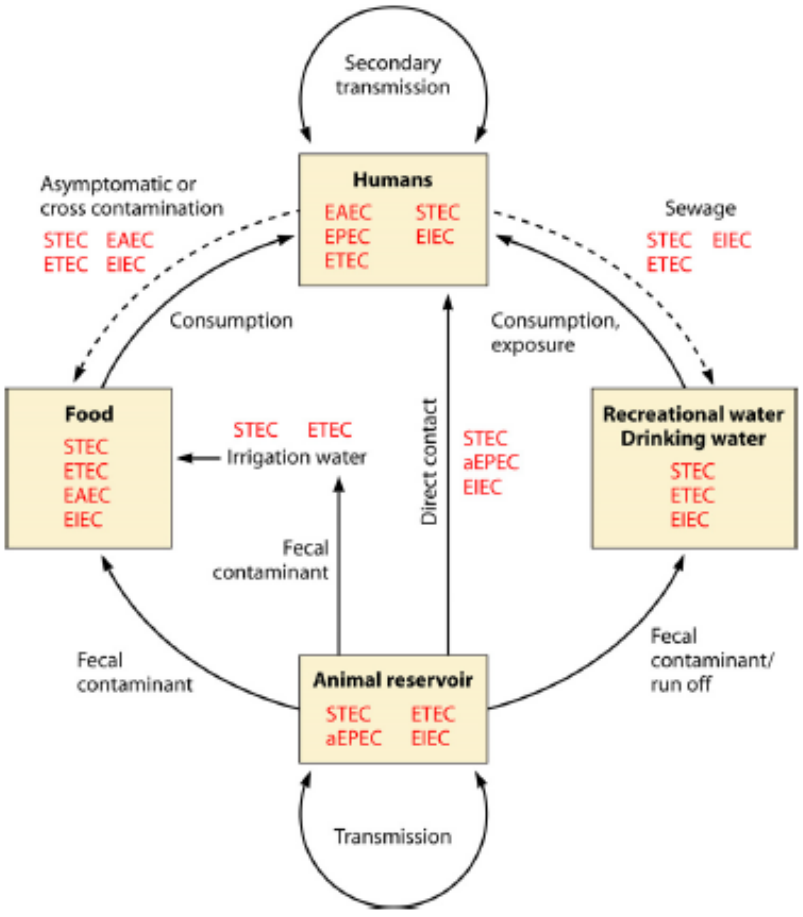

Figure 2.2 *Schematic overview of potential ways of transmission and reservoirs for pathogenic E. coli (Croxen et al., 2013).*

2.1.3.1 Structure of the *E. coli* genome

E. coli is characterized by dynamic genome structure that originated the metabolic and phenotype diversity own by the different strains cited above. Based on comparative genomics approach, it is possible to divide *E. coli*'s genome in two components: the "core genome" and the "flexible/dispensable genome".

The "core genome" consists in the genes that are present in all the strains of *E. coli* such as housekeeping genes involved in replication, transcription, and translation. This core genome is like a genomic backbone that define the basic metabolic functions of the species.

The "flexible/dispensable" genome embraces genes that are present in only few *E. coli* strains. These genes are responsible for strain specific characteristics like the diverse metabolic and phenotypical traits that confer adaptation to several environments or peculiar pathogenic abilities (Medini et al., 2005). They show high rates of nucleotide sequence variability, that is a characteristic of mobile elements like plasmids, phages or genomic islands (GEIs). Virulence genes are part of the flexible genome pool, and for this reason, *E. coli* that acquires these mobile elements, may become pathogenic and able to colonize different body districts and causing infections (Medini et al., 2005).

The total of the genes of a species is called "pangenome" and is the combination of the core and the flexible/dispensable genome (Tettelin et al., 2005). A recent comparison of 186 *E. coli* genomes found approximately 1,700 homolog gene clusters shared in all genomes and a pangenome of about 16,400 gene clusters (Kaas et al., 2012).

Horizontal gene transfer (HGT) through conjugation, homologous recombination, genome reduction events, insertion sequence (IS)-elements, transposons, and integrons are the main responsible for genetic flexibility in bacterial species (Dobrindt et al., 2004; Hacker et al., 2003). These elements enhance homologous recombination and can lead to large-scale genomic rearrangements. Moreover, through conjugation, these accessory elements can be transfer between strains. Consequently, genomic diversity can be propagated vertically within a population by clonal proliferation (Schubert et al., 2009; Jackson et al., 2011;). For example, *E. coli* is deeply in contact with the gut microbiome's community, and the bacterial diversity in that environment can be considered a pool of genes that can be acquired by HGT (Tenaillon et al., 2010).

Pathogenic islands (PAI) are a subgroup of genomic islands (GEIs) that are originated by genome integration and loss of mobile function events of former lysogenic bacteriophages and plasmids (Hacker et al., 2003). In GEIs and PAIs can be stored a lot of novel genes for environment adaptation and survival strategies. Also, virulence factors (VFs) can be over-represented and clustered on PAIs. For this reason, with horizontal gene transfer, it is possible to propagate virulence determinants between different bacterial strains and species. With the same mechanism, also antibiotic resistance genes can be transferred because they are usually encoded in integrons and resistance islands. PAIs are integration sites, and that's why a concentration of virulence genes in specific genomic regions can be easily and simultaneously acquired. On the other hand, by using the same mechanism, GEIs or PAIs can be also deleted in a single step (Tenaillon et al., 2010).

Over the evolutive process, extensive gene acquisition by HGT and gene loss resulted in the above-described pathotypes with distinct pathogenic capabilities, and independently by phylogenetic lineages (Dobrindt, 2005). It was demonstrated that commensal *E. coli* strains usually have smaller genome sizes than pathogenic strains, and this might be related to virulence-associated genes (Sims and Kim, 2011; Chaudhuri and Henderson, 2012).

Intestinal and extraintestinal *E. coli* pathogen (IPEC and ExPEC respectively), are different in their genetic composition as well as in their phylogenetic evolutive past. IPEC pathotypes evolved clonally by a common serotype under intestinal niches adaptation. Subsequently, early HGT events caused subsequent divergence of these clones, adding specific pathogenic capabilities (Didelot et al., 2012). New combinations of VFs gave the bacterium the capacity to colonize new niches and allow these *E. coli* clones to cause a broad spectrum of diseases. ExPEC differ from IPEC because they derived from different serotypes, and they do not have a distinctive repertoire of VFs that can cause specific type of disease (Köhler and Dobrindt, 2011). In fact, ExPEC are characterized to have different combinations of VFs and genome sequencing projects showed considerable genome diversity among them. These VFs combinations are pathotype-specific genes including toxins, iron acquisition systems, adhesins, lipopolysaccharides (LPS), polysaccharide capsules, proteases, and invasins. They are all genes frequently found on mobile elements easily identified with molecular techniques like by PCR (Dobrindt, 2005; Moriel et al., 2010; Köhler and Dobrindt, 2011; Lu et al., 2011).

2.1.3.2 *E. coli* phylogroup classification

Ochman and Selanders in 1984 created the "ECOR: *E. coli* reference strain collection" with the use of multi-locus enzyme electrophoresis (MLEE) on a set of 72 reference strains of *Escherichia coli* isolated from different hosts (both animals and humans) and from different geographical locations (Ochman and Selander, 1984). At the beginning ECOR classified *E. coli* strains in five major phylogenetic lineages: A, B1, B2, D, and E. Surprisingly even now, in the age of genomics, this partition is still in use and true in most of the cases. With this classification, most of *E. coli* commensal strains are grouped in the phylogroup A and B1. At early stages of *E. coli* evolution, phylogroup B2 and D diverged simultaneously. Many of ExPEC and AIEC strains are contained in B2 phylogroup. Phylogroup D is divided in two clades: D1 and D2, in which are present UPEC, EAEC, ExPEC and environmental strains. Phylogroup E is in a separate clade than the others and it contains O157:H7 EHEC and O55:H7 EPEC strains. In this collection it is also possible to notice that *Shigella*, the ''*E. coli* pathotype'', is phylogenetically close to groups A, B1, and E (Chaudhuri et al., 2010).

MLST (multi-locus sequence typing), feature frequency profiles, and whole genome phylogeny of the core genome of several *E. coli* strains, have confirmed the overall topology described above (Sims and Kim, 2011; Chaudhuri and Henderson, 2012). These observations suggested the need of a simple method for assign a phylogroup to clinical *E. coli* isolates.

At the beginning of the century, subtractive libraries were created for two *E. coli* strains belonging to different phylogenetic groups and an unknown 14.9 kb fragment was characterized because deeply associated with neonatal meningitis (Bonacorsi, et al., 2000). These findings suggested that certain DNA fragments or genes can be specific for a specific phylogroup and because of that identified. Three candidate markers were studied in order to develop a method for phylogroup assignment:

- *chuA*, a gene required for heme transport in enterohemorrhagic O157:H7 E. coli (Bonacorsi et al., 2000);
- *yjaA*, a stress response protein;
- *TspE4.C2*, a lipase.

Based on the presence or the absence of these three fragments, Clermont demonstrated that it was possible to assign isolated *E. coli* strains to the phylo-groups, A, B1, B2 or D (Clermont et al., 2000).

Recently, two other phylogroups were identified: group F and C. Phylogroup F is a sister group phylogroup B2, and phylogroup C has been suggested to be a close related B1 phylogroup (Jaureguy et al., 2008; Moissenet et al., 2010; Clermont et al., 2011a).

To sum up, *E. coli sensu strictu* can be classified in seven recognised phylogroups: A, B1, C, B2, D, E, F. The first tree groups are mainly commensal *E. coli* strains while the others are principally pathogenic strains. Because two more phylogroups were added, in 2013 Clermont and colleagues implemented their previous triplex-PCR for phylogroup identification. *ArpA* target gene was added to the other tree target genes, for differentiate strains belongings to phylogroup D to F. It was demonstrated that *ArpA* gene is present in all *E. coli* strains with exceptions of strains belonging to phylo-groups B2 and F (Clermont et al., 2004). Moreover, for differentiate phylogroup E to D and A to C three additional allele-specific PCR primer pairs were also designed (Lescat et al., 2012). By scoring the presence/absence of the genes *arpA/chuA/yjaA/TspE4.C2* it is possible to assign the phylogroup to isolated *E. coli* (Figure 2.3, Table 2.2) (Clermont et al., 2013).

Quadruplex genotype					
arpA (400 bp)	*chuA* (288 bp)	*yjaA* (211 bp)	TspE4.C2 (152 bp)	Phylo-group	Next step
+	–	–	–	A	
+	–	–	+	B1	
–	+	–	–	F	
–	+	+	–	B2	
–	+	+	+	B2	
–	+	–	+	B2	Could be confirmed by testing *ibeA* gene[a]
+	–	+	–	A or C	Screen using C-specific primers. If C+ then C, else A
+	+	–	–	D or E	Screen using E-specific primers. If E+ then E, else D
+	+	–	+	D or E	Screen using E-specific primers. If E+ then E, else D
+	+	+	–	E or clade I	Screen using E-specific primers. If E– then clade I, confirm using cryptic clade primers[b]
–	–	+	–	Clade I or II	Confirm using cryptic clade primers[b]
–	(476)[c]	–	–	Clade III, IV or V	Confirm using cryptic clade primers[b]
–	–	–	+	Unknown	Perform MLST
–	–	+	+	Unknown	Perform MLST
+	–	+	+	Unknown	Perform MLST
+	+	+	+	Unknown	Perform MLST
–	–	–	–	Unknown	Confirm *Escherichia* identification using *uidA* or *gadA/B*[d], if positive screen using cryptic clade primers[b] and/or perform MLST

a. Gordon and colleagues (2008).
b. Clermont and colleagues (2011b).
c. The quadruplex PCR reaction will result in strains belonging to cryptic clade III, IV or V yielding a 476 bp PCR product. If this outcome eventuates then such strains should be screened using the cryptic clade detection primers (Clermont *et al.*, 2011b).
d. McDaniels and colleagues (1996).

Table 2.2 *Criteria for phylogroup assignment according to Clermont et al., 2013.*

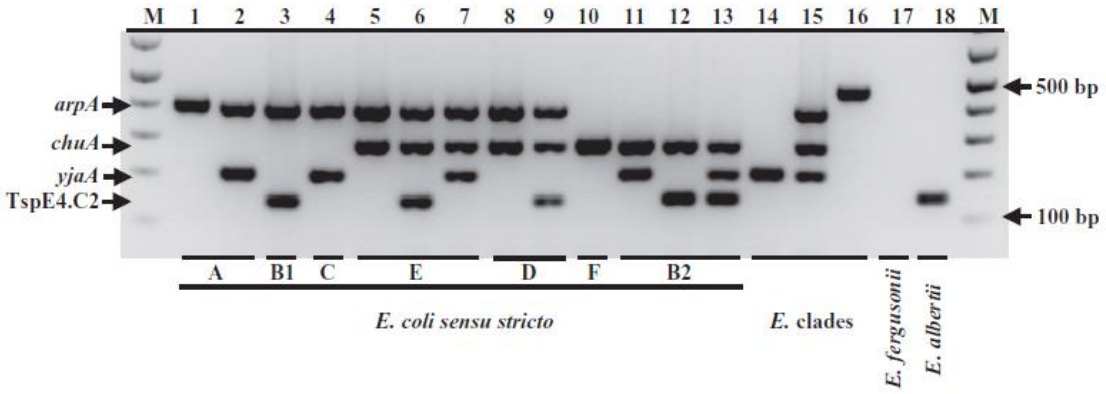

Figure 2.3 *Quadruplex PCR profiles according to Clermont phylo-typing (2013).*

2.1.4 Klebsiella

Klebsiella genus was named in memory of Edwin Klebs (1834–1913), a German bacteriologist who described for the first time this bacterium, by Trevisan in 1885 (Trevisan, 1885).Bacteria belonging to this genus are capsulated rod cells and can be present as single isolated cell,s in pairs or short chains. They are not motile; they can use citrate and glucose as sole carbon source, and they are positive for catalase.

The genus *Klebsiella* consist of 14 species: *Klebsiella aerogenes, Klebsiella granulomatis, Klebsiella grimontii, Klebsiella huaxiensis, Klebsiella kielensis, Klebsiella michiganensis, Klebsiella milletis, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella quasivariicola, Klebsiella senegalensis, Klebsiella steroids, Klebsiella variicola*.

In particular *K. pneumoniae* can be divided in 3 subspiecies: *K. pneumoniae* subsp. *ozaenae, K. pneumoniae* subsp. *pneumoniae* and *K. pneumoniae* subsp. *rhinoscleromatis*. *K. quasipneumoniae* is composed by two subspiecies: *K. quasipneumoniae* subsp. *quasipneumoniae* and *K. quasipneumoniae* subsp. *similipneumoniae*.

Klebsiella species are ubiquitous in nature, they can be found in water, soil and animals, and they can colonise medical devices and the healthcare environment (Podschun and Ullmann 1998; Podschun *et al.*2001). *Klebsiella* spp. can be isolated from the human intestinal tract and in minor concentration also from the nasopharynx. In particular, *Klebsiella* spp. are typically opportunistic pathogens that colonise mucosal surfaces without causing diseases. However, from mucosae, *Klebsiella* spp. can start to spread to other tissues causing life-threatening infections including pneumonia, bloodstream infections and sepsis (Paczosa and Mecsas,

2016). *Klebsiella* infections are severe problem among neonates, elderly and immunocompromised individuals. *Klebsiella* spp. are a severe cause of both nosocomial and community acquired infections (Magill *et al.*, 2014). Among all *Klebsiella* species, *K. pneumoniae* is one of the most common causes of nosocomial infections worldwide. Nowadays there's a high prevalence of antibiotic-resistant strains, and it has become a serious public health concern (Paczosa and Mecsas, 2016).

Several virulence factors were identified as being important for *K. pneumoniae*: lipopolysaccharide (LPS), siderophores, fimbriae, OMPs, porins, efflux pumps, iron transport systems, and genes involved in allantoin metabolism. These virulence factors determine which kind of infections *K. pneumoniae* could cause. *K. pneumoniae* is an extremely resilient bacterium, and its success as a pathogen seems to follow the statement of "the best defence for a pathogen is a good defence" rather than "the best defence for a pathogen is a good offense." In fact, it can evade many components of the immune system and survive, rather than actively suppress, and with this strategy it can grow in many different sites inside the hosts (Paczosa and Mecsas, 2016). For example, in *K. pneumoniae* are not present type III secretion systems for inject toxins into immune cells in order to inactivate the phagocytic capability of these cells as pathogenic *Yersinia* commonly does (Trosky et al., 2008). In contrast, *K. pneumoniae* appears to evade phagocytosis thanks to the capsule that makes really hard to bound and taken up by phagocytes and prevent also to be killed by bactericidal serum factors (Domenico et al., 1994; Rahn et al., 2003).

K. pneumoniae virulent strains can express capsular types K1 and K2. It has been demonstrated that the 63,4% of *K. pneumoniae* isolated form liver abscess and the 85,7% of those isolated from complicated endophthalmitis is a K1 serotype that are also highly resistant to neutrophil phagocytosis (Hirche et al., 2005). Fang et al., have identified virulence gene *magA* that is a component of K1 capsule formation. Although non-K1 serotypes have been also observed in *K. pneumoniae* liver abscess (KP-LA), but they were less frequently encountered. Serotype K2 is the second most commonly isolated serotype in KP-LA in Asia and US (Siu et al., 2011; Shen et al., 2013)

Nowadays, the vast majority of *K. pneumoniae* are resistant to ampicillin due to the presence of a chromosomal gene encoding a penicillin-specific β-lactamase (Hæggman et al., 1997). In addition, as consequence of the acquisition of multidrug-resistant plasmids, nosocomial isolates are usually resistant to numerous other antibiotics. In fact, *K. pneumoniae*

usually carry plasmids encoding extended-spectrum β-lactamases and carbapenemases and infections with this kind of strains is associated with higher rates of treatment failure and death (Tumbarello et al., 2006; Ben-David et al., 2012). In particular, hypervirulent *K. pneumoniae* (hvKp) are strains that are resistant to the majority of antibiotics commonly used today. This strain usually express extended spectrum β-lactamases (ESBL), a variety of carbapenemases, and the colistin resistance gene *mcr-1*. Treatment of such superbugs is very difficult due to the paucity of therapeutic options available and the toxicity of last-resort drugs. However, these pathogens have been largely isolated in healthcare environment and are of relatively low virulence, because they cause invasive infection only in opportunistic settings or after extensive antimicrobial exposure. For such strains, the combination of virulence and antimicrobial resistance is highly problematic and leaves few polymyxins available or no other options of treatment (Russo et al., 2018).

Klebsiella oxytoca can cause community-acquired infections similar to those produced by *K. pneumoniae* but is a less common pathogen. *K. oxytoca* and *K. pneumoniae* can be distinguished because the first one can produce indole and the second not. *K. oxytoca* is characterized by a similar antibiotic resistance profile to *K. pneumoniae*. Many strains of *K. oxytoca* produce a chromosomally mediated beta-lactamase (known as K1 β-lactamases) that hydrolyses extended-spectrum cephalosporins and aztreonam (Arakawa et al., 1989). Although the majority of *K. oxytoca* isolates produce only low levels of the K1 β-lactamase, 10-20% of the clinical isolates have a mutational hyperproduction the enzyme (Livermore 1995). *K. oxytoca* isolates have been found also with different types of ESBLs, such as TEM, SHV, IRT, plasmid-mediated ESBLs, and plasmid-mediated *ampC* type β-lactamases (Jacoby et al., 1991; Granier et al., 2002; Philippon et al., 2002).

2.1.5 Enterobacter

The *Enterobacter* genus was described for the first time in 1960 by Hormaeche and Edwards (Hormaeche and Edwards, 1960). The genus includes 20 species: *E. aerogenes, E. amnigenus, E. arachidis, E. asburiae, E. cancerogenus, E. cloacae, E. cowanii, E. gergoviae, E. helveticus, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. oryzae, E. pulveris, E. pyrinus, E. radicincitans, E. soli,* and *E. turicensis*. Additionally, *E. cloacae* is divided in two subspecies, *E. cloacae* subsp. *cloacae* and *E. cloacae* subsp. *dissolvens*. *E.*

hormaechei is divided into three subspecies: *E. hormaechei* subsp. *oharae, E. hormaechei* subsp. *hormaechei* and *E. hormaechei* subsp. *steigerwaltii* (Hoffmann et al. 2005b).

On the basis of the 16S rRNA gene sequence comparison, it was determined that the genus *Enterobacter* is polyphyletic, but most of the species could not be resolved (Mezzatesta et al., 2012). Therefore, it has been determined that multilocus sequence typing analysis (MLST) is a more appropriate way for the phylogenetic classification of *Enterobacter* species (Brady et al., 2013).

Enterobacter spp. can be found in both terrestrial and aquatic environments: in soil, sewage, water, vegetable and fruits (Egamberdieva et al. 2008; Madhaiyan et al. 2010). Some species are plant associated, such as *E. more*, that is a common phytopathogens and *E. radicincitans, E. oryzae*, and *E. ludwigii* that are plant growth-promoting bacteria (Pawlicki-Jullian et al. 2010; Peng et al. 2009; Manter et al. 201; de Melo Pereira et al., 2012;). *Enterobacter* spp. can be also isolated form human and animal intestinal tract and they are significant human pathogens as well (Hoffmann et al., 2005a).

Enterobacter spp. achieved reputation of pathogen in 1976 after a nationwide outbreak of septicemia in 378 patients in 25 different hospitals due to contaminated intravenous solutions (Maki et al., 1976). In fact, they can easily grow in glucose-containing parental fluids and keep on causing sporadic outbreaks of this type (Verschraegen et al., 1988). *Enterobacter* spp. are well adapted to survive on skin and dry surfaces. Numerous outbreaks have been described, including infections due to contaminated enteral feedings, hydrotherapy water in a burn unit humidifiers and respiratory therapy equipment (Mayhall et al., 1979; Wang et al., 1991; Simmonsm et al., 1989). *Enterobacter* spp. are the fourth most common cause of Gram-negative bloodstream infection and are found to be increasing in a population-based study in the USA (Al-Hasan et al., 2011). In particular, *E. cloacae* and *E. hormaechei* are the most frequently isolated in nosocomial bloodstream infection (Mezzatesta et al. 2012).

Enterobacter species have numerous virulence factors including adhesions, endotoxin, and siderophores to acquire iron (Eisenstein et al., 2000). The incidence of nosocomial infections increases broad resistance to third generation cephalosporins, penicillins and quinolones. Luckily a number of antibacterial molucules still remain effective for treatment: fourth generation cephalosporins and carbapenems are the most attractive options (Al-Hasan et al., 2011).

2.1.6 Citrobacter

The genus *Citrobacter* was firstly described in 1932 by Werkman and Gillen (1932). A total of 11 species belong to this genus: *C. freundii, C. koseri, C. amalonaticus, C. farmeri, C. youngae, C. braakii, C. werkmanii, C. sedlakii, C. rodentium, C. gillenii, and C. murliniae*.

Citrobacter species are usually found in the intestinal tracts of animals and humans. However, they are also present in soil, water, and food probably due to fecal environmental contamination. *Citrobacter* spp. are opportunistic human pathogens that can cause a wide range of infections such as urinary tract infections, gastroenteritis, wound infections, pneumonia, brain abscesses, septicemia, meningitis, and endocarditis (Doran, 1999; Borenshtein and Schauer, 2006). In particular, *C. koseri* usually causes sepsis and meningitis leading to brain abscesses (Martinez-Lage et al., 2010; Lin et al., 2011; Vaz Marecos et al., 2012). Several of the *Citrobacter* infections are nosocomially acquired, but they can also be community acquired. The 0.8% of Gram-negative infection are caused by *Citrobacter*, but in hospital settings they might account for the 3-6% of all nosocomial infection from Enterobacteriaceae (Lavigne et al., 2007; Jones et al., 2010). The infection may happen as sporadic cases or nosocomial outbreaks.

2.1.7 Cronobacter

Cronobacter was proposed as a new genus in 2007 after a clarification of the taxonomic relationship of a group found among strains of *Enterobacter sakazakii* (Iversen et al., 2007). The strains belonging to this genus are positive for Voges–Proskauer test, citrate utilization, esculin and arginine hydrolysis, and negative for methyl red test, H_2S production, urea hydrolysis, lysine decarboxylation, and b-D-glucuronidase. They can growth different temperatures (6–45°C), pH (5-10) and NaCl concentration should be up to 7 % (w/v). They produce acid from D-glucose, D-sucrose, D-raffinose, D-melibiose, D-cellobiose, D-mannitol, D-mannose, L-rhamnose, L-arabinose, D-xylose, D-trehalose, galacturonate, and D-maltose (Rosenberg, Octavia and Lan, 2014).

Seven species are known: *C. condimenti, C. dublinensis, C. malonaticus, C. muytjensii, C. sakazakii, C. turicensis,* and *C. universalis*. In particular, *C. dublinensis* has three subspecies: subsp. *dublinensis*, subsp. *lausannensis*, and subsp. *lactariid*.

Cronobacter spp. are environmental organism, has been isolated from different foods and has emerged as a pathogen in recent years (Grimont and Grimont, 2006). *Cronobacter* spp. are also recognized as opportunistic pathogen that can cause bacteremia, necrotizing enterocolitis and meningitis, mostly in neonates. In 2012, due to contamination of the manufacturing environment, *Cronobacter* sp. was isolated in a powdered infant formula, becoming a very serious pathogen for new-born (Yan et al., 2012). It was reported that *Cronobacter* infections lead to death in the 40% of the infant infected (Yan et al., 2012). Moreover, *Cronobacter* spp. can also infect adults, and mostly immunocompromised patients (Tsai et al., 2013).

2.1.8 Hafnia

In 1954 the genus *Hafnia* was described for the first time by Moller (Moller 1954). The strains belonging to this genus are typically positive for Voges–Proskauer test, methyl red, lysine, and ornithine. Conversely, they are negative for arginine dihydrolases. *Hafnia* spp. can grow in a range of temperatures between 4 and 44°C, in media with the 2-5% of NaCl and pH between 4,9 and 8,25 (Greipsson and Priest 1983).

Two species of this genus exist: *H. alvei* and *H. paralvei*. They are distinct but closely related on the 16S rRNA gene tree. They differ for metabolic characteristic that include malonate assimilation, esculin hydrolysis, and the fact that *H. alvei* have β-galactosidase activity while *H. paralvei* not (arabinose and salicin (Janda and Abbott, 2006; Huys et al., 2010).

Members of the genus *Hafnia* are usually isolated from the gastrointestinal tract of humans and animals, including mammals, birds, reptiles, fish and insects and from foods, in particular in meat and dairy products (Janda and Abbott, 2006). *Hafnia* can cause animal infections and it have been found implicated human cases of bacteraemia, gastroenteritis and respiratory tract infections. There are some controversies about their status of primary human or animal pathogens because these organisms are usually found together with other pathogens and/or opportunists at the site of infection (Janda and Abbott, 2006).

2.1.9 Moraganella

The genus *Morganella* was described in 1943 by Fulton. The strains belonging to this genus are positive for indole, ferment salicin and produce gas when galactose and glucose are utilized as carbon source. they do not produce H_2S and ferment lactose or sucrose. They are not characterized by proteolytic activity.

Two species belong to this genus: *M. morganii* and *M. psychrotolerans*. In particular, *M. morganii* is divided into two subspecies and seven biogroups; *M. morganii* subsp. *morganii* containing biogroups A, B, C, and D and *M. morganii* subsp. *sibonii* containing biogroups E, F, and G.

M. psychrotolerans is a psychrotolerant species that grow at 0-35°C. *Morganella* spp. can tolerate up to 7,5% of NaCl and pH value from 4,6 to 9,2. *M. morganii* was isolated from the feces of healthy humans and animals (Manos and Belas, 2006). It can cause a broad range of infections in humans including sepsis, liver, renal and tube-ovarian abscesses, skin and soft tissue infections, bacteraemia, and pericarditis (Chou et al., 2009; Chang et al., 2011; Chen & Lin 2012). It is also a pathogen for animals as well, causing lethal infections in chickens and pneumonia in rabbits and piglets (Ono et al., 2001; Roels et al., 2007; Zhao et al., 2012).

2.1.10 Serratia

In 1823 Bizio described for the first time a red-pigmented bacterium on polenta and named it *Serratia marcescens* (Breed and Breed 1924). The strains belonging to this genus are able to grow on minimal medium, produce acids when they grow on maltose, salicin, trehalose, and they can hydrolyse ONPG. They rarely ferment lactose, and produce an extracellular DNase.

There are 15 species that belong to this genus: *S. entomophila, S. ficaria, S. fonticola, S. glossinae, S. grimesii, S. liquefaciens, S. marcescens, S. nematodiphila, S. odorifera, S. plymuthica, S. proteamaculans, S. quinivorans, S. rubidaea, S. symbiotica,* and *S. ureilytica*. The species that produce the typical red pigment called prodigiosin, from which *Serratia* spp. was identified, are *S. nematodiphila, S. marcescens, S. rubidaea*, *S. plymuthica*, and *S. fonticola*; the other species do not produce it.

These microorganisms are widespread in the environment. It has been found that *Serratia* spp. can exist as a symbiont of plants, insects and animals or as free-living form. Species like *S. fonticola* and *S. urilytica* can be present in water sources (Bhadra et al., 2005). *Serratia* spp. are not common component of the human gut microbiota (Donnenberg, 2015).

Potential virulence factors of *Serratia* spp. have not received a great deal of attention, but certain strains can be both mannose-sensitive (probably because of type 1 fimbriae) and mannose-resistant, and they may adhere to uroepithelial cells (Parment et al., 1992; Livrelli et al., 1996; Herte and Schwarz, 2004). *Serratia* spp. survive under harsh conditions, including after the use of different disinfectants, some of them have been the sources of outbreaks (Hejazi and Falkiner 1997). Infections caused by *S. marcescens* probably start after exogenous contamination and spread through the hospital on the hands of the personnel (Acar, 1986). Healthy humans are infected by *Serratia* only occasionally, but hospitalized patients are more susceptible. *S. marcescent*, *S. liquefaciens* and *S. rubidae* are the species that can cause nosocomial infections (Gill et al., 1981; Farmer et al., 1985). Clinically, *Serratia* infections symptoms are similar to those caused by other opportunistic pathogens: it can cause respiratory tract infections in intubated patients, urinary tract infections in patients with indwelling catheters, surgical wound infections, and septicaemia. However, meningitis, brain abscesses, and intraabdominal infections are not frequent (Maki et al., 1973; von Graevenitz, 1980). In addition, *S. marcescens* has a particular association with infections in injection drug users (Yu, 1979; Donnenberg, 2015). *Serratia* was found resistant to first-generation cephalosporins because of an inducible, chromosomal AmpC β-lactamase and to ampicillin (Mahlen et al., 2003) Moreover, many isolates possess plasmids encoding for cephalosporins, penicillins, carbapenems, and aminoglycosides resistance (Hejazi & Falkiner 1997; Acar, 1986).

2.1.11 Antimicrobial resistance and emergence of multidrug resistance

From the last years, a main global healthcare problem is antimicrobial resistance (WHO, 2014). Since the introduction of the penicillin, a large number of bacteria have developed different mechanisms to protect themselves from antibiotics and they can also transmit the antimicrobial resistance acquired to other species (Von Baum et al., 2005). The inappropriate use and the increased consumption of antimicrobial agents accelerated this phenomenon.

Moreover, the everlasting migration of people between countries as well as international tourism and business travel contributed in the acquisition and spread of multidrug resistant strains (Van der Bij et al., 2012). In animals, antimicrobial resistance was also observed. In fact, in particular for intensive animal farming, antibiotics are used for therapy, and for prophylaxis of infectious diseases (Szmolka et al., 2013). Resistant bacteria from animals can infect humans by direct contact and via animal origin food products, but they can even spread in the environment.

In the last decade we assisted to multidrug resistant bacteria phenomena. Multidrug resistance is defined when a bacterium is resistant to three or more antimicrobial classes (Magiorakos et al., 2012). Multi-resistant strains originated principally by the spread of genes located on mobile genetic elements, including plasmids, integrons and transposons. In addition, the combination of these genes with chromosomally encoded resistance genes results in bacteria that are resistant to all main classes of available antimicrobials (Kaper et al., 2004; Johnson et al., 2009). Antibiotic resistance associated with enterobacteriaceae was reported for the first time in the early '80s in Germany and France, and nowadays there's growing numbers of enterobacteriaceae strains found capable to produce extended-spectrum β-lactamases (ESBLs) (Wachino and Arakawa, 2012; D'Andrea et al., 2013; Ghafourian et al., 2014).

Extended spectrum β-lactamases (ESBLs) are enzymes that can hydrolyse extended spectrum cephalosporins such as ceftazidime, ceftriaxone, cefotaxime, cefepime, and render the bacteria resistant to their activity. There are three important types of ESBLs: TEM, SHV and CTX-M (Ghafourian et al., 2014). TEM and SHV types are usually carried by *E. coli* and *K. pneumoniae* but they are also found in *Enterobacter*, *Proteus*, *Morganella* and *Salmonella* species (Haeggman et al., 2004) These two enzymes have evolved from a mutation present on a common plasmid-encoded penicillinase. For what concern CTX-M enzymes, they are an example of plasmid-acquired β-lactamase genes. CTX-M family, described for the first time in Japan in 1982, hydrolyse mainly cefotaxime and are usually predominant among community-acquired infections. CTX-M are generally identified among the enterobacteriaceae isolated from the gut microbiota (Jenkins et al., 2017). Woerther et al., in 2013 suggest that CTX-M carriage is emerging as a global pandemic problem because they demonstrated that intercontinental travel of people and food may have accentuate the globalization of the problem of CTX-M enzymes.

It has been reported that many outbreaks of ESBL-producing bacteria are reported in Intensive Care Units (ICUs) in which unrelated strains of the same species or from different species are involved due to the dissemination of the ESBL coding plasmid. The risk factor of being infected by an ESBL-producing enterobacteriaceae increases with the length of the hospital recovery, admission to the ICU or for the pre-existence of comorbidities (Mahlen, 2011). In addition, in CTX-M-producing bacteria present also co-resistance to trimethoprim–sulfamethoxazole, tetracycline, gentamicin, and ciprofloxacin (Jenkins et al., 2017).

In 1980s carbapenems, such as imipenem, meropenem and ertapenem, were developed to treat infections caused by ESBL-producing strains. They are derivatives of thienamycin and have broad-spectrum activities, however, three distinct mechanisms have been described for carbapenem resistance (Papp-Wallace et al., 2011):

- Decreased outer-membrane permeability due to a protein mutation;
- Carbapenemases production;
- Impaired affinity of the penicillin-binding proteins for carbapenems;

In 1991 in Japan the first acquired carbapenemase, IMI-1, was isolated in *Serratia marcescens*. Three classes of carbanemases, encoded either on chromosomes or plasmids, have been described successively (Tzouvelekis et al., 2012):

- Class A: KPC "*Klebsiella pneumoniae* carbapenemase"; isolated for the first time in USA in 2001 from *K. pneumoniae*; plasmid encoded;
- Class B: metallo-β-lactamas;
    - IMP-carbapenemases (metallo-β-lactamase), firstly described in Japan in 1990s; It has been found in *E. coli, S. flexneri, S. marcescens, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia rettgeri, Citrobacter* spp., *Pseudomonas* spp. *and Acinetobacter* spp.;
    - VIM (Verona integron-encoded metallo-β-lactamase) present mainly in *P. aeruginosa* rather than Enterobacteriaceae.
    - NDM-1 (New Delhi metallo-βlactamase), described for the first time in New Delhi in 2009.
- Class D: OXA (oxacillinase), present mainly in *Acinetobacter* spp. OXA-48 firstly emerged in *K. pneumoniae* but found also in *E. coli* and *C. freundii* (Tzouvelekis et al., 2012).

Classes A and B are mostly identified in enterobacteriaceae and they consist in β-lactamase enzymes that hydrolyse β-lactams and compromise the treatment with carbapenems. Their diffusion has rapidly increased over the last decade among different enterobacteriaceae species because the carbapenemases are often located on mobile genetics elements.

For what concern quinolone resistance, in Enterobacteriaceae it is often caused by chromosomal mutations in DNA gyrase and topoisomerase IV. However, in *E. coli, Klebsiella* spp., *Enterobacter* spp., *C. freundii*, and *Providencia stuartii* it has been described a plasmid-quinolone mediated resistance associated with the presence of *qnr* genes, *aac(6')-Ib-cr* encoding a variant of aminoglycoside acetyltransferase, or *qepA* that encodes for a efflux pump (Denton, 2007; Strahilevitz et al., 2009). Often, quinolone resistance genes are co-carried with ESBL genes (Strahilevitz et al., 2009).

In the last ten years, in *E. coli* emerged the "high-risk clone", that facilitates persistence and further dissemination of resistance traits around the world. This is the case of the phylogroup B2 O25:H4 ST131 clone with IncFII plasmids. This strain is characterized by a Cefotaximase-Munich extended-spectrum β-lactamase (CTX-M-15) and is also an emerging MDR bacterium (Coque et al., 2008; Banerjee and Johnson, 2014). This strain doesn't only infect humans, but also animals like pets and livestock (Liebana et al., 2013; Rubin & Pitout 2014). Clone O25:H4 ST131 is characterized by fluoroquinolones and expanded spectrum cephalosporins resistance but it can also recruit carbapenemase genes (Banerjee & Johnson 2014; Cai et al., 2014).

The European Antimicrobial Resistance Surveillance Network (EARS-Net) declare that in 2018, more than half (58.3%) of the *E. coli* new isolates and more than a third (37.2%) of the *Klebsiella pneumoniae* strains were resistant to at least one of the antimicrobial groups under regular surveillance (i.e. aminopenicillins, fluoroquinolones, third generation cephalosporins, aminoglycosides and carbapenems). In the European population, the most common *E. coli* resistant strains were insensitive to aminopenicillins (57.4%), followed by fluoroquinolones (25.3%), third generation cephalosporins (15.1%), aminoglycosides (11.1%). Resistance to carbapenems remained rare. For what concern *K. pneumoniae*, the highest resistance percentage reported was for third generation cephalosporins (31.7%), followed by fluoroquinolones (31.6%), aminoglycosides (22.7%) and carbapenems (7.5%) (European Centre for Disease Prevention and Control, 2019).

2.1.12 Virulence and virulence factors

In the gut microbiota different species of the Enterobacteriaceae family are present, included those that can cause morbidity and mortality on compromised hosts, and pathogens capable of starting illnesses in individuals in perfect health. There is a variable pathogenic potential among these species, that reflect the variable expression of specific virulence factors that are implicated in the disease process.

A strong debate has been done about the precise definition of a virulence factor, but the "Molecular Koch's postulates" articulated by Stanley Falkow better explain the topic: " a trait is considered to be a virulence factor if it is found specifically in strains of a microbe that cause disease; if mutation of a gene encoding the factor results in less severe infection in a suitable model of the disease; and if restoration of a wild-type allele of the gene to the mutant (genetic complementation) results in reinstatement of the original disease severity" (Falkow, 1988).

Many virulence factors have been described for the Enterobacteriaceae family (Schmidt and Hensel, 2004; Toth et al., 2006; Croxen and Finlay, 2010; Chen et al., 2012). Several virulence factors are encoded on pathogenicity islands (PAI) that can be transferred intra-enterobacteriaceae and, similarly, analogous virulence mechanisms are shared across species and genera, and across human/animal and plant pathogens (Hacker and Kaper 2000; Schmidt and Hensel 2004). Among enterobacteriaceae, common virulence factors are flagella, LPS, ECA, (enterobacterial common antigen), fimbriae/adhesins, protein secretion systems and iron acquisition. The first three features are possibly present in a common ancestral enterobacteriaceae because they are always omnipresent in the family.

Fimbriae and adhesins are involved in the adherence to epithelial cells and to extracellular matrix proteins (Soto and Hultgren 1999) and different kinds of them can be found in enterobacteriaceae. In fact, there are six major phylogenetic clades and nine subclades of chaperone/usher assembly classes of fimbriae (Nuccio et al. 2007).

For what concern iron acquisition systems, there are many strategies used by enterobacteriaceae, and their possession can enhance their virulence. Enterobactin and aerobactin are the most common siderophores in enterobacteriaceae and they are found in both animal and plant pathogens (Toth et al., 2006). In *Yersinia* have been discovered for the first time the high-pathogenicity island (HPI) that encodes for genes involved in the siderophore yersiniabactin synthesis and is widely distributed in enterobacteriaceae such as *Escherichia*

coli, Klebsiella, Citrobacter, Salmonella, and *Enterobacter hormaechei* and it is associated with increased virulence (Carniel, 2001; Paauw et al., 2010).

Virulence factors are a particular feature that determine the thin line between its role as commensal or pathogenic organism. Commensalism is a relationship between two species in which one obtains benefits from the other without harming or benefiting it. This is the case of the relationship between *E. coli* and the host (Rastegar Lari et al., 1990; Conway et al., 2004; Schamberger et al., 2004). Furthermore, *E. coli* must face variable environments, and for this reason many different adaptive strategies are needed. The selective pressure in the host is intense and the plasticity of the genome is the adaptative answer. Clusters of genes, as well as genomic islands, including pathogenic islands (PAI), usually are only found in a specific subset of strains for help them colonizing specific environments (Hacker et al., 2000). There is now growing evidence that virulence genes evolved and are maintained by selection for other roles that they have in commensal bacteria (Le Gall et al., 2007; Levin et al., 1996). Commensal *E. coli* found in the gut, showed that the phylogroups responsible for long term colonization are frequently B2 and D, in which are also present adhesins (P fimbriae and type 1 fimbriae), capsular antigens (K1 or K5), α-hemolysins, and siderophore system aerobactin. The more these strains persists in the colon, the more accumulation of PAI are detected. Moreover, it was shown that phylogroups B2 and D have the superior capacity to persist in the infantile colonic microbiota. It seems that owning typical ExPEC virulence traits helps B2 phylogroup to improve its fitness to evolve and persist in the intestinal niche (Schierack et al., 2008; Nowrouzian et al., 2009; Diard et al., 2010; Ostblom et al. 2011).

The *pks* genomic island that encodes for colibactin, a hybrid polyketide/non-ribosomal peptide that causes DNA damage and cell cycle arrest of eukaryotes, is often present in phylogroup B2 *E. coli* strains (Nougayrède et al., 2006). Colibactin genes are predominantly detected in pathogenic isolates of *E. coli, Klebsiella pneumonia, Enterobacter aerogenes* and *Citrobacter koseri*, but recently, they have been found in low frequency also in commensal *E. coli*. The fact that pks island are a characteristic of extraintestinal pathogens may imply that colibactin contributes to fitness or virulence during extraintestinal infection (Johnson et al., 2008; Putze et al., 2009; Krieger et al., 2011). Moreover, *pks* island together with PAIs island, are frequently detected in *E. coli* isolates from intestinal biopsies of patients suffering from colon cancer. A screening of the gut microbiota of Swedish infants from birth to 18 months of age demonstrate that when pks are present in *E. coli* isolates, the capacity to persist in the

intestine increases. To sum up, these findings suggest that in B2 strains, the presence of *pks* island contributes to long term gut-colonizing capacity (Nowrouzian and Oswald, 2012).

In pathogenic *E. coli* strains, horizontally acquired genes that support fitness and competitiveness also code for metabolic traits. *E. coli* had adapted to the intestinal environment, utilizing different energy sources. Moreover, after a comparative analysis of the metabolic versatility of pathogenic and non-pathogenic *E. coli* present in mice, it was demonstrated that pathogenic *E. coli* can use sugars or other carbon sources not used by other commensal strains. This is a strategy that guarantee colonization advantages to pathogenic *E. coli* strains because they can simultaneously consume various C-sources available because they are not consumed by the commensal intestinal microbiota (Anfora and Welch 2006; Anfora et al., 2007; Fabich et al., 2008). Analogously, studies conducted on different animal models revealed that the metabolism of short chain fructooligosaccharides and deoxyribose contribute to avian and human pathogenic *E. coli* to outcompete the commensal microbiota and this guarantee and ease colonization of the intestine. Moreover, phosphotransferase system (PTS) and non-PTS sugar transporters can enhance metabolic capabilities and modulate ExPEC virulence (Le Bouguénec and Schouler 2011; Porcheron et al., 2012; Schouler et al., 2009).

On the other hand, in commensal strains, these virulence genes are necessary for an efficient intestinal colonization. Virulence genes may have evolved to adapt to the intestinal environment, and they were selected for a commensal lifestyle. Therefore, iron uptake systems, bacteriocins, toxins, proteases, flagella, adhesins, and extracellular polysaccharides can be rather considered fitness traits and they promote host colonization (Tenaillon et al., 2010). As a whole, it is not possible to cleary separate ExPEC and commensal *E. coli* strains (Tenaillon et al., 2010; Köhler and Dobrindt, 2011; Diard et al., 2010).

2.1.13 Biofilm production

The word “biofilm” was introduced to describe the biological assemblage created by bacteria in a specific environment (Costerton et al., 1987). Accordingly, biofilm is a structured community of microorganisms that lives together encapsulated within a self-developed protective matrix. Biofilm provides advantages to bacterial communities: it can guarantee interaction and symbiosis among bacteria of different species and the host, as it happens for the colonization of the squid by the luminescent bacteria *Aliivibrio fischeri* (formerly *Vibrio*

fischeri) (Bais et al., 2004; Yaron & Romling 2014). Biofilms are also implicated in different chronic bacterial infections. For instance, in the lungs of people affected by the genetic disease cystic fibrosis, a highly mucoid biofilm matrix that makes the bacteria less susceptible to antibiotic treatment it is present (Sherrard et al., 2014). The cause of infections in indwelling medical devices, such as catheters and artificial joint implants, is the biofilm produced by pathogenic bacteria (Hall et al., 2014).

The biofilm matrix produced by most organisms is formed by lipids, exopolysaccharides, and extracellular proteins, many of which exhibit amyloid-like properties (Branda et al., 2005; Flemming and Wingender, 2010). Biofilm have a remarkably role in evolution because upon their creation it allows the propagation and the survival of bacteria in the environment (Davey and O'Toole, 2000). The integrity and rigidity of protein fibres forms a scaffold onto which the cells and other matrix components (such as exopolysaccharides) are attached, offering success opportunities in environment colonization (Barnhart and Chapman 2006; Branda et al., 2006; Borlee et al., 2010). Certain matrix components provide a full protection of their inhabitants, like BslA, a biofilm-surface layer protein of *Bacillus subtilis* that confers hydrophobicity to the biofilm, or the cellulose produced by *E. coli*, that avoids community desiccation (Kobayashi and Iwano, 2012; Hobley et al., 2013).

Strains belonging to the genera *Escherichia, Citrobacter, Enterobacter, Klebsiella*, and *Proteus* can cause biofilm-related infections such as biliary tract infections, bacterial prostatitis, and catheter-associated urinary tract infections, among the most common secondary nosocomial diseases (Bouza et al., 2001). The gut is a primary source for dissemination and transmission of those potential pathogens to susceptible sites. These bacteria can form biofilm on different surfaces, including plastic and glass, and floating pellicle biofilms at an air–liquid interface (Danese et al., 2000; Hung et al., 2013; Serra et al., 2013b). In biofilms, cell-to-cell adhesion is mediated by a set of protein components called autotransporter adhesins, such as the outer membrane protein Antigen 43 (Ag43). This bond between microbial proteins is based on strong hydrogen bonds and salt bridges and forms a head-to-tail conformation, creating a 'velcro-like' structure that results in the auto-aggregation of cells. Two other autotransporters work as adhesins: *AIDAI* and *TibA*. The former, produced by some diarrheagenic *E. coli* strains, in the glycosylated form can adhere to different mammalian cells (Benz and Schmidt, 2001). The latter is produced by some enterotoxigenic *E. coli*, but glycosylation is not required for aggregation (Sherlock et al., 2005). Understanding the molecular mechanism of these

interactions, will provide future pharmacological targets to block cell-to-cell interaction during the infection process.

2.1.13.1 Curli fibres

In the biofilm matrix of Enterobacteriaceae the main protein constituents are curli fibres, that are composed of two proteins, *CsgB* that starts the polymerization of the fibre and *CsgA* that constitute the fibre itself (Hammar et al., 1996; Bian & Normark 1997) (Figure 10). Curli fibres are rich in β-sheet structure and can be classified as 'amyloid-like' proteins (Shewmaker et al., 2009). Structurally, amyloid fibres are composed of polypeptides rich in β-sheet secondary structures where individual β-strands are primarily aligned perpendicular to the fibre axis (Eanes et al., 1968, Bonar et al., 1969). Curli are highly resistant to denaturation and detergent solubilization (Collinson et al., 1991; Hammar et al., 1996; Chapman et al., 2002).

Genes associated to curli production are located in a single genetic locus and are clustered in the divergently oriented *csgDEFG* and *csgBA* operons (Arnqvist et al., 1994; Hammar et al., 1995). *csgBA* operon encodes for the structural components of curli fibres and is strictly dependent on *csgD* transcription regulator (Hammar et al., 1995). The expression of both *csgDEFG* and *csgBA* operons is induced by a combination of environmental conditions, i.e. low growth temperature (32°C), low osmolarity, and slow growth (Olsen et al., 1993). Curli operons are silent in different *E. coli* laboratory strains, as well as in some environmental isolates (Romling et al., 1998a; Bokranz et al., 2005; Castonguay et al., 2006). *csgD*, beside its role of *csgBA* operon activator, can also regulate a lot of other genes involved in biofilm formation and production of cell-surface-associated structures (Latasa et al., 2005; Gibson et al., 2006). *csgD* stimulates also cellulose production activating the transcription of the *adrA* gene. AdrA protein is involved in the activation of the cellulose biosynthetic machinery through its diguanylate cyclase activity that catalyse the production of the signal molecule cyclic di-GMP (c-di-GMP) (Paul et al., 2004).

Thanks to the capability of Congo Red dye to bind these β-sheet rich structures, curli production can be qualitatively scored by colony staining when growth occurs in presence of the dye (Khurana et al., 2001; Eisert et al., 2006).

2.1.13.2 Cellulose

Cellulose can be another important biofilm's component (Ljungdahl and Eriksson, 1985). It is a (1 → 4)-β-linked linear glucose synthesized by some species of bacteria with protective, architectural and regulatory function in biofilm formation (Solano et al., 2002; Ude et al., 2006; Gualdi et al., 2008). Curli and cellulose works together in order to protect *E. coli* against environmental stresses (Gualdi et al., 2008). For instance, biofilm can protect *E. coli* from predation by both the nematode *Caenorhabditis elegans* and by the predatory bacterium *Myxococcus xanthus* (DePas et al., 2014).

Detection and quantification of cellulose are carried out using the fluorophore calcofluor white (CF) that is a capable to bind glycosidic bonds β (1-3) and β (1-4) of exopolysaccharides (Ramos et al., 2006). The fluorescence emitted by CF binding to exopolysaccharides forming biofilm can be observed after UV light exposition (Flores-Encarnación et al., 2016). Also Crystal Violet dye can be used for biofilm detection because it binds to negatively charged surface molecules and to polysaccharides of the extracellular matrix (Li et al., 2003). Crystal Violet assay allows quantitation of biofilm by spectrophotometer after staining and solubilization of unbound dye with an acetone/ethanol solution (Sicard et al., 2018).

2.1.13.3 Flagellum

On agar plates, *E. coli* and *S. enterica* can form architecturally complex macrocolonies that are structural similar to those of *Bacillus subtilis* and *Vibrio cholerae* when they produce biofilm (Serra et al., 2013b, Zogaj et al., 2003; Branda et al., 2001). The molecular mechanisms involved in macrocolony formation involves curli fibres and cellulose, but also the bacterial flagellum (Hung et al., 2013; Serra et al., 2013a; Serra et al., 2013b). The role of flagellum in biofilm formation has been revealed thanks to the analysis of *E. coli* K12 strain W3110 macrocolonies. This common laboratory strain does not produce cellulose because of a point mutation in the *bcsQ* gene. In *E. coli* K12 W3110 macrocolonies can be identified three distinct regions: the inner wrinkled region, corresponding to the spot were cells were firstly inoculated; the middle zone, where wrinkles seems concentric rings, and the outer edge, that forms a narrow and smooth zone (Figure 2.4). This morphology depends by both the production of flagella and their ability to rotate. When a mutation on *fliC* (flagellar filament protein) or *motA* (flagellar motor protein) occured, *E. coli* was unable to create a ringed colony morphology as describes

above. Microscopy analysis revealed that the cells in the lower portion, the nearest to the agar, were characterized by a network of flagella filaments, suggesting that flagella filaments may act as structural biofilm matrix components. In *E. coli* K12 W3110, a mutation in the gene *motA* lead to flagella unable to rotate, with a final structure less entangled, suggesting that flagella rotation is necessary to tether together the cells and guarantee the formation of the network of flagella filaments that are at the base of the macrocolony formation (Serra et al., 2013b).

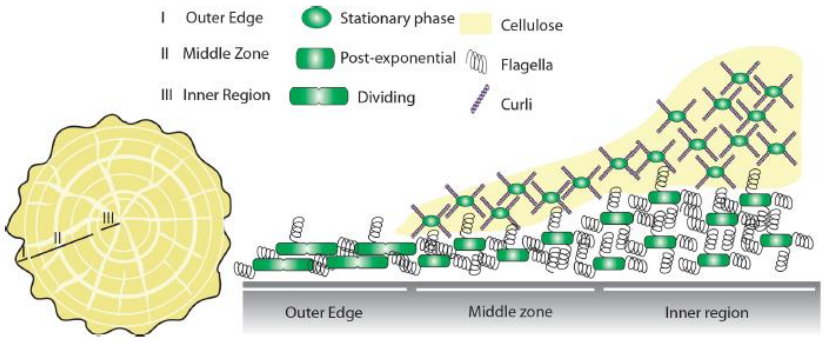

Figure 2.4 *Escherichia coli biofilm structure (Hobley et al., 2015).*

The analysis of floating pellicle biofilms formed at the air–liquid interface revealed that the main role of flagella is to drive the initial state of cells aggregations, without a role as structural component. The natural environment likely selects the diverse role of flagella as physical propulsors to cell aggregation and/or structural component of the biofilm matrix (Hung et al. 2013; Serra et al., 2013b).

2.2 AIM OF THE PROJECT

Research interest mainly focused on the characterization of virulent *E. coli* and Enterobacteriaceae isolated from clinical specimens, with strains isolated from healthy subjects generally investigated in comparative studies with patients affected by specific diseases (Johnson, et al., 2000; Kudinha, et al., 2013; Lee et al., 2019). The few studies focused on the intestinal Enterobacteriaceae population of healthy subjects have been focused on antibiotic resistance profile, without performing a thorough genetic and phenotypic analysis of the strains (Mathai et al., 2015; Zhou et al., 2015; Wang et al., 2017; González et al., 2019).

This research project focused on the deep characterization of the *E. coli* and non-*E. coli* Enterobacteriaceae (NECE) isolated from the fecal samples of 20 healthy adults, with the aim to determine whether a relationship between genotyping, phylogroups, genetic determinants, and functional features can be established among the isolates.

The relative amounts of *E. coli* and NECE from the 20 healthy human population, were determined by enumeration onto selective media and by qPCR. Isolates were characterized by ERIC-PCR and RAPD-PCR for biotype identification, and every biotype further analyzed by PFGE genotyping. Moreover, *E. coli* strains have been clustered according to the generally accepted phylotype classification known as "Clermont method" (Clermont, et al., 2000; Clermont et al., 2013). This classification includes *E. coli* strains in one of the seven phylogroups A, B1, B2, C, D, E, or F, and it gives a preliminary information about the putative commensal or pathogenic behaviour of the strain. Following partial 16S rRNA gene sequencing, conclusive taxonomic attribution of Enterobacteriaceae has been obtained by MALDI-TOF analysis.

Thirty-four virulence determinants were searched in the set of s *E. coli* isolates (*afa/draBC, cdtB, chuA, cnf1, cvaC, fimH, focG, fyuA, gafD, hlyD, hlyF, hra, ibeA, iha, ire, iroN, iss, iutA,*

KpsMTII, kpsMTIII, malX (PAI), ompT, papAH, papC, papEF, pic, rfc, sat, sfa, sfaS,traT, usp, vat, yfcV), together with the *pks* island claimed as a concurrent cause in the development of human colorectal cancer (Johnson et al. 2015; Wassenaar et al., 2018).

For *Klebsiella* spp., the presence of nineteen virulence determinants was screened (*ybtS, mrkD, entB, rmpA, K2, kfu, allS, and magA, kpn, ycfM, irp-1, irp-2 and hlyA, fimH-1, iutA, fyuA, iroN, traT* and *cnf-1*), whereas *Enterobacter* spp. biotypes were screened for the presence of the genes *mrkD, kfu, entB, ybtS, allS, irp-1 and irp-2, fimH, fyuA, iroN* and *traT*, on the basis of *in silico* primer annealing (El Fertas-Aissani et al., 2013; Compain et al., 2014; Johnson et al. 2015).

Horizontal gene transfer (HGT) mostly resides on conjugation, responsible for genetic flexibility of bacterial species (Hacker et al. 2003; Dobrindt et al. 2004). Through this way, accessory elements such as pathogenic islands (PAI), *pks*, o virulence genes can be transferred between strains. For this reason, the presence of *traD* gene was searched in all the strains, as marker of conjugative elements and the strains were challenged as recipients in conjugation experiments with *E. coli* as donor. Furthermore, since *E. coli* gut population can produce colicins against pathogen colonizers, the genes encoding E7 colicin (*colE7*), and the corresponding immunity protein (*immE7*) have been searched (Chak et al., 1991; Frost et al., 1994).

Biofilm has an important role in the propagation and survival of bacteria in the environment. Phenotypical characterization of the isolates included the evaluation of their capability to form biofilms on polystyrene surfaces, and the presence of structures involved in adherence like curli.

Antibiotic resistance is a global healthcare problem and is it important to evaluate how this mechanism is spread among the Enterobacteriaceae isolated from healthy humans. Thanks to the collaboration with Professor Giovanna Gentilomi at Policlinico St. Orsola-Malpighi in Bologna, it was possible complete the profiling of the isolates through the evaluation of the antibiotic resistance exploiting the Vitek2 semi-automated system.

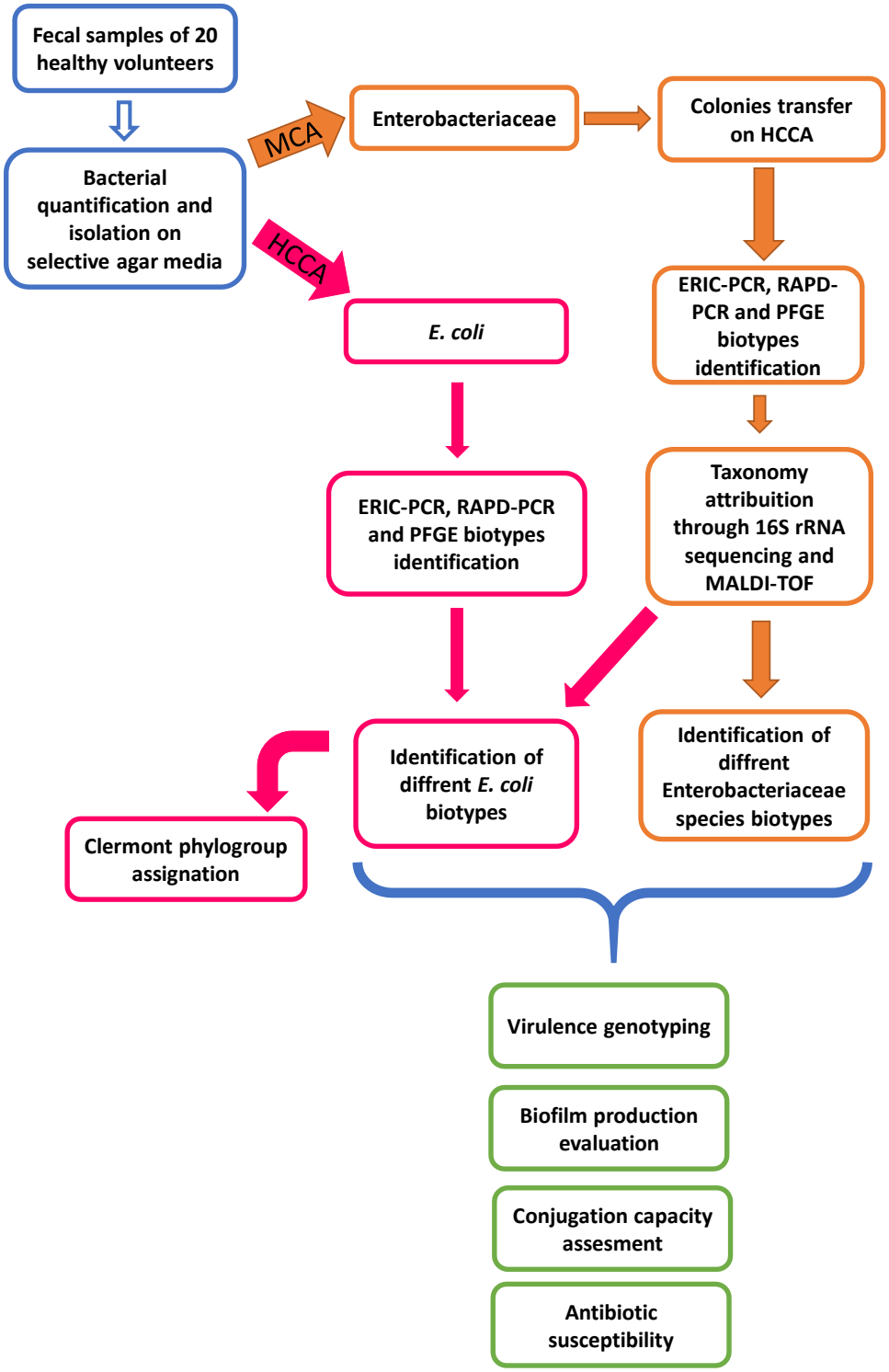

Figure 2.5 *Experimental design. MCA: McConkey Agar; HCCA: Hi Chrome Coliform Agar.*

2.3 MATERIALS AND METHODS

2.3.1 Isolation and enumeration and of *E. coli* and non-*E. coli* enterobacteriaceae (NECE)

Fresh fecal samples were collected from 20 healthy adult subjects, 10 males and 10 females aged 35 to 45, following a western omnivore diet, and who had not been treated with prebiotics and/or probiotics for 1 month and antibiotics for 3 months. The subjects were enrolled among the employees of the University of Modena and Reggio Emilia and their relatives and were not in relationship with the researchers. All human clinical samples were collected after the subjects gave written informed consent regarding their participation in the study. All personal data used in the study were anonymized.

Fresh fecal samples were homogenized (10% w/v) and serially diluted in isotonic Buffered Peptone Water (Sigma, Steinheim, Germany), then spread on HiCrome Coliform Agar (HCCA, peptone 3 g/L, sodium chloride 5 g/L, dipotassium hydrogen phosphate 3 g/L, potassium dihydrogen phosphate 1.7 g/L, sodium pyruvate 1 g/L, L-tryptophan 1 g/L, sodium lauryl sulphate 0.1 g/L**,** chromogenic mixture 0.2 g/L**, a**gar 12 g/L; Sigma-Aldrich) and on MacConkey Agar (Pancreatic digest of Gelatin 17g/L, Peptones (meat and Casein) 3g/L, Lactose 10g/L, Bile Salts 1.5g/L, Sodium Chloride 5g/L, Agar 13.5g/L, Neutral red 0.03g/L, Crystal Violet 1mg/L, 10 g/L Lactose, BD Difco™) and incubated.

HCCA can discriminate between *E. coli* and NECE and contains sodium lauryl sulfate for gram-positive inhibition. The chromogenic mixture holds two chromogenic substrates, Salmon-GAL and X-glucuronide. The β-D-galactosidase produced by coliforms cleaves Salmon-GAL, resulting in the salmon to red coloration, whereas the β-D-glucuronidase produced only by *E. coli* cleaves X-glucuronide, an the double action of these two enzymes forms dark blue to violet colored colonies (Fig. 2.6).

MacConkey Agar is a selective and differential medium used for the isolation and differentiation of non-fastidious gram-negative rods belonging to the family Enterobacteriaceae, based on the ability to ferment lactose. Bile salts and crystal violet inhibit the growth of Gram-positive bacteria. Lactose is the sole carbohydrate of the medium and lactose fermenting bacteria (i. e. *E. coli, Enterobacter* spp., and *Klebsiella* spp.) produce sufficient acid which causes precipitation of the bile salts around the growth, with a pink halo surrounding individual colonies. Non-lactose fermenting bacteria (*Proteus* spp., *Pseudomonas aeruginosa, Salmonella* spp. and *Shigella* spp.) form yellowish, white, or colorless and translucent colonies.

Blue colonies isolated in HCCA putatively ascribed to *E. coli* were checked for indole production with Kovac's reagent (Sigma). Colonies that exhibited positive reactions were recognized as *E. coli*. NECE isolated from HCCA and MCA were collected and subjected to further analysis for taxonomic classification.

E. coli and NECE were maintained and propagated in Luria Bertani medium (LB: 1% tryptone, 0.5% yeast extract, 0,5% sodium chloride) aerobically at 37 °C.

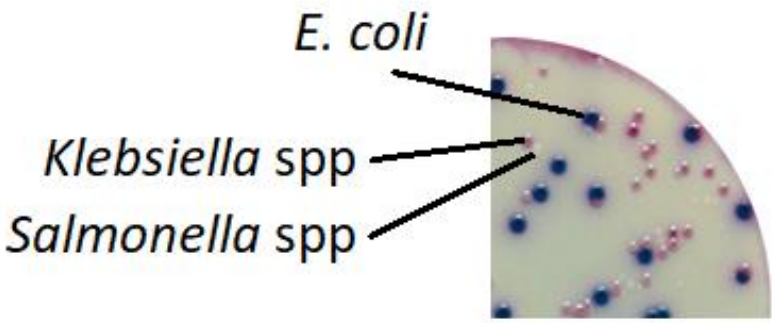

Figure 2.6 *Morphology of colonies belonging to E. coli, KLebsiella, and Salmonella on HCCA.*

2.3.2 Strain biotypization by ERIC-PCR and RAPD-PCR

For each volunteer, 96 blue colonies in HCCA identified as putative *E. coli*, and up to 48 pink colonies in HCCA classified as putative NECE were subjected to ERIC-PCR and RAPD-PCR for biotype assignation (Wilson and Sharp 2006; Quartieri et al. 2016). The ERIC-PCR was performed in 15 µL of total mixture consisting of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA, 2 µM of primer ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and 2 µM of primer ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), 100 µM of each

dNTP, 0.75 U Taq polymerase (DreamTaq, Thermo Fisher Scientific), and 50 ng of gDNA from the isolates.

The thermocycle was the following: 95°C for 3 min; 30 cycles at 95°C for 30 seconds, 52°C for 45 seconds, and 72°C for 4 min; 72°C for 5 min. The PCR products were electrophoresed for 1 h at 160 V in a 25 × 25 cm 2% (w/v) agarose gel in TAE buffer.

The RAPD-PCR was carried out in 15 µL of a mixture consisting of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 0.5 µM of M13-RAPD primer (5'-GAGGGTGGCGGTTCT-3'), 100 µM of each dNTP, 0.75 U Taq polymerase (DreamTaq, Thermo Fisher Scientific), and 50 ng of gDNA from the isolates. The thermocycle was the following: 94°C for 4 min; 45 cycles at 94°C for 1 min, 34°C for 1 min, and 72°C for 2 min; 72°C for 7 min.

PCR products were electrophoresed for 2 h at 160 V in a 25 × 25 cm 2% (w/v) agarose gel in TAE buffer. Fingerprints were digitally captured with Gel Doc XR+ (Bio-Rad, USA), then analyzed with Gene Directory 2.0 software (Syngene, UK), subsequently, also in this case they were clustered into biotypes with similarity level of 75% using the Pearson correlation coefficient.

2.3.3 Taxonomy assignment

To assign each NECE biotypes to a species, the hypervariable regions V1, V2 and V3 of the 16S rRNA gene was amplified, sequenced, and compared with Genbank database. Amplification was performed with 16S 500f (5'-TGGAGAGTTTGATCCTGGCTCAG-3') and 16S 500R (5'-TACCGCGGCTGCTGGCAC-3') primers within 50 µL of PCR Master Mix (Thermo Fisher Scientific), containing 0,2 µM of each primer, and 50 ng of DNA. The thermocycle was the following: 94°C for 4 min; 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; 72°C for 7 min. PCR products were electrophoresed for 1 h at 160 V in a 25 × 25 cm 2% (w/v) agarose gel in TAE buffer.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) technology has become a tool of choice in microbiological laboratories for prompt highly accurate identification and classification of bacterial species (Sauer and Kliem, 2010). The isolates at species level identification was performed in our laboratories, using the MALDI Biotyper 3.1 system (Bruker Daltonik, Germany. Sample preparation for MALDI-TOF MS was performed as previously described with minor modifications (Mencacci et al., 2013). Briefly, colonies of fresh overnight culture derived from 83 isolates were placed on a MALDI

sample slide (Bruker-Daltonics, Bremen, Germany) and dried at room temperature. The sample was then overlaid with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried at room temperature. A MALDI-TOF MS measurement was performed with a Bruker MicroFlex MALDI-TOF MS (Bruker-Daltonics) using FlexControl software and a DH5-α Escherichia coli protein extract (Bruker-Daltonics) was placed on the calibration spot of the sample slide for external calibration. Spectra collected in the positive-ion mode within a mass range of 2,000 to 20,000 Da were analyzed using a Bruker Biotyper (Bruker-Daltonics) automation control and the Bruker Biotyper 3.1 software and library (a database with 5,627 entries). High confidence species identification was accepted, if the log(score) was ≥2.00, low confidence species identification log(score) values (≥1.70 and < 2.00) were accepted if the three best matches showed the same species name. Any results with log(score) < 1.70 were considered as an unacceptable identification.

2.3.4 q-PCR

For qPCR quantification, feces were suspended in PBS pH 7.8 (10% w/w). Bacterial gDNA was extracted with QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany), quantified with NanoPhotometer P-Class (Implen GmbH, Munchen, Germany), diluted to 2.5 ng/µL in TE buffer pH 8, and subjected to qPCR analysis with primers targeting eubacteria and *E. coli*

The set of primers Eco-F (5'-GTTAATACCTTTGCTCATTGA-3') and Eco-R (5'-ACCAGGGTATCAATCCTGTT-3') and Eub-F (5'-GCAGGCCTAACACATGCAAGTC-3' and Eub-R (5'-CTGCTGCCTCCCGTAGGAGT-3') were used for *E. coli* and eubacteria respectively. (Malinen et al. 2003; Castillo et al., 2005).

Standard curves were constructed using the DNA from a known concentration of an overnight culture of *E. coli* N4i pOX38:Cm spiked onto a fecal sample (Starčič Erjavec et al. 2015). After the homogenization of the sample, the total DNA was extracted with QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Standard sample were serially diluted in a range from 10^9 to 10^5 CFU/g of fecal sample.

The mixture contained 10 µL of SsoFast EvaGreen Supermix, 4 µL of each 2 µM primer, and 2 µL of template. qPCR reactions were carried out with the CFX96 Real-Time System (Bio-Rad Laboratories, Redmond, WA, USA) according to the following program: 98°C for 2 min; 45 cycles at 98°C for 0.05 min, 60°C for 0.05 min, and 95°C for 1 min; 65°C for 1 min.

2.3.5 Phylogroup assignement according to Clermont

Quadruplex PCR reactions described by Clermont et al. (2013). were carried out in a 20 μL volume containing 2 μL of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 2 U Taq polymerase (DreamTaq, Thermo Fisher Scientific) 2 μM each dNTP, 3 μL of DNA extract (at approximately 100 ng/mL) and the appropriate primers. The primer used are: 20 pmol for primers chuA.1b 5′-ATGGTACCGGACGAACCAAC-3′ and chuA.2 5′-TGCCGCCAGTACCAAAGACA-3′ targeting *chuA* gene; 20 pmol for primer yjaA.1b 5′-CAAACGTGAAGTGTCAGGAG-3′ and primer yjaA.2b 5′-AATGCGTTCCTCAACCTGTG-3' necessary for *yiaA* gene amplification; 20 pmol for primer TspE4C2.1b 5′-CACTATTCGTAAGGTCATCC-3′ and primer

TspE4C2.2b 5′-AGTTTATCGCTGCGGGTCGC-3′ that target *TspE4.C2* gene; 40 pmol for primer AceK.f 5′-AACGCTATTCGCCAGCTTGC-3′ and primer ArpA1.r 5′-TCTCCCCATACCGTACGCTA-3′ for *arpA* gene. The combination of the presence/absence of genes *chuA, yjaA, TspE4.C2* and *arpA* gives the phylogroup assignation as A, B1, B2 or F. For distinguish between E or D phylogroup or C or A two control PCR are needed.

"Group E" PCR mix (20 μL) volume contained 2 μL of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 2 U Taq polymerase (DreamTaq, Thermo Fisher Scientific) 2 μM each dNTP, 3 μL of DNA extract (at approximately 100 ng/mL) and 20 pmol of both primers ArpAgpE.f 5′-GATTCCATCTTGTCAAAATATGCC-3' and ArpAgpE.r 5′-GAAAAGAAAAAGAATTCCCAAGAG-3′ for *arpA* gene amplification and 12 pmol of both primers trpBA.f 5′-CGGCGATAAAGACATCTTCAC-3′ and trpBA.r 5′-GCAACGCGGCCTGGCGGAAG-3′ that amplifies *trpA* gene, internal control. The presence of both genes' amplicon (*arpA* and *trpA*) means that the isolate belongs to E phylogroups otherwise, if it is only present *trpA* is a D phylogroup (Clermont et al., 2013).

"Group C" PCR mix (20 μL) volume contained 2 μL of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 2 U Taq polymerase (DreamTaq, Thermo Fisher Scientific) 2 μM each dNTP, 3 μL of DNA extract (at approximately 100 ng/mL) and 20 pmol of both primers trpAgpC.1 5′-AGTTTTATGCCCAGTGCGAG-3′ and trpAgpC.2 5′-TCTGCGCCGGTCACGCCC-3′ needed for *trpA* gene amplification, and 12 pmol of both primers trpBA.f 5′-CGGCGATAAAGACATCTTCAC-3′ and trpBA.r 5′-GCAACGCGGCCTGGCGGAAG-3′ that amplifies *trpA* gene, internal control. In this case the presence of both genes' amplicon (*trpA.C* and *trpA*) means that the isolate belongs to C phylogroups otherwise, if it is only present *trpA* internal control is a A phylogroup (Clermont et al., 2013).

PCR reactions were performed under the following conditions: denaturation 4 min at 94°C, 30 cycles of 5 s at 94°C and 20 s at 57°C (group E) or 59°C (quadruplex and group C), and a final extension step of 5 min at 72°C.

2.3.6 PFGE

PFGE was performed on *E. coli* strains and NECE species according to the PulseNet protocol designed for *Escherica coli* (http://www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). The genomic DNA was digested with 50 U of *Xba*I at 37 °C for 3 h. Macrorestriction fragments were resolved by counter-clamped homogeneous electric field electrophoresis in a CHEF-DRIII apparatus (Bio-Rad, USA). The run time was 24 h at 6.0 V/cm, with initial and final switch times of 2.2 s and 54.2 s, respectively. PFGE images were digitally captured with Gel Doc XR+ (Bio-Rad, USA), and analyzed with GelCompare II 6.0 software (Applied Maths NV, Belgium). Dice's coefficients were calculate based on band profile, setting the position tolerance at 1% and the optimization at 1%. A similarity dendrogram was derived using the unweighted pair group method with arithmetic means (UPGMA). Strains were ascribed to the same pulsotype if PFGE profile possessed >85% similarity.

2.3.7 Virulence genotyping of *E. coli*

The *E. coli* isolates were screened by multiplex-PCR for carriage of 34 genes associated to virulence factors: *afa/draBC, cdtB, chuA, cnf1, cvaC, fimH, focG, fyuA, gafD, hlyD, hlyF, hra, ibeA, iha, ire, iroN, iss, iutA, KpsMTII, kpsMTIII, malX (PAI), ompT, papAH, papC, papEF, pic, rfc, sat, sfa, sfaS, traT, usp, vat,* and *yfcV*. Primer sequences, pool of primers, and amplification reactions were set up according to Johnson et al. 2015.

Gene's primers were divided in 6 groups (Primer pool 1 to 6).

The reaction was conducted in 25 µL of a mixture consisting of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 4 mM of MgCl2, 2,5 mM of dNTPs, 1,25 U of Taq polymerase (DreamTaq, Thermo Fisher Scientific), 0,6 µM of Primer pool.

The thermocycle was the following: 95°C for 12 min; 30 cycles at 94°C for 30 seconds, 63°C for 30 seconds, and 68°C for 3 min; 72°C for 10 min.

Pool	*Primer*	*Forward Primer sequence 5'-3'*	*Reverse primer sequence 5'-3'*
1	*malX (PAI)*	*GGACATCCTGTTACAGCGCGCA*	*TCGCCACCAATCACAGCCGAAC*
	papAH	*ATGGCAGTGGTGTCTTTTGGTG*	*CGTCCCACCATACGTGCTCTTC*
	fimH	*TCGAGAACGGATAAGCCGTGG*	*GCAGTCACCTGCCCTCCGGTA*
	kpsMT III	*TCCTCTTGCTACTATTCCCCCT*	*AGGCGTATCCATCCCTCCTAAC*
	papEF	*GCAACAGCAACGCTGGTTGCATCAT*	*AGAGAGAGCCACTCTTATACGGACA*
	yfcV	*ACATGGAGACCACGTTCACC*	*GTAATCTGGAATGTGGTCAGG*
	ireA	*GATGACTCAGCCACGGGTAA*	*CCAGGACTCACCTCACGAAT*
	ibeA	*AGGCAGGTGTGCGCCGCGTAC*	*TGGTGCTCCGGCAAACCATGC*
2	*cnf1*	*ATCTTATACTGGATGGGATCATCTTGG*	*GCAGAACGACGTTCTTCATAAGTATC*
	fyuA	*TGATTAACCCCGCGACGGGAA*	*CGCAGTAGGCACGATGTTGTA*
	iroN	*AAGTCAAAGCAGGGGTTGCCCG*	*GACGCCGACATTAAGACGCAG*
	sfa	*CTCCGGAGAACTGGGTGCATCTTAC*	*CGGAGGAGTAATTACAAACCTGGCA*
	iutA(aerJ)	*GGCTGGACATCATGGGAACTGG*	*CGTCGGGAACGGGTAGAATCG*
	hra	*CGAATCGTTGTCACGTTCAG*	*TATTTATCGCCCCACTCGTC*
3	*pic*	*GGGTATTGTCCGTTCCGAT*	*ACAACGATACCGTCTCCCG*
	hlyD	*CTCCGGTACGTGAAAAGGAC*	*GCCCTGATTACTGAAGCCTG*
	rfc	*ATCCATCAGGAGGGGACTGGA*	*AACCATACCAACCAATGCGAG*
	ompT	*ATCTAGCCGAAGAAGGAGGC*	*CCCGGGTCATAGTGTTCATC*
	iss	*CAGCAACCCGAACCACTTGATG*	*AGCATTGCCAGAGCGGCAGAA*
	papC	*GTGGCAGTATGAGTAATGACCGTTA*	*ATATCCTTTCTGCAGGGATGCAATA*
4	*vat*	*AGAGACGAGACTGTATTTGC*	*GTCAGGTCAGTAACGAGCAC*
	gafD	*TGTTGGACCGTCTCAGGGCTC*	*CTCCCGGAACTCGCTGTTACT*
	cvaC	*CACACACAAACGGGAGCTGTT*	*CTTCCCGCAGCATAGTTCCAT*
	cdtB	*GAAAATAAATGGAACACACATGTCCG*	*AAATCTCCTGCAATCATCCAGTTA*
	focG	*CAGCACAGGCAGTGGATACGA*	*GAATGTCGCCTGCCCATTGCT*
	traT	*GGTGTGGTGCGATGAGCACAG*	*CACGGTTCAGCCATCCCTGAG*
5	*iha*	*CTGGCGGAGGCTCTGAGATCA*	*TCCTTAAGCTCCCGCGGCTGA*
	afa	*GGCAGAGGGCCGGCAACAGGC*	*CCCGTAACGCGCCAGCATCTC*
	sfaS	*GTGGATACGACGATTACTGTG*	*CCGCCAGCATTCCCTGTATTC*
	hlyF	*TCGTTTAGGGTGCTTACCTTCAAC*	*TTTGGCGGTTTAGGCATTCC*
6	*sat*	*GCAGCTACCGCAATAGGAGGT*	*CATTCAGAGTACCGGGGCCTA*
	kpsMT II	*GCGCATTTGCTGATACTGTTG*	*AGGTAGTTCAGACTCACACCT*
	usp	*ACATTCACGGCAAGCCTCAG*	*AGCGAGTTCCTGGTGAAAGC*
	chuA	*TTATCCCCTGCGTAGTTGTGAATC*	*TGCCGCCAGTACCAAAGACA*

Table 2.3 *Set of primers used to detect genetic determinants of virulence in E. coli*

2.3.8 Virulence genotyping of NECE

All the isolates ascribed to the genus *Klebsiella*, were screened by multiplex-PCR for carriage of 19 genes associated to virulence factors: *ybtS, mrkD, entB, rmpA, K2, kfu, allS, magA, kpn, ycfM, irp-1, irp-2, hlyA, fimH, iutA, fyuA, iroN, traT*, and *cnf-1*.

PCR reactions were set up according to El Fertas-Aissani et al., 2012; Compain et al., 2014; and to Johnson et al. 2015 (Table 2.4). A blast search of primer annealing was carried out, and search of the genes by PCR was performed in other species when both the primers properly annealed (> 90% identity). The reactions were conducted in 25 µL of a mixture consisting of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 4 mM of MgCl2, 2,5 mM of dNTPs, 1,25 U of Taq polymerase (DreamTaq, Thermo Fisher Scientific), 0,6 µM of Primer pool. The thermocycle was the following: 95°C for 12 min; 30 cycles at 94°C for 30 seconds, annealing temperature last 30 seconds at 63°C for *hlA, cnf.1, fyuA, iroN, fimH, iutA, irp1, traT* and *ent*; 60° for *ybtS, mrkD, rmpA, K2, kfu, allS,* and *magA*; 57° for *kpn, ycf.M* and *irp2*; then, 68°C for 3 min; 72°C for 10 min.

Primer name	***Primer sequence 5'-3'***	***Reference article***
ybtS_for	GACGGAAACAGCACGGTAAA	Compain et al., 2014
ybtS_rev	GAGCATAATAAGGCGAAAGA	
mrkD_for	AAGCTATCGCTGTACTTCCGGCA	Compain et al., 2014
mrkD_rev	GGCGTTGGCGCTCAGATAGG	
entB_for	GTCAACTGGGCCTTTGAGCCGTC	Compain et al., 2014
entB_rev	TATGGGCGTAAACGCCGGTGAT	
rmpA_for	CATAAGAGTATTGGTTGACAG	Compain et al., 2014
rmpA_rev	CTTGCATGAGCCATCTTTCA	
K2_for	CAACCATGGTGGTCGATTAG	Compain et al., 2014
K2_rev	TGGTAGCCATATCCCTTTGG	
kfu_for	GGCCTTTGTCCAGAGCTACG	Compain et al., 2014
kfu_rev	GGGTCTGGCGCAGAGTATGC	
allS_for	CATTACGCACCTTTGTCAGC	Compain et al., 2014
allS_rev	GAATGTGTCGGCGATCAGCTT	
magA_for	GGTGCTCTTTACATCATTGC	Compain et al., 2014
magA_rev	GCAATGGCCATTTGCGTTAG	
kpn_for	GTATGACTCGGGGAAGATTA	El Fertas-Aissani et al., 2012
kpn_rev	CAGAAGCAGCCACCACACG	
ycfM_for	ATCAGCAGTCGGGTCAGC	El Fertas-Aissani et al., 2012
ycfM_rev	CTTCTCCAGCATTCAGCG	
irp-1_for	TGAATCGCGGGTGTCTTATGC	El Fertas-Aissani et al., 2012
irp-1_rev	TCCCTCAATAAAGCCCACGCT	
irp-2_for	AAGGATTCGCTGTTACCGGAC	El Fertas-Aissani et al., 2012
irp-2_rev	TCGTCGGGCAGCGTTTCTTCT	
hlyA_for	AACAAGGATAAGCACTGTTCTGGCT	El Fertas-Aissani et al., 2012
hlyA_rev	ACCATATAAGCGGTCATTCCCGTCA	
fimH-1_for	TCGAGAACGGATAAGCCGTGG	Johnson et al. 2015.
fimH-1_rev	GCAGTCACCTGCCCTCCGGTA	
iutA_for	GGCTGGACATCATGGGAACTGG	Johnson et al. 2015.
iutA_rev	CGTCGGGAACGGGTAGAATCG	
fyuA_for	TGATTAACCCCGCGACGGGAA	Johnson et al. 2015.
fyuA_rev	CGCAGTAGGCACGATGTTGTA	
iroN_for	AAGTCAAAGCAGGGGTTGCCCG	Johnson et al. 2015.
iroN_rev	GACGCCGACATTAAGACGCAG	
traT_for	GGTGTGGTGCGATGAGCACAG	Johnson et al. 2015.
traT_rev	CACGGTTCAGCCATCCCTGAG	
cnf-1_for	AAGATGGAGTTTCCTATGCAGGAG	Johnson et al. 2015.
cnf-1_rev	CATTCAGAGTCCTGCCCTCATTATT	

Table 2.4 *Set of primers used to detect genetic determinants of virulence in NECE*

2.3.9 Detection of *traD*, *colE7*, and *immE7* genes

A triplex-PCR was utilized search in *E. coli* the genes *colE7*, *immE7*, and *traD*, utilizing the set of primers colE7-F / colE7-R2 (5'-AAGTCAGATGCTGATGTTGC-3' / 5'-ATAGACACCACCATTTTGGC-3'), immE7-F / immE7-R2 (5'-GCAACTGATGATGTGTTAGATG-3' / 5'-TGTTTAAATCCTGGCTTACCG-3'), and traD-F / traD-R2 (5'-GATAAATACATGATCTGGTGCGG-3' / 5'-TTGATGGAAAGCAGTGTGTC-3'), specifically developed in this study. The amplification reaction was carried out in a final volume of 50 µL containing 5 µL of 10X Dream Taq Green Buffer, 5 µL dNTP Mix (2 mM each), 0.25 µL Dream Taq, 9 µL primer mix (2 µM each), 50 ng template DNA. The thermocycle was: 95 °C for 5 min; 30 cycles at 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 1 min; 72 °C for 7 min.

2.3.10 Detection of *pks* island in *E. coli*

Polyketide synthases *pks* pathogenicity island was searched by duplex-PCR, amplifying *clbB* and *clbN* genes located at the 5′ and 3′ regions of the island, according to Sarshar et al., 2017. The reaction was conducted in 20 µL of a mixture consisting of Dream Taq Buffer (Thermo Fisher Scientific, Waltham, MA), 10 mM of dNTPs, 1,25 U of Taq polymerase (DreamTaq, Thermo Fisher Scientific), 10 µM of primers ClbB-F GATTTGGATACTGGCGATAACCG and ClbB-R CCATTTCCCGTTTGAGCACAC for *clbB* gene amplification and 10 µM of primers ClbN-F GTTTTGCTCGCCAGATAGTCATTC and ClbN-R CAGTTCGGGTATGTGTGGAAGG for *clbN* gene amplification. The thermocycle was the following: 94°C for 5 min; 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 3 min; 72°C for 10 min.

2.3.11 Antibiotic susceptibility

Antimicrobial susceptibility was tested for all the strains isolated with Vitek2 semi-automated system (bioMerieux, France). Minimum inhibitory concentrations (MICs) were interpreted using EUCAST clinical breakpoints (European Committee on Antimicrobial Susceptibility Testing – www.eucast.org) and were expressed as mg/L: for amikacin S ≤ 8 and R > 16; for amoxicillin/clavulanic acid S ≤ 8 and R > 8; for cefotaxime 1 ≤ 8 and R > 2; for ceftazidime S ≤ 8 and R > 8; for ciprofloxacin S ≤ 0.5 and R > 1; for gentamicin S ≤ 2 and R > 4; for piperacillin + tazobactam S ≤ 8 and R > 16 and for trimethoprim/sulfamethoxazole S ≤

40 and R > 80 (S, susceptible; R, resistant, as established by EUCAST breakpoints, version 6.0, 2016).

2.3.12 Biofilm production and phenotype assays

Biofilm formation was assayed by crystal violet (CV) staining as described Sicard et al. 2018. *E. coli* and NECE were cultured in LB without NaCl and in M9 medium (BD Difco, Sparks, USA) containing 4 g/L glucose and 0.25 g/L yeast extract (hereinafter referred to as LBWS and M9glu, respectively). Specific biofilm formation (SBF) index was calculated as the ratio between CV absorbance at 570 nm and culture turbidity at 600 nm, setting a threshold of 1. The data herein reported are means of 3 independent experiments, each carried out in triplicate.

Curli and cellulose formation were assayed in LBWS agar plates supplemented with congo red (CR) or calcofluor white (CF), respectively, according to Vogeleer et al. (2015). Red colonies were positive to CR. Emission of fluorescence as result of UV exposure (315-400 nm) was consistent with binding of CF.

2.3.13 Solid mating conjugation experiments

To assess the capability of both β-glucuronidase-negative *E.coli* and NECE to behave as recipients in conjugation, solid mating conjugation experiments were carried out using *E. coli* N4i pOX38:Cm (N4i: EcN immE7 Gmr; pOX38:Cm: Tra+ RepFIA+ Cmr) as a donor strain (Starčič Erjavec et al. 2015, Maslennikova et al. 2018). The donor was cultured aerobically a 37 °C in LB supplemented with 15 µg/mL gentamycin and 20 µg/mL chloramphenicol, while the recipients were cultured in LB. Overnight cultures of donor and recipients were seeded in their respective media (1% v/v) and aerobically incubated for 2 h. 1 mL of recipient culture was centrifuged (10,000 × g for 5 min at 4°C) and resuspended in 400 µL of donor culture. The whole volume was spread onto an LB-agar plate and incubated at 37 °C for 4 h, then the cells were collected from the surface with 1 ml of PBS, serially diluted, and spread onto HCCA supplemented with 20 µg/mL chloramphenicol. In this way it will be possible to differentiate transconjugant strain (β-glucuronidase-negative *E. coli* or NECE resulting in pink colonies with acquired chloramphenicol resistance) from donor colonies (*E. coli* N4i pOX38:Cm that result in blue colonies). In this media, recipient will not growth due to their chloramphenicol sensitivity.

2.3.14 Statistical analysis

Dice's coefficient was utilized for gauging the distance between strains, based on the sets of binary data, i.e. the presence / absence of genes or phenotypic properties. Distance matrices between strains were utilized for computation and display of UPGMA dendrograms and Principal Coordinates Analysis plots. Co-occurrence of genetic determinants and phenotypic properties was evaluated by means of contingency tables and Cramér's V metrics, considering as limit of significance $P < 0.01$ and $P < 0.05$. Statistical analyses were performed with the SPSS Statistics 21 software (IBM Corp., NY, USA).

2.4 RESULTS AND DISCUSSION

2.4.1 *E. coli* and NECE enumeration and isolation

The fecal samples of the 20 healthy subjects were properly diluted and plated on HCCA and incubated for 24 hours at 37 °C aerobically. After incubation, 96 blue colonies per sample were randomly chosen and tested for indole production. All the blue colonies tested were positive to Kovac's reagent, confirming the assignment to the species *E. coli*. From the fecal samples of the 20 volunteers, the *E. coli* concentration obtained on HCCA ranged between $1.3 \cdot 10^5$ to $9.3 \cdot 10^7$ cfu/g (Table 2.5).

On the basis of q-PCR results, the ratio between *E. coli* and total bacteria ranged from $5.6 \cdot 10^{-6}$ and $1.9 \cdot 10^{-3}$ with a mean of $2.8 \cdot 10^{-5}$. (Figure 2.7). *E. coli* concentration determined by plate counting and molecular techniques did not significantly differ ($P > 0.05$, paired samples t-test).

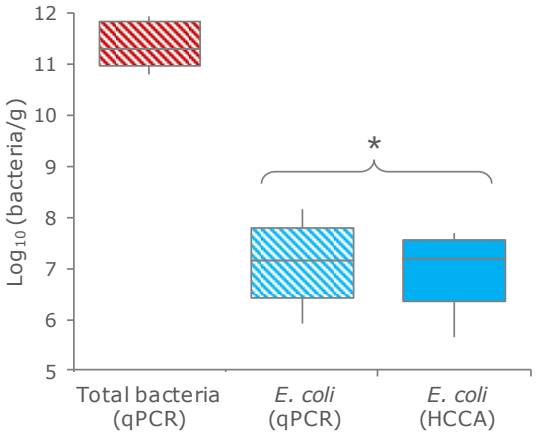

***Figure 2.7**: Counts of E. coli and total bacteria in the feces of 20 subjects. Boxes indicate the median and the 25th and 75th percentiles; whiskers indicate the 10th and 90th percentiles. * indicates means that did not significantly differ (P > 0.05, paired samples t-test)*

The same fecal samples were serially diluted and plated also on McConkey agar for the isolation of NECE. 48 random pink colonies for each volunteer were streaked on Hi Chrome Coliform Agar (HCCA) to discriminate between *E. coli* and NECE. From fecal samples of volunteers 3, 7, 10, 12, 15, and 20 no pink or white colonies were isolated, taking into account the overwhelming number of *E. coli*. From the other 14 samples, a total of 357 pink colonies putatively ascribed to non-*E. coli* enterobacteriaceae were isolated. This set of clones was tested for indole production by Kovac's analysis, since *E. coli* are expected to be positive and NECE negative. Summing up, from this pipeline emerged that NECE isolated on HCCA ranged between $<10^4$ to 10^8 cfu/g (Table 2.5).

Subject	qPCR counts, cfu/g		HCCA counts, cfu/g			
	Total bacteria	*E. coli*	*E. coli*	NECE	Total Enterobacteriaceae (E. coli + NECE)	% NECE
1	$6.8\cdot10^{11}$	$5.3\cdot10^{7}$	$3.6\cdot10^{7}$	$4.5\cdot10^{6}$	$4.0\cdot10^{7}$	11.2
2	$3.1\cdot10^{10}$	$5.8\cdot10^{6}$	$2.0\cdot10^{6}$	$9.0\cdot10^{4}$	$2.1\cdot10^{6}$	4.2
3	$8.5\cdot10^{11}$	$3.0\cdot10^{8}$	$3.3\cdot10^{7}$	-	$3.3\cdot10^{7}$	-
4	$1.9\cdot10^{11}$	$2.4\cdot10^{6}$	$3.3\cdot10^{5}$	$1.0\cdot10^{4}$	$3.4\cdot10^{5}$	2.9
5	$7.2\cdot10^{11}$	$6.2\cdot10^{7}$	$6.3\cdot10^{7}$	-	$6.3\cdot10^{7}$	-
6	$8.6\cdot10^{11}$	$3.2\cdot10^{7}$	$1.9\cdot10^{7}$	$1.1\cdot10^{7}$	$3.0\cdot10^{7}$	36.5
7	$9.1\cdot10^{10}$	$8.6\cdot10^{5}$	$2.8\cdot10^{6}$	-	$2.8\cdot10^{6}$	-
8	$9.0\cdot10^{10}$	$4.9\cdot10^{5}$	$4.4\cdot10^{5}$	$1.0\cdot10^{4}$	$4.5\cdot10^{5}$	2.2
9	$6.7\cdot10^{10}$	$2.9\cdot10^{6}$	$1.3\cdot10^{7}$	$5.4\cdot10^{5}$	$1.3\cdot10^{7}$	3.9
10	$2.2\cdot10^{11}$	$1.1\cdot10^{8}$	$4.1\cdot10^{7}$	-	$4.1\cdot10^{7}$	-
11	$4.7\cdot10^{11}$	$6.5\cdot10^{7}$	$9.3\cdot10^{7}$	$1.2\cdot10^{8}$	$2.2\cdot10^{8}$	57.8
12	$9.2\cdot10^{11}$	$6.0\cdot10^{7}$	$3.7\cdot10^{7}$	-	$3.7\cdot10^{7}$	-
13	$9.0\cdot10^{10}$	$5.2\cdot10^{6}$	$2.9\cdot10^{6}$	-	$2.9\cdot10^{6}$	-
14	$3.2\cdot10^{11}$	$3.6\cdot10^{7}$	$7.0\cdot10^{6}$	$1.4\cdot10^{6}$	$8.4\cdot10^{6}$	17
15	$4.3\cdot10^{11}$	$1.0\cdot10^{7}$	$3.2\cdot10^{7}$	-	$3.2\cdot10^{7}$	-
16	$1.8\cdot10^{11}$	$1.2\cdot10^{8}$	$5.4\cdot10^{7}$	-	$5.4\cdot10^{7}$	-
17	$7.9\cdot10^{10}$	$2.0\cdot10^{8}$	$3.2\cdot10^{7}$	$1.7\cdot10^{6}$	$3.4\cdot10^{7}$	5.1
18	$2.4\cdot10^{11}$	$1.3\cdot10^{7}$	$4.5\cdot10^{6}$	$8.5\cdot10^{4}$	$4.6\cdot10^{6}$	1.9
19	$6.8\cdot10^{11}$	$1.7\cdot10^{7}$	$1.5\cdot10^{7}$	$1.2\cdot10^{6}$	$1.6\cdot10^{7}$	7.6
20	$8.1\cdot10^{10}$	$7.8\cdot10^{6}$	$2.0\cdot10^{7}$	-	$2.0\cdot10^{7}$	-

Table 2.5 *qPCR and/or plate counts concentration of total bacteria, E. coli and NECE in the feces of 20 healthy subjects. The ratio between NECE and total enterobacteriaceae (E.coli + NECE) is reported as %. – indicates values laying below the limit of detection o 10^4 cfu/g.*

For each sample, *E. coli* clearly dominated over the NECE, with a mean ratio of NECE / *E. coli* (when detected) of 7.5% ± 1.1. In some cases, NECE were not recovered in HCCA plates, being outnumbered by *E. coli*. Excluding the outlier sample of volunteer 11, where NECE outnumbered *E. coli* and reached $1.3 \cdot 10^8$ CFU/g, an exceptionally high load, the restricted range of the index calculated as the ratio of NECE / on total enterobacteriaceae (including *E. coli*) (Figure 2.8) indicated that generally a low charge of NECE was associated to a high charge of *E. coli*, and *vice versa*, likely as a consequence of chemical-physical and nutritional features of the gut that lower or strengthen these phylogenetically related species.

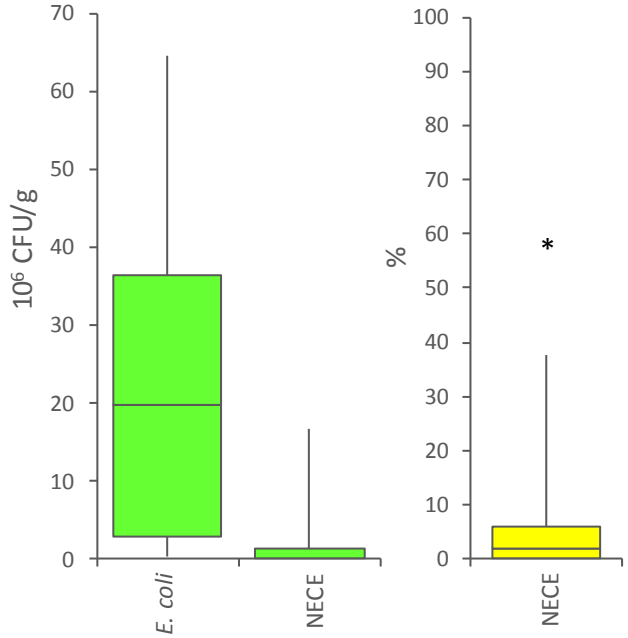

Figure 2.8 *Green boxplot: counts of E. coli and total bacteria in the feces of 20 subjects. Yellow boxplot: Ratio between NECE and total enterobacteriaceae (E.coli + NECE) reported as percentage. Boxes indicate the median and 25th and 75th percentiles; whiskers indicate the 10th and 90th percentiles. * indicates means that did not significantly differ (P > 0.05, paired samples t-test).*

2.4.2 *E. coli* fingerprinting by ERIC-PCR, RAPD-PCR, and PFGE

E. coli isolates were subjected to ERIC-PCR to identify the different biotypes. For each volunteer, 96 colonies were randomly chosen and analysed. 31 different biotypes were identified, resulting in a collection of 51 *E. coli* strains because, when the same biotype was shared among different hosts, an isolate with the same ERIC-PCR profile per each hosts was included (Figure 2.9; Table 2.6). The collection encompassed also 10 β-glucuronidase-negative

strains obtained during the NECE isolation, that were ascribed to *E. coli* by MALDI-TOF analysis. A minimum of 1 and a maximum of 6 different biotypes were identified for each host, with most of the samples (14 out of 20) characterized by a dominant one that represented more than 80% of the isolates. 7 biotypes were common to diverse hosts.

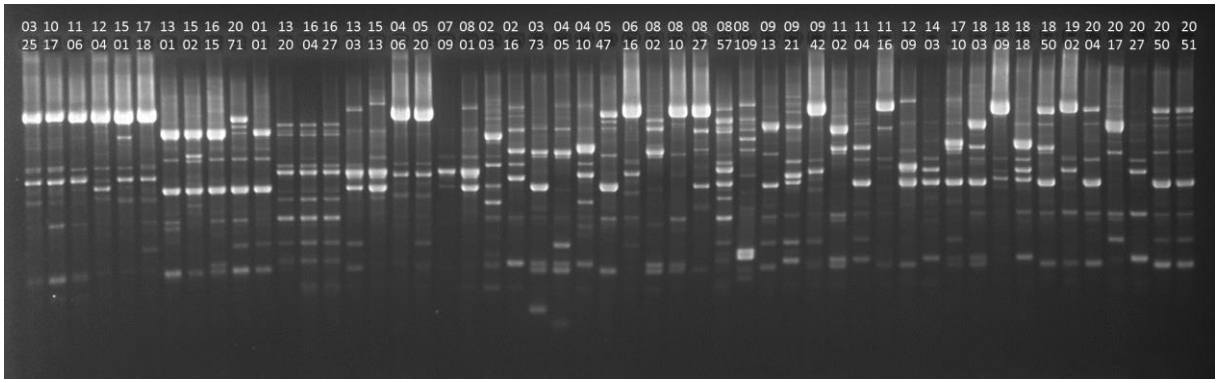

Figure 2.9 *ERIC-PCR fingerprinting of the E. coli isolates.*

RAPD-PCR identified 27 different biotypes, still maintaining common ones among different hosts (Figure 2.10; Table 2.6). ERIC-PCR and RAPD-PCR likely were not be able to discriminate different strains of the same species.

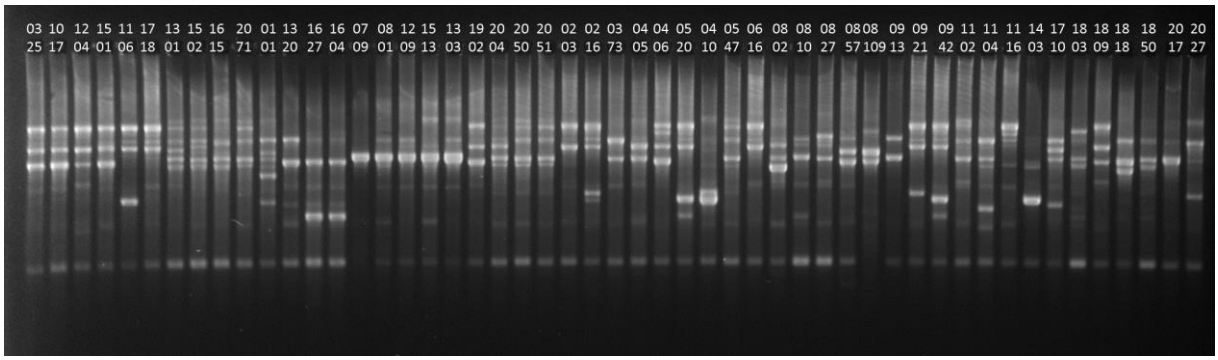

Figure 2.10 *RAPD-PCR fingerprinting of the selected E. coli isolates.*

*Xba*I-PFGE differentiated several biotypes shared by diverse volunteers, that were not discriminated by ERIC-PCR and RAPD-PCR, resulting in a total of 44 different pulsotypes, (Table 2.6; Figure 2.11).

E. coli	β-glu	ERIC-PCR			RAPD-PCR profile	*Xba*I-PFGE profile	Clermont
		profile	relative abundance	Log (CFU/g)			Phylo-group
01.01	+	E01	100%	7.56	R01	P01	B1
02.03	+	E02	8%	5.22	R02	P02	B2
02.16	+	E03	92%	6.28	R03	P03	F
03.25	+	E04	99%	7.52	R04	P04	B2
03.73	+	E05	1%	5.53	R03	P05	A
04.05	+	E06	5%	4.22	R05	P06	A
04.06	+	E07	70%	5.37	R06	P07	B2
04.10	+	E08	25%	4.92	R07	P08	E
05.20	+	E07	96%	7.78	R02	P09	B2
05.47	-	E09	4%	6.40	R08	P10	B1
06.16	+	E07	100%	7.28	R07	P11	B2
07.09	+	E10	100%	6.45	R04	P12	D
08.01	+	E11	18%	4.91	R09	P12	D
08.02	+	E12	5%	4.37	R10	P13	A
08.10	+	E13	5%	4.37	R11	P14	A
08.27	-	E14	36%	5.20	R11	P15	B1
08.57	-	E15	34%	5.18	R12	P16	F
08.109	-	E16	2%	3.86	R13	P17	F
09.13	+	E01	92%	7.09	R14	P18	C
09.21	+	E17	4%	5.73	R12	P19	F
09.42	+	E07	4%	5.73	R12	P09	B2
10.17	+	E04	100%	7.62	R12	P04	B2
11.02	+	E18	50%	7.67	R15	P20	A
11.04	+	E19	9%	6.91	R10	P21	A
11.06	+	E07	37%	7.54	R16	P22	B2
11.16	+	E20	4%	6.57	R17	P23	B2
12.04	+	E13	71%	7.43	R18	P24	B2
12.09	+	E11	29%	7.04	R18	P25	D
13.01	+	E01	3%	4.98	R19	P26	B1
13.03	+	E11	1%	4.47	R11	P27	D
13.20	-	E21	97%	6.46	R13	P28	F
14.03	+	E22	100%	6.85	R13	P29	E
15.01	+	E07	9%	6.45	R13	P30	B2
15.02	+	E01	87%	7.45	R11	P31	B1
15.13	+	E11	4%	6.15	R20	P32	D
16.04	+	E21	1%	5.74	R21	P33	F
16.15	+	E01	1%	5.74	R22	P34	B1
16.27	-	E21	98%	7.73	R23	P33	F
17.10	+	E23	87%	7.45	R23	P35	A
17.18	+	E07	13%	6.63	R24	P36	B2
18.03	+	E24	39%	6.25	R25	P37	B1
18.09	+	E13	41%	6.28	R25	P24	B2
18.18	+	E25	19%	5.93	R13	P38	E
18.50	-	E26	1%	4.70	R21	P39	B1
19.02	+	E07	100%	7.19	R14	P40	B2
20.04	+	E27	12%	6.39	R26	P41	B1
20.17	+	E28	5%	6.01	R12	P42	C
20.27	+	E29	53%	7.03	R27	P43	E
20.50	-	E30	22%	6.65	R04	P44	B1
20.51	-	E31	4%	5.91	R06	P44	B1
20.71	-	E09	4%	5.91	R06	P44	B1

Table 2.6 *ERIC-PCR, RAPD-PCR, XbaI-PFGE biotypes and Clermont phylogroups assigned to E. coli strains.*

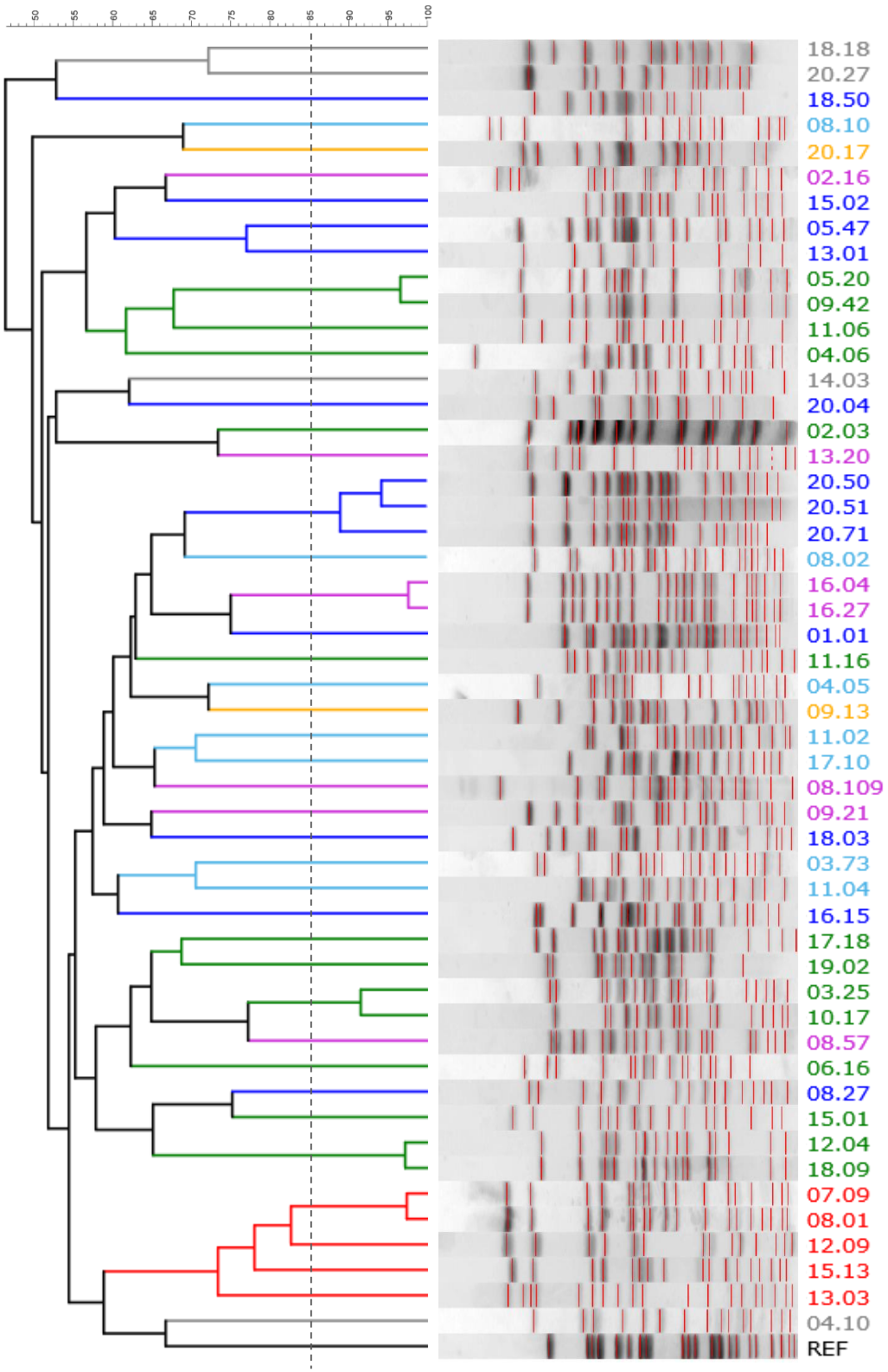

Figure 2.11 *XbaI-PFGE pattern of E. coli strains: UPGMA dendrogram derived from Dice's coefficients, calculated based on band profile. Strains were ascribed to the same pulsotype if PFGE profile if were characterized by >85% similarity. Strains are colored based on their phylogroup: A, cyan; B1, blue; B2, green; C, yellow; D, red; E, grey; F, pink.*

2.4.3 NECE fingerprinting by ERIC-PCR, RAPD-PCR, PFGE, and taxonomic classification

The isolates putatively attributed to NECE were clustered by ERIC-PCR in 32 different biotypes (Figure 2.12).

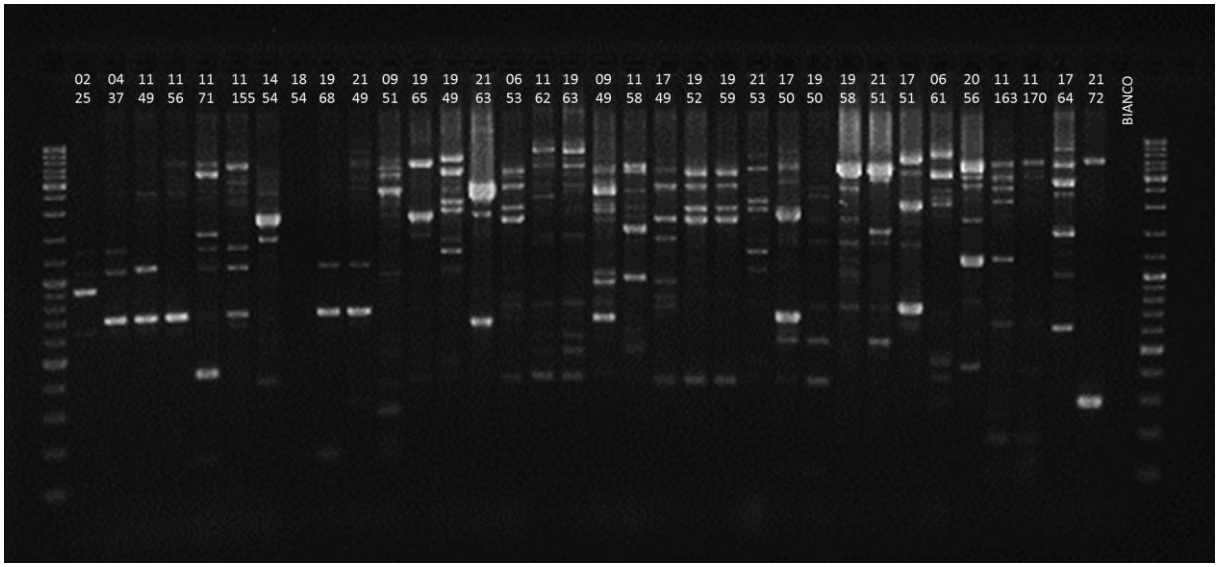

Figure 2.12 *ERIC-PCR fingerprinting of NECE isolates*

RAPD-PCR performed on the same set of isolates recognized 30 different biotypes (Figure 2.13), less than ERIC-PCR.

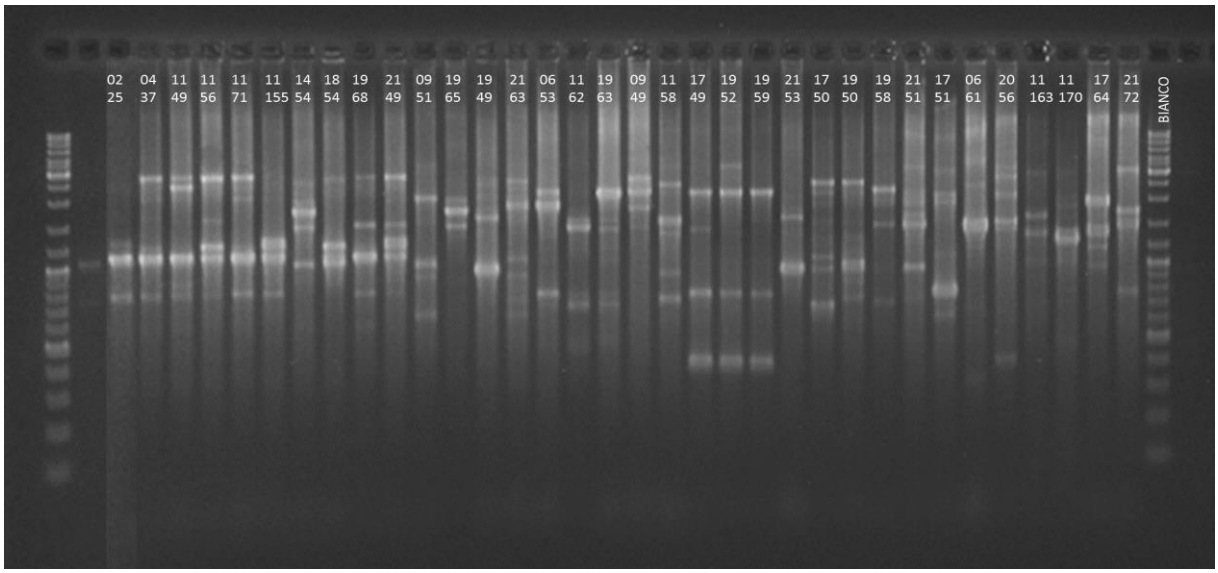

Figure 2.13 *RAPD-PCR fingerprinting of NECE isolates*

The taxonomic classification of the 32 selected biotypes was carried out by MALDI-TOF typing and by partial sequencing of the 16S rRNA genes (Table 2.7). With MALDI-TOF, the 32 strains were ascribed to 11 different species. The genus *Klebsiella* encompasses 15/32 strains, 11 *K. pneumoniae* and 4 *K. oxytoca*. *Enterobacter* was the second most represented genus (10/32), encompassing 6 *E. cloacae*, 1 *E. aerogenes*, 1 *E. kobei*, and 2 *E. cloacae* recently ascribed to the new genus *Cronobacter* (Iversen et al., 2007). The other strains belonged to the species *C. freundii* (3), *Citrobacter amalonaticus*, *Hafnia alvei*, *Morganella morganii*, and *Serratia liquefaciens.*

For each strain the partial 16S rRNA gene sequence was aligned with the SILVA rRNA database (https://www.arb-silva.de/) with the use of SINA (v1.2.11) aligner tool, resulting generally in a taxonomic identification at genus level. In most cases (27/32), the genus assigned by SILVA database corresponded with the genus attributed by MALDI-TOF. However, SILVA database assigned the genus *Raoultella* to the strain 01.72CA that was identified as *Enterobacter aerogenes* by MALDI-TOF. The genus *Raoultella* was named after the French bacteriologist Didier Raoult in 2001 after a reclassification of three *Klebsiella* species *K. ornithinolytica*, *K. terrigena*, and *K. planticola* as *R. ornithinolytica*, *R. terrigena, and R. planticola*, respectively (Drancourt et al. 2001). This reclassification does not explain the inconsistent assignment by the two methods. In four cases, alignment to SILVA database did not allow genus identification for the strains 09.51CA *K. oxytoca*, 11.58CA *Enterobacter kobei*, 14.54CA *K.a pneumoniae* and 17.50CA *C. freundii.*

The lower number of biotypes identified by RAPD-PCR (30/32) was due to the similar profiles obtained for 2 strains of *K. pneumoniae* (02.25MC and 11.71CA), and for 2 strains belonging to the species *K. pneumoniae* (19.49CA) and *E. cloacae* (01.53CA).

Volunteer	***Strain ID***	***MALDI-TOF taxonomy assignation***	***SILVA database taxonomy assignment***
1	01.49CA	*Klebsiella pneumoniae*	*Klebsiella*
1	01.51CA	*Citrobacter freundii*	*Citrobacter*
1	01.53CA	*Enterobacter cloacae*	*Enterobacter*
1	01.63CA	*Klebsiella oxytoca*	*Klebsiella*
1	01.72CA	*Enterobacter aerogenes*	*Raoultella*
2	02.25MC	*Klebsiella pneumoniae*	*Klebsiella*
4	04.37CA	*Klebsiella pneumoniae*	*Klebsiella*
6	06.53MC	*Enterobacter cloacae*	*Enterobacter*
6	06.61MC	*Hafnia alvei*	*Hafnia-Obesumbacterium*
9	09.49CA	*Serratia liquefaciens*	*Serratia*
9	09.51CA	*Klebsiella oxytoca*	*Enterobacteriaceae*

Volunteer	*Strain ID*	*MALDI-TOF taxonomy assignation*	*SILVA database taxonomy assignment*
11	11.49CA	*Klebsiella pneumoniae*	*Klebsiella*
11	11.55MC	*Klebsiella pneumoniae*	*Klebsiella*
11	11.56CA	*Klebsiella pneumoniae*	*Klebsiella*
11	11.58CA	*Enterobacter kobei*	*Enterobacteriaceae*
11	11.62CA	*Enterobacter cloacae*	*Enterobacter*
11	11.63MC	*Cronobacter spp (= Enterobacter cloacae)*	*Cronobacter*
11	11.70MC	*Cronobacter spp (= Enterobacter cloacae)*	*Cronobacter*
11	11.71CA	*Klebsiella pneumoniae*	*Klebsiella*
14	14.54CA	*Klebsiella pneumoniae*	*Enterobacteriaceae*
17	17.49CA	*Enterobacter cloacae*	*Enterobacter*
17	17.50CA	*Citrobacter freundii*	*Enterobacteriaceae*
17	17.51CA	*Citrobacter amalonaticus*	*Citrobacter*
17	17.64MC	*Morganella morganii*	*Morganella*
18	18.54MC	*Klebsiella pneumoniae*	*Klebsiella*
19	19.49CA	*Klebsiella oxytoca*	*Klebsiella*
19	19.50CA	*Citrobacter freundii*	*Citrobacter*
19	19.52CA	*Enterobacter cloacae*	*Enterobacter*
19	19.58CA	*Klebsiella pneumoniae*	*Klebsiella*
19	19.63CA	*Enterobacter cloacae*	*Enterobacter*
19	19.65CA	*Klebsiella oxytoca*	*Klebsiella*
19	19.68MC	*Klebsiella pneumoniae*	*Klebsiella*

Table 2.7: *MALDI-TOF and 16S sequencing (SILVA database) taxonomy assignment of the 32 NECE strains.*

In volunteer n° 2, 4, 14 and 18, a single strain of *Klebsiella pneumoniae* was identified. In volunteer 6 and 9 two different biotypes of NECE were found: in volunteer n° 6 *Hafnia alvei* and *Enterobacter cloacae,* and in volunteer n° 9 *Serratia liquefaciens* and *Klebsiella oxytoca.* The high load of NECE in sample 11 was represented by 4 different strains of *Klebsiella pneumoniae,* 2 different biotypes of *Cronobacter, Enterobacter kobei* and *Enterobacter cloacae*. NECE isolated in volunteers n° 17 were ascribed to *Enterobacter cloacae*, *Citrobacter freundii, Citrobacter amalonaticus,* and *Morganella morganii.* Moreovane, in volunteer 19 it was possible to isolate 7 different biotypes of NECE ascribed to to *Citrobacter freundii, Klebsiella pneumoniae* (2), *K. oxytoca* and *E. cloacae* (2), and *Klebsiella pneumoniae* (2). For what concern volunteer 1 it was possible to isolate 5 different biotypes ascribed to *Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter freundii, Enterobacter cloacae* and *Enterobacter aerogenes* (Table 2.7).

The barplot of Figure 2.14 clearly represents the prevalence of *E. coli* over NECE in all the samples, with the exception of sample 11. The plot showed that generally a single strain of *E. coli* prevailed among the others and allowed the comparison of the relative amount of single biotypes of *E. coli* and NECE for each strain.

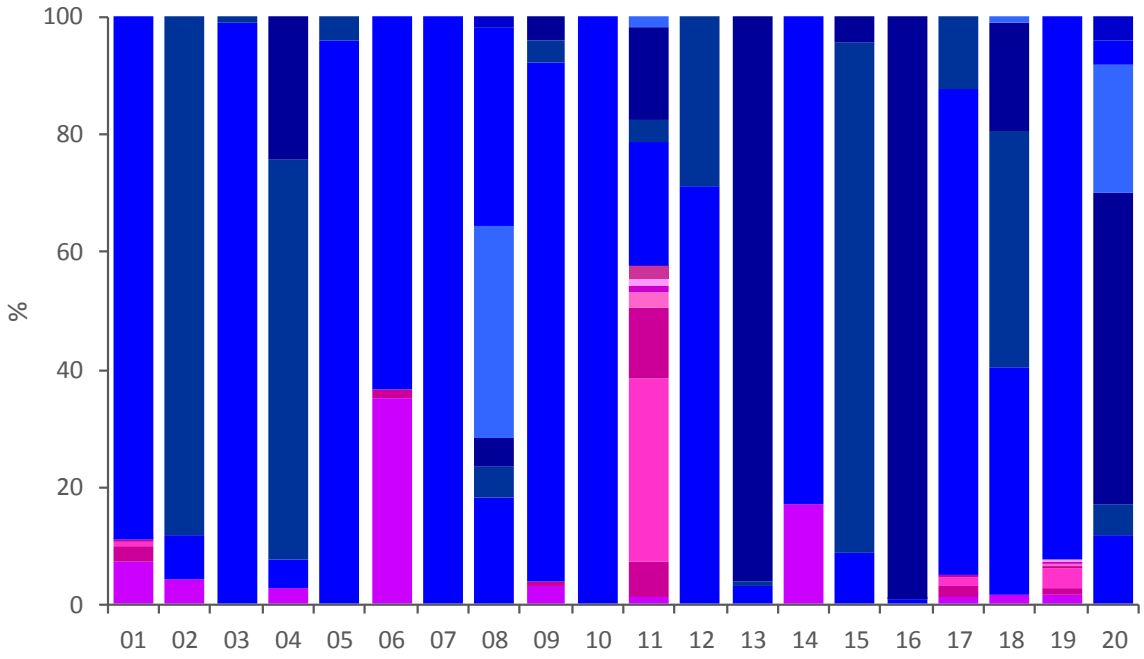

Figure 2.14 *Relative amounts of E. coli and NECE biotypes in the fecal samples of the 20 healthy volunteers. Within each bar, the diverse blu sections represent different E. coli, the pink-fuxia ones the NECE strains*

Fingerprinting by *Xba*I-PFGE was performed on the collection of the 32 NECE. Interestingly, strains belonging to the same species generally did not cluster together (Figure 2.15). The shuffling of strains ascribed to the same species was common to all the species, and suggested a wide diversity within the same species, according to the big dimensions of the corresponding pangenomes, but also a notable synteny among the different genera and species of Enterobacteriaceae, likely due to the adaptation to the same lifestyle.

Enterobacteriaceae represented for long time the sole family within the order Enterobacteriales (class Gammaproteobacteria). However, in 2016 Adelou et al., taking into account phylogenetic analyses and genomic features, proposed to rename the order Enterobacteriales into the order Enterobacterales that included seven families: Enterobacteriaceae, Erwiniaceae fam nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov. and Budviciaceae fam. nov. Attempts to catch markers and fingerprinting methods to describe the phylogenetic relationship within enterobacteriaceae have been made (Adelou et al., 2016). Among them, PFGE is clearly not

able to properly discriminate species within this family, nor either ERIC-PCR or RAPD-PCR, according to a wide intra-species variability, but also to the inter-genus similarity likely due to genetic exchanges associated to the shared ecological niches.

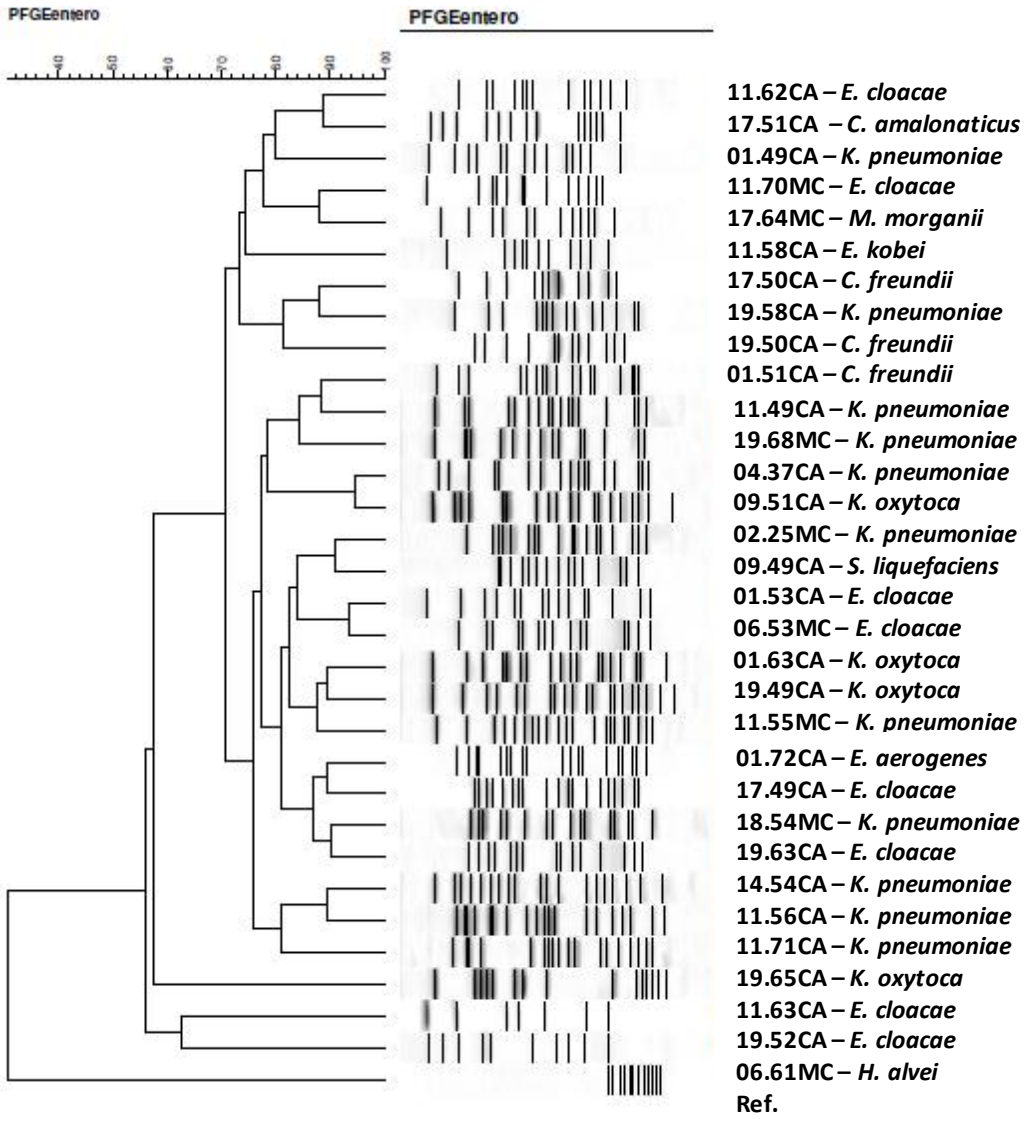

Figure 2.15: *XbaI-PFGE pattern of non-E. coli Enterobacteriaceae species: UPGMA dendrogram derived from Dice's coefficients, calculated based on band profile. Strains were ascribed to the same pulsotype if PFGE profile if were characterized by >85% similarity.*

2.4.4 Phylogroup assignement to *E. coli* according to Clermont

The *E. coli* isolates were tested for phylogroup assignment following the quadruplex-PCR method described by Clermont et al. 2013. The combination of the presence/absence of genes

chuA, yjaA, TspE4.C2, arpA, and *trpA* provided the phylogroup assignation as A, B1, B2, C, D, E and F, on the basis of the framework proposed by Clermont et al. 2013 (Table 2.6). Most of the strains belonged to phylogroup B2 (14), followed by B1 (12), F (7), A, D, E, and C (12, 7, 7, 5, 4, and 2 strains). The strains of phylogroup B2 generally corresponded to the highest absolute *E. coli* charges, and were the most abundant within *E. coli* population, accounting for the all colonies recovered from several samples. Conversely, B1 strains were detected at lower concentrations and represented a minor subpopulation (Table 2.6). This result is consistent with the fact that B2 strains showed an enhanced ability to persist in the intestinal microbiota, as demonstrated in infants (Nowrouzian et al., 2005; Ostblom et al., 2011). B1 strains, on the contrary, showed lower performance in colonization, generally representing minor *E. coli* subpopulations within each sample.

Phylogroups classification and PFGE pulsotypes were consistent, but the strains belonging to the same phylogroups were spread over different clades of the PFGE dendrogram. Only in the case of phylogroup D, the isolates clustered together. PFGE analysis targeted the whole genome, most of which is composed of accessory genes that can be acquired by horizontal gene transfer. The PFGE fingerprint of *E. coli* results from the digestion of a genome, the size of which, including plasmids and prophages, ranges approximately from 4.6 to 5.9 Mbp, encoding from 4200 to 5500 genes (Leimbach, et al., 2013). This huge difference is mainly due to the genes associated with bacteriophage elements and involved in virulence or resistance to antimicrobials. The phylotypes recognized in this study were shuffled in PFGE, likely because of the genome evolution, mainly due to recombination and horizontal transfer that broke the old phylotype relationships, thereby emphasizing more recent changes. On the other hand, MLST typing, the gold-standard approach for *E. coli* classification, allows a phylotyping-consistent analysis, because MLST is based on the conserved nature of the housekeeping genes of the core genome (Robins-Browne et al., 2016).

2.4.5 Virulence factors in *E. coli* and in NECE

Thirty-four genetic determinants encoding for virulence factors that potentially enhance the risk of infections of *E. coli* were screened by PCR in the set of the 51 *E. coli* strains (Table 2.8). Most isolates were positive to *finH, fyuA, ompT, traT, chuA, and kpsMTII* (47, 36, 35, 35, 32, and 27 strains, respectively). On the other hand, *afa/draBC, cncn, facG, hlyD, ibeA, rfc, sfa,* and *sfaS* recurred rarely (≤ 5 strains), while *cdtB, gafD, pic,* and *vat* were absent in all the strains (Table 2.8). On the basis of virulence factors, all the strains belonging to the phylotypes B2, D

(with an exception), E, and F clustered together (Figure 2.16). Strains ascribed to the phylotypes C were grouped in another cluster that included also the majority of B1 and most of the A isolates, with a single B1 strain not included (Figure 2.16).

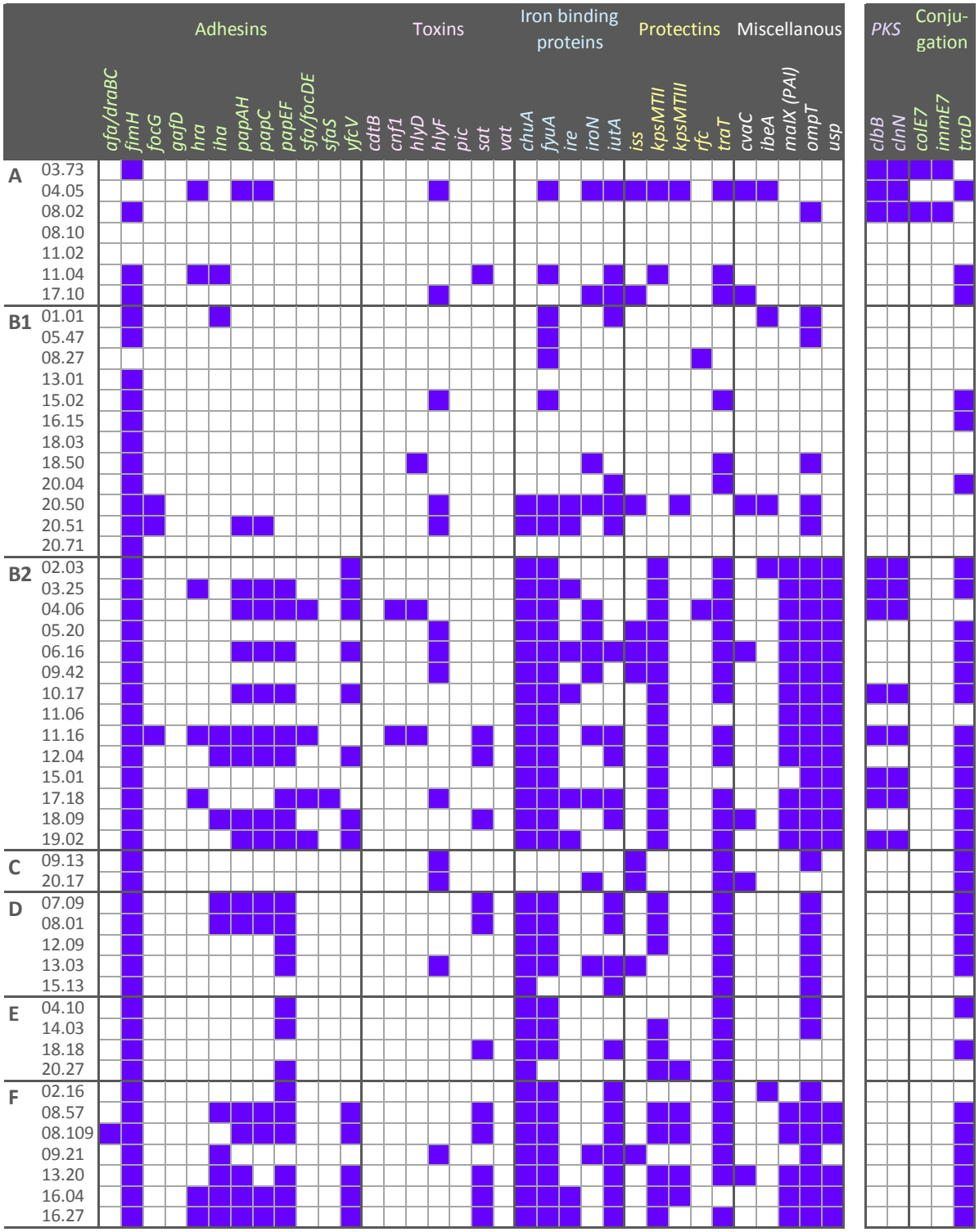

Table 2.8 *Presence of genetic determinants in the E. coli strains, ordered on the basis of the phylotype.*

The great variability of pathogenicity-associated features suggests that the genetic determinants of virulence had a role in shaping the genome of the isolates. A significant co-occurrence was observed in a set of B2 and F strains for: *pap* genes (*papAH*, *papC*, *papEF*), encoding proteins of the fimbria favouring the ascension of the urinary tract and promoting colonization and infection; *sat* gene, which is associated with a cytopathic secreted autotransported toxin exerting effect; *kpsMTII*, the marker of K2 capsular polysaccharides, which plays a main role in pathogenesis, *chuA* (heme receptor), *yfcV* (Yfc fimbria), along with *fyuA*, *malX*, *usp*, and *ompT* (Table 2.8). Among them, twelve strains, belonging to the phylogroups B2 (03.25, 04.06, 06.16, 10.17, 12.04, 18.09, and 19.02) and F (08.57, 08.109, 13.20, 16.04, and 16.27), presented a pattern of virulence genes similar to those strains responsible for urinary tract infections, on the basis of the presence of the genes *chuA* (heme receptor), *yfcV* (Yfc fimbria), *fyuA* (*Yersinia* siderophore receptor) and *ompT* (aspartyl protease) (Spurbeck et al., 2012). This reinforces the idea that potentially pathogenic *E. coli* inhabit the gut of healthy subjects without start infections, although they can act as etiologic agents of extra-intestinal infections. This highlights the absence of a clear distinction between commensal *E. coli* and pathogens.

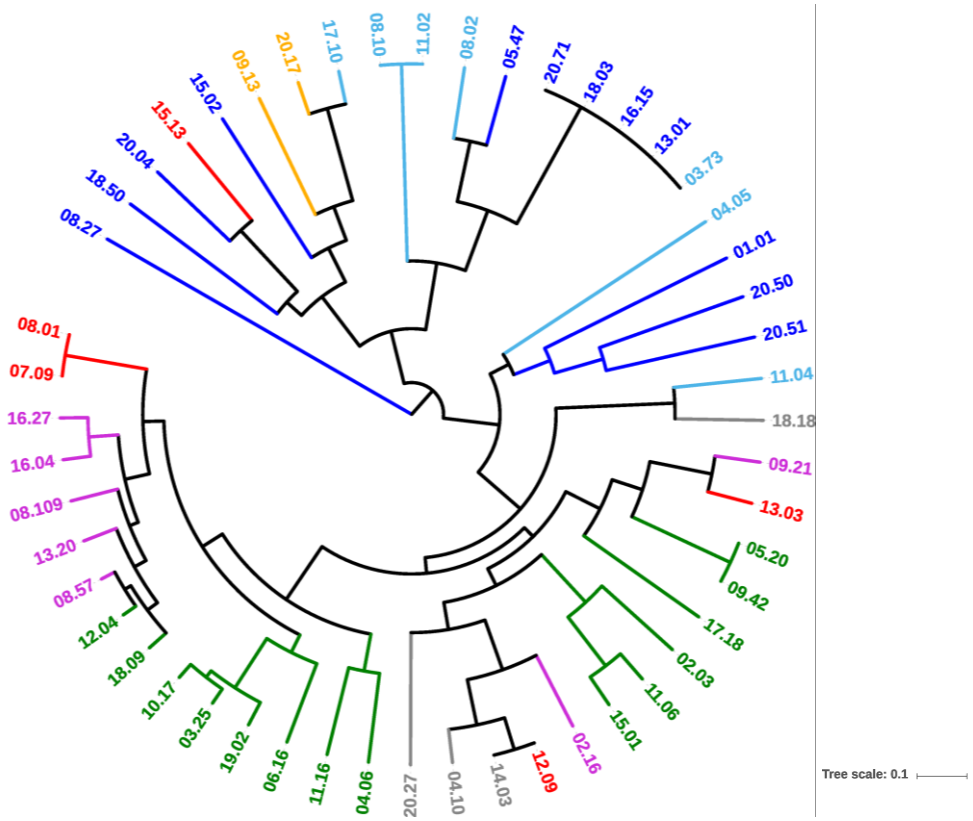

Figure 2.16 *UPGMA dendrogram of E. coli strains, computed from Dice's distance matrix of virulence determinants. Strains are colored based on their phylogroup: A, cyan; B1, blue; B2, green; C, yellow; D, red; E, grey; F, pink.*

Capsular polysaccharides represent a class of macromolecules contributing to the surface properties. They are involved in important biological processes including adhesion and resistance to the host's immune responses, such as complement-mediated killing and phagocytosis (Sarowska et al., 2019). In this study, 27 out of 51 carried the gene *kpsMTII*, among which, six strains carried also *kpsMTIII*, encoding for another capsular antigen. Macromolecules on bacteria's surface confers ultrastructural stability, that is important for recognition and interaction with the environment, and form a defensive barrier against the host's immune system. All but one strain was negative to *afa/draBC* gene present in the *afa* operons, encoding for Dr-family of adhesins (Servin, 2005). Like capsular polysaccharides, curli are involved in *E. coli* adhesion and defence against complement-mediated killing by the host, and thereby can contribute to the pathogenic onset (Biesecker et al., 2018).

E. coli pathogenicity islands serve as integration sites for genetic elements. Thus, virulence genes and AR determinants can be rapidly and simultaneously acquired, changing commensal strains into major risk agents (Kaushik et al., 2018). Nineteen stains, belonging mostly to B2 or F isolates, harboured a PAI island, which produced a positive result for the maltose- and glucose-specific component IIa of the phosphoenolpyruvate dependent phosphotransferase system (*malX*). Furthermore, all the isolates carried the gene *usp*, encoding for a putative bacteriocin generally located on the PAI island (Bag et al., 2019). The gene *hlyF*, encoding for the toxin α-emolysin, was present in several strains spread over most of the phylotypes (Murase et al., 2016). Moreover, 9 strains harboured the *iss* gene, associated to an increased survival of bacteria in the serum and generally carried by the big virulence plasmid ColV. All the *iss* gene-postive strains were also positive to *traD* gene, according to the fact that ColV plasmids are usually conjugative (Gophna et al., 2003).

Genetic determinants encoding virulence factors for NECE are not deeply characterized. For *K. pneumoniae* a specific group of 13 genes involved in virulence has been identified, encompassing: *ybtS, mrkD, entB, rmpA, K2, kfu, allS, magA, kpn, ycfM, irp-1, irp-2, hlyA* (El Fertas-Aissani et al., 2012; Compain et al., 2014; Johnson et al. 2015). Because of the relatedness within Enterobacteriaceae, the set of *K. pneumoniae* primers have been blasted against the DNA of *K. oxytoca, Citrobacter* spp., and *Enterobacter* spp., to *in silico* evaluate the presence of cognate genes. Likewise, the 34 set of primers amplifying the *E. coli* virulence genetic determinants have been blasted against the DNA of the genera *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp., revealing the possible presence of 6/34 genes (*fimH, iutA, fyuA, iroN, traT*, and *cnf-1)* in some of these genera (Table 2.9).

In *K. pneumoniae* were generally detected the genes *entB* and *irp-2* enconding for the siderophores enterobactin, (10/11) and yersiniabactin (8/11) (Table 2.9). Most of *K. pneumonia* harboured also the adhesin coding gene *mrkD* (10/11). Other genes encoding for adhesins such as *ycfM* and *kpn* were frequently detected (9/11 and 6/11, respectively). On the other hand, the presence of the genes *irp-1, ybtS, kfu*, and *allS* was rare. Genes *irp-1* and *ybtS are* associated with yersiniabactin siderophore and they were detected a single strain. *kfu* encoding a protein that mediates the uptake of ferric iron, recurrent in hypervirulent strains, was detected in two strains (Hsieh et al., 2008).

entB, enconding for the siderophores enterobactin was present also in the 3 *K. oxytoca* strains, that resulted negative to all the other overmentioned genes frequently detected in *K. pneumoniae*, with the exception of *K. oxytoca* 01.63 that succeeded in amplification of *YbtS*.

All the strains of *E. cloacae* harbored the gene *irp-2*, and 2 of them also *mrkD*. Among the genes of *E. coli* potentially present in *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp., only *fimH* was detected in 4/6 *E. cloacae* and in *E. kobei*, albeit the *in silico* search did not provided consistent results of primer pairing in the latter species.

Taxonomy identification			allS	entB	hlyA	irp-1	irp-2	K2	kfu	kpn	mag	mrkl	rmp	ybtS	ycfM	cnf-1	fimH	fyuA	iroN	iutA	traT
Strain ID	**MALDI-TOF**	**SILVA database**																			
17.51CA	*Citrobacter amalonaticus*	*Citrobacter*																			
01.51CA	*Citrobacter freundii*	*Citrobacter*																			
17.50CA	*Citrobacter freundii*	*Enterobacteriaceae*																			
19.50CA	*Citrobacter freundii*	*Citrobacter*																			
11.63MC	*Cronobacter spp (= Enterobacter cloacae)*	*Cronobacter*																			
11.70MC	*Cronobacter spp (= Enterobacter cloacae)*	*Cronobacter*																			
01.72CA	*Enterobacter aerogenes*	*Raoultella*																			
01.53CA	*Enterobacter cloacae*	*Enterobacter*																			
06.53MC	*Enterobacter cloacae*	*Enterobacter*																			
11.62CA	*Enterobacter cloacae*	*Enterobacter*																			
17.49CA	*Enterobacter cloacae*	*Enterobacter*																			
19.52CA	*Enterobacter cloacae*	*Enterobacter*																			
19.63CA	*Enterobacter cloacae*	*Enterobacter*																			
11.58CA	*Enterobacter kobei*	*Enterobacteriaceae*																			
06.61MC	*Hafnia alvei*	*Hafnia-Obesumbacterium*																			
01.63CA	*Klebsiella oxytoca*	*Klebsiella*																			
09.51CA	*Klebsiella oxytoca*	*Enterobacteriaceae*																			
19.49CA	*Klebsiella oxytoca*	*Klebsiella*																			
19.65CA	*Klebsiella oxytoca*	*Klebsiella*																			
01.49CA	*Klebsiella pneumoniae*	*Klebsiella*																			
02.25MC	*Klebsiella pneumoniae*	*Klebsiella*																			
04.37CA	*Klebsiella pneumoniae*	*Klebsiella*																			
11.49CA	*Klebsiella pneumoniae*	*Klebsiella*																			
11.55MC	*Klebsiella pneumoniae*	*Klebsiella*																			
11.56CA	*Klebsiella pneumoniae*	*Klebsiella*																			
11.71CA	*Klebsiella pneumoniae*	*Klebsiella*																			
14.54CA	*Klebsiella pneumoniae*	*Enterobacteriaceae*																			
18.54MC	*Klebsiella pneumoniae*	*Klebsiella*																			
19.58CA	*Klebsiella pneumoniae*	*Klebsiella*																			
19.68MC	*Klebsiella pneumoniae*	*Klebsiella*																			
17.64MC	*Morganella morganii*	*Morganella*																			
09.49CA	*Serratia liquefaciens*	*Serratia*																			

Table 2.9 *Presence of genetic determinants of virulence in NECE strains determine by PCR. Purple: presence; white: absence; grey: assessed the absence by in silico analysis.*

2.4.6 Other genetic determinants affecting fitness and pathogenicity to *E. coli*: *pks* island, *traD* gene, *colE7* and *immE7*

The *E. coli* strains were screened for the presence of a 54-kb polyketide synthases *pks* pathogenicity island, that encodes for the genotoxin colibactin. The genes *clbB* and *clbN*, utilized as markers, were found in 11 out of 51 strains, 3 ascribed to phylotype A and 8 to B2.

The presence of *traD* was investigated to establish the potential in conjugative DNA exchange. Most isolates were positive to *traD* (32/51), including the majority of *pks*-positive strains (Table 2.8). *traD* was differently distributed among the phylogroups: most B2, C, D, and F strains harboured the gene, while only a minority within A and B1 did. When a same subject hosted more biotypes, some isolates bore *traD* while others did not. The genes *colE7* and *immE7*, respectively encoding the bactericidal nuclease Colicin E7 and the corresponding immunity protein, were searched. Only two strains harboured both the genes (Table 2.8).

The fitness and competitiveness of the bacteria can be improved by bacteriocin production, which is generally associated with the counterpart immunity protein, thereby protecting the producing host cell from the lethal action of the bacteriocin. Colicins are plasmid encoded toxins produced by *E. coli* under conditions of stress and able to kill related bacteria competing for niches and nutrients. The genes encoding Colicin E7 and its immunity counterpart have been used to develop bacterial conjugation-based antimicrobial agents (Starčič Erjavec, et al., 2015; Maslennikova et al., 2018). The antibacterial activity of the this bacterial "kill"– "anti-kill" antimicrobial system has been determined, thereby offering new perspectives in the development of *E. coli* targeted antimicrobials. For this reason, the strains isolated in this study were screened for the presence of the *colE7* and *immE7* genes, in order to investigate the susceptibility of commensal strains to the action of recombinant antimicrobials. Most of the strains were negative for both the genes, only two of them carried them, suggesting that, if necessary, they could be potentially vulnerable to this new approach.

2.4.7 Conjugation

The 10 β-glucuronidase-negative *E. coli* strains, six belonging to phylotype B1 and four to phylotype F, were challenged as conjugation recipients for receiving pOX38:Cm plasmid from *E. coli* N4i. Conjugation frequencies were calculated on a ration between the concentration of

transconjugant obtained on the total concentration of recipient cells. (Table 12). Plasmid transfer succeeded in 8 strains out of 10, confirming their receptive aptitude for the genetic exchange and DNA shuffling of commensal *E. coli*. In particular, all the six *E. coli* strains belonging to B1 phylotype received the plasmid, while the strains belonging to F phylotypes only in two cases. Interestingly, 2 of the 8 transconjugant strains harboured *traD*, as a putative marker of conjugative plasmid it is expected to exclude the acquirement of another plasmid through conjugation.

Specie	***Strain ID***	***phylo-group***	***Conjugation efficiency [T/R]***
E. coli	05.47	B1	$3.3 \cdot 10^{-1}$
E. coli	08.57	F	$1.6 \cdot 10^{-1}$
E. coli	08.109	F	$2 \cdot 10^{-1}$
E. coli	08.27	B1	$5.6 \cdot 10^{-3}$
E. coli	13.20	F	0.0
E. coli	16.27	F	00
E. coli	18.50	B1	$8.9 \cdot 10^{-3}$
E. coli	20.51	B1	$6.2 \cdot 10^{-2}$

Table 2.10: *The conjugation frequency, reported as the average of three distinct conjugation experiments, was calculated as the number of CFU E. coli transconjugants per CFU recipient strain.*

Also the 32 different NECE strains were challenged as conjugation recipients for receiving pOX38:Cm plasmid from *E. coli* N4i (Table 13). Only 2 strains of *K. pneumoniae*, and single strains of *Cronobacter* and *Citrobacter amalonaticus* succeeded in plasmid acquisition.

Strain ID	***Species***	***Conjugation efficiency [T/R]***
02.25CA	*Klebsiella pneumonie*	$1.8 \cdot 10^{-5}$
04.37CA	*Klebsiella pneumonie*	$1.8 \cdot 10^{-4}$
11.163MC	*Cronobacter spp.*	$1.2 \cdot 10^{-2}$
17.51CA	*Citrobacter amalonaticus*	$1.7 \cdot 10^{-2}$

Table 2.11: *The conjugation frequency, reported as the average of three distinct conjugation experiments, was calculated as the number of CFU NECE transconjugants per CFU recipient strain.*

2.4.8 Production of curli and cellulose, and biofilm formation

The production of curli was assessed by observing the colonies grown in CR-containing LB agar plates. *E. coli* strains belonging to B2 and F phylogroup presented the lowest attitude to bind CR dye: only 2 out of 14 B2 strain and 1 out of 7 F strain gave positive results. However,

C, D, and E strains seemed to be inclined to produce curli (Table 2.12). The occurrence of cellulose-like extracellular components was analysed growing the isolates on LB plates supplemented of CF. The ability to bind CF of *E. coli* was less frequent than that of CR, in fact it was observed only in 9 isolates out of 51.

Among NECE strains, *Citrobacter* spp., *E. cloacae*, *H. alvei* and *K. oxytoca* 19.49 produced both cellulose structures and curli, on the basis of CR and CF binding. The two other strains of *K. oxytoca* did not produce any of these envelop structures. *Cronobacter*, phylogenetically related to *E. cloacae*, produced curli but not cellulose extracellular components. *K. pneumonia*, with a sole exception, did not form curli, whereas the production of extracellular cellulose was detected in some strains (5/11). *E. kobei* and *M. morganii* bound CR, whereas *S. liquefaciens* resulted positive to cellulose production (Table 2.13).

Species	***Strain ID***	***phylo-group***	***Curli***	***Cellulose***
E. coli	01.01	B1	-	-
E. coli	02.03	B2	-	-
E. coli	02.16	F	+	+
E. coli	03.25	B2	-	-
E. coli	03.73	A	-	-
E. coli	04.05	A	+	-
E. coli	04.06	B2	-	-
E. coli	04.10	E	+	+
E. coli	05.20	B2	-	+
E. coli	05.47	B1	-	-
E. coli	06.16	B2	-	-
E. coli	07.09	D	+	-
E. coli	08.01	D	+	-
E. coli	08.02	A	-	-
E. coli	08.10	A	+	-
E. coli	08.27	B1	-	-
E. coli	08.57	F	-	-
E. coli	08.109	F	-	-
E. coli	09.13	C	+	+
E. coli	09.21	F	-	-
E. coli	09.42	B2	-	-
E. coli	10.17	B2	-	-
E. coli	11.02	A	-	-
E. coli	11.04	A	-	-
E. coli	11.06	B2	-	-
E. coli	11.16	B2	-	-
E. coli	12.04	B2	+	-
E. coli	12.09	D	-	-

Species	***Strain ID***	***phylo-group***	***Curli***	***Cellulose***
E. coli	13.01	B1	+	-
E. coli	13.03	D	+	+
E. coli	13.20	F	-	-
E. coli	14.03	E	+	-
E. coli	15.01	B2	-	+
E. coli	15.02	B1	+	+
E. coli	15.13	D	+	-
E. coli	16.04	F	-	-
E. coli	16.15	B1	+	-
E. coli	16.27	F	-	-
E. coli	17.10	A	-	-
E. coli	17.18	B2	-	-
E. coli	18.03	B1	-	-
E. coli	18.09	B2	+	-
E. coli	18.18	E	-	+
E. coli	18.50	B1	-	-
E. coli	19.02	B2	-	-
E. coli	20.04	B1	+	-
E. coli	20.17	C	+	-
E. coli	20.27	E	+	+
E. coli	20.50	B1	-	-
E. coli	20.51	B1	-	-
E. coli	20.71	B1	-	-

Table 2.12 *Production of curli and cellulose-like extracellular components in E. coli.*

Strain ID	***Species***	***Curli***	***Cellulose***
17.51CA	*Citrobacter amalonaticus*	+	+
01.51CA	*Citrobacter freundii*	+	+
17.50CA	*Citrobacter freundii*	+	+
19.50CA	*Citrobacter freundii*	+	+
11.63MC	*Cronobacter* spp. *(Ent. cloacae)*	+	-
11.70MC	*Cronobacter* spp. *(Ent. cloacae)*	+	-
01.72CA	*Enterobacter aerogenes*	-	-
01.53CA	*Enterobacter cloacae*	+	+
17.49CA	*Enterobacter cloacae*	+	+
19.52CA	*Enterobacter cloacae*	+	+
06.53MC	*Enterobacter cloacae*	+	+
11.62CA	*Enterobacter cloacae*	+	+
19.63CA	*Enterobacter cloacae*	+	+
11.58CA	*Enterobacter kobei*	+	-
06.61MC	*Hafnia alvei*	+	+

Strain ID	Species	Curli	Cellulose
01.63CA	*Klebsiella oxytoca*	-	-
09.51CA	*Klebsiella oxytoca*	-	-
19.49CA	*Klebsiella oxytoca*	+	+
19.65CA	*Klebsiella oxytoca*	-	+
01.49CA	*Klebsiella pneumoniae*	-	-
02.25MC	*Klebsiella pneumoniae*	-	-
04.37CA	*Klebsiella pneumoniae*	-	+
11.49CA	*Klebsiella pneumoniae*	-	-
11.55MC	*Klebsiella pneumoniae*	-	+
11.56CA	*Klebsiella pneumoniae*	-	+
11.71CA	*Klebsiella pneumoniae*	-	-
14.54CA	*Klebsiella pneumoniae*	-	-
18.54MC	*Klebsiella pneumoniae*	-	-
19.68MC	*Klebsiella pneumoniae*	-	+
19.58CA	*Klebsiella pneumoniae*	+	+
17.64MC	*Morganella morganii*	+	-
09.49CA	*Serratia liquefaciens*	-	+

***Table 2.13**: Production of curli and cellulose-like extracellular components in NECE.*

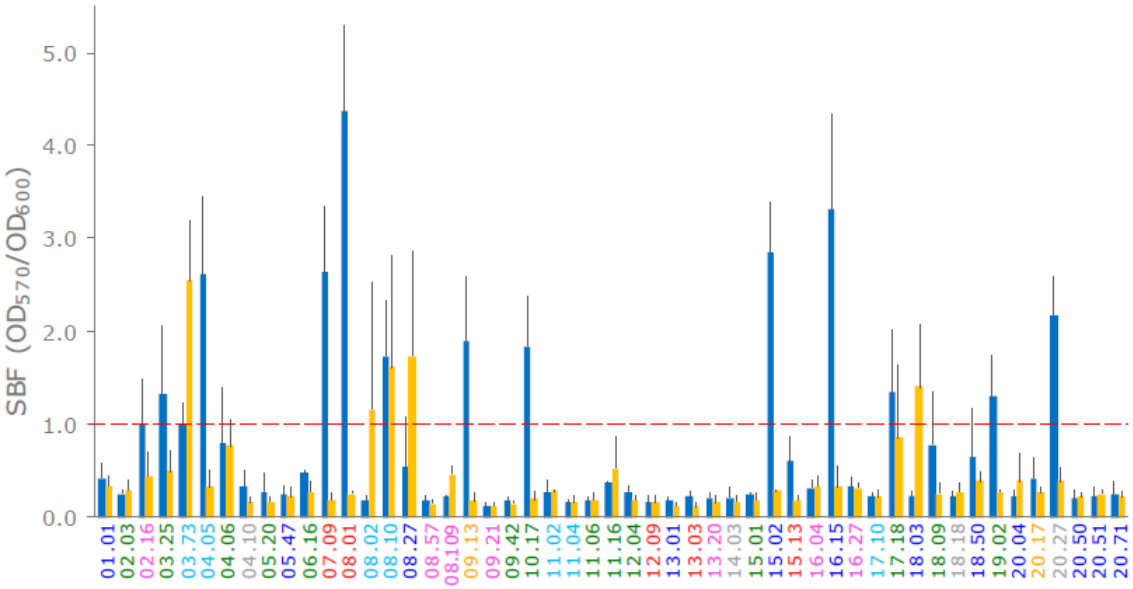

***Figure 2.17:** Formation of biofilm by E. coli strains on LBWS (blue bars) and M9glu (yellow bars). The specific biofilmformation index (SBF) is calculated as the ratio between the crystal violet absorbance at 570 nm and the culture turbidity at 600 nm, setting a threshold of 1 for biofilm producers (red dashed line). The reported data are means ± standard deviations of at least three independent experiments, each carried out in triplicate. The names of the strains are colored based on their phylogroups: A, cyan; B1, blue; B2, green; C, yellow; D, red; E, grey; and F, pink (hereafter referred to as LBWS and M9glu, respectively).*

E. coli and NECE strains were assayed for biofilm formation in LBWS and M9glu. For what concern *E. coli*, most of them did non form biofilm. For this species, formation occurred more frequently in LBWS than in M9glu (13/51 and 5/51 strains, respectively). Only two strains ascribed to phylotype A 03.73 and 08.10 produced biofilm in both the media. For 5 other strains the score of biofilm formation ranged between 0.6 and 0.8. All but one of these strains presented very high standard deviations suggesting that biofilm formation was sensitive to environmental conditions hardly controlled by the operator (Figure 2.17).

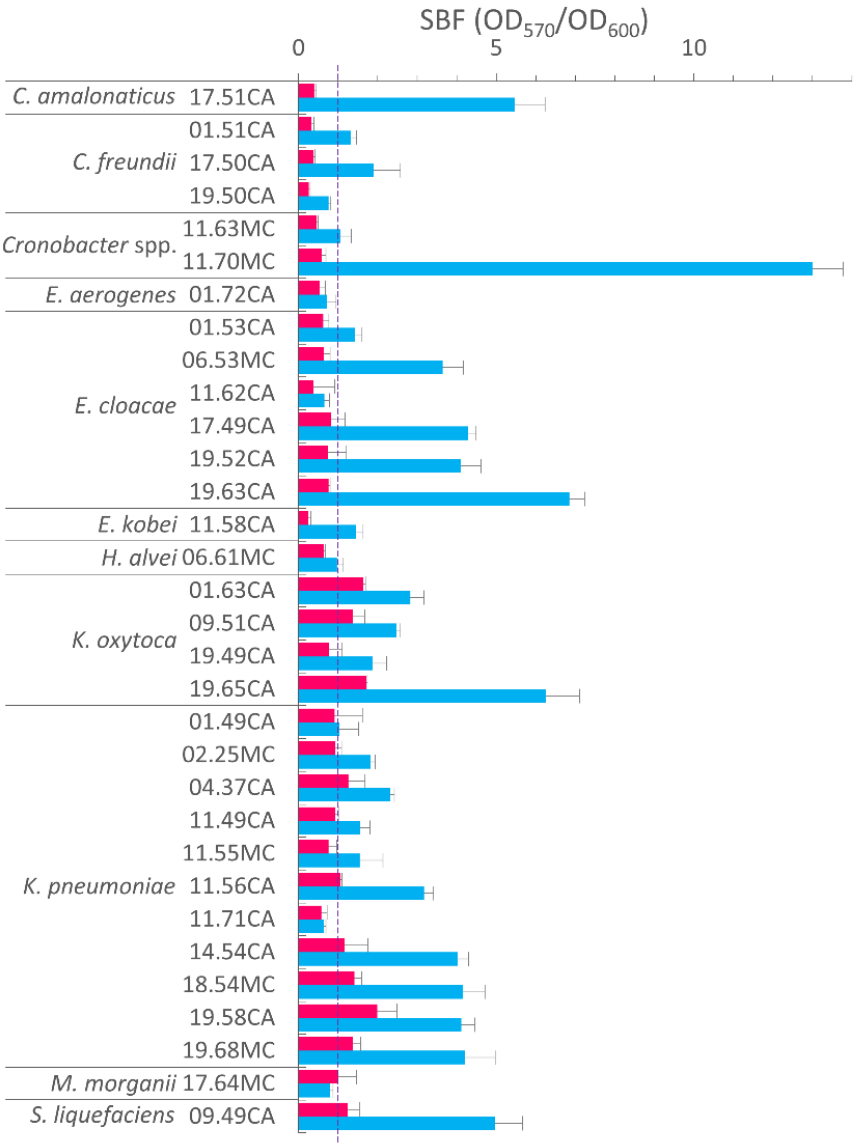

Figure 2.18: *Biofilm formation of NECE on LBWS (pink bars) and M9glu (ble bars). The specific biofilm formation index (SBF) is calculated as the ratio between the crystal violet absorbance at 570 nm and the culture turbidity at 600 nm, setting a threshold of 1 for biofilm producers (red dashed line). The reported data are means ± standard deviations of at least three independent experiments, each carried out in triplicate.*

Among NECE, 7 strains (*C. freundii* S19.50CA, *E. cloacae* 11.62CA, *Cronobacter* 11.63 CA, *H. alvei* 06.62MC, *K. pneumoniae* 11.71CA, *M. morganii* 17.64MC, *E. aerogenes* 21.72CA) did not show any biofilm production in both media LBW and M9glu.

The other (27/32), showed strong biofilm production on M9glu. All *Cronobacter* and *K. oxytoca* strains produced biofilm on M9glu, likewise most of *Citrobacter*, *Enterobacter*, and *K. pneumonia.* Biofilm production on LBWS was way less frequent among the strains than on M9glu, with an opposite response to growth medium of NECE compared to *E. coli* (Figure 2.18). According to the specific biofilm formation index (SBF) calculated as the ratio between crystal violet absorbance at 570 nm and culture turbidity at 600 nm, particularly high was biofilm production in M9glu by *Cronobacter* 11.70MC.

2.4.9 Antibiotic susceptibility

Phenotypic antibiotic susceptibility tests were carried out on the 51 *E. coli* strains. Nearly all the isolates were extensively sensitive to the whole set of tested antibiotics including, amoxicillin plus clavulanic acid and cephalosporins, the most used antibiotics for the first treatment of infections. (49/51). Only 2 strains recovered from the same fecal sample, belonging to the phylogroups B2 (17.18) and C (17.10), were resistant to gentamycin. *E. coli* 17.10 was resistant also to ciprofloxacin.

Also NECE resulted generally sensitive to all the tested antibiotics. Resistance was observed only toward amoxicillin plus clavulanic acid in all the 15 strains belonging to the genera *Citrobacter, Enterobacter*, *Hafnia, Morganella,* and *Serratia.* Among *K. pneumoniae*, 2 out of 11 strains reached the Eucast breakpoint ("The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. http://www.eucast.org), suggesting that they were resistant to normal antimicrobial dosing, but clinical response is possible with higher doses or if the antimicrobial concentrates at the site of infection. However, the absence in *E. coli* and NECE of the antibiotic resistance (AR) phenotype does not exclude the presence of AR genes, which may be expressed *in vivo* or can be involved in the diffusion and spread of these genes (Suzuki et al., 2014).

All the amoxacillin plus clavulanic acid resistant NECE have been isolated from fecal samples obtained by volunteers 1, 6, 9, 11, 17, and 19. From sample 17 have been isolated the two sole *E. coli* strains resistant to the same association of antibiotics. This result confirms the genetic exchange of AR genes among strains sharing the same niche, with strains belonging to different species and genera isolated from the same fecal sample (i.e. 1, 6, 17, and 11) presenting similar AR susceptibility.

There's nowadays a growing emergence of resistant bacteria occurring worldwide attributed to the massive use of antibiotics. However, in healthy subjects in which an endogenous *E. coli* population is present, they are still sensitive to a wide range of antibiotics, and on the contrary the pathogenic strains isolated from different clinical specimens are often resistant to major antibiotics (Sahuquillo et al., 2011; Cole et al., 2019; Morley et al., 2019). The scarce relevance of commensal *E. coli* as an antibiotic-resistance carrier disagrees with other studies but is consistent with a recent analysis of metagenomes that investigated antibiotic resistance genes in the gut microbiota of healthy people (Dyar et al., 2012; Gurnee et al., 2015; Bag et al., 2019; Purohit et al., 2019). The results show that commensal *E. coli* may not pose a threat by itself in terms of antibiotic resistance, but major differences are registered among countries (Feng et al., 2018; Pormohammad et al., 2019). Interestingly, the resistance to amoxicillin plus clavulanic acid, generally absent in *E. coli* isolates, recur frequently in NECE.

2.4.10 Co-occurrence and PCoA analysis of genetic and functional features in *E. coli* strains

A co-occurrence analysis was applied to the whole set of genetic and function observations for *E. coli* isolates (Table 2.14).The presence of *pks* islands and several virulence determinants was significantly associated ($p < 0.05$ or < 0.01) to certain genotypic classifications (such as the ERIC profile, the pulsotype, and the phylogroup). In particular, the genes encoding some adhesins (*iha, papAH, papC, papF,* and *sat*), iron binding proteins (*chuA, fyuA,* and *iutA*), protectins (*kpsMTII* and *traT*), and *malX, ompT, usp,* and *traD* were significantly associated to phylogroups and presented a significantly positive tendency to co-occur within the same strains ($p < 0.05$ or 0.01).

With regards to the phenotypic features involved in bacterial adhesion, the production of curli was significantly associated with the phylogroup ($p < 0.01$). Although biofilm formation was not frequent, curli presented a significant co-occurrence with the biofilm in the LBWS medium.

A Principal Coordinate Analysis (PCoA) was applied to assist in the identification of clusters, taking into account both their genetic and functional features (Figure 2.19). The first two coordinates were the most informative, accounting for approximately 28.9% and 10.2% of the total observed variances.

In the PCoA plot, PCo1 separated B2 and F strains from B1, C, and E ones (negative and positive PCo1, respectively). F strains were grouped in two clusters. With respect to PCo2, most of the A, C, D, and E strains lie in the positive quadrants.

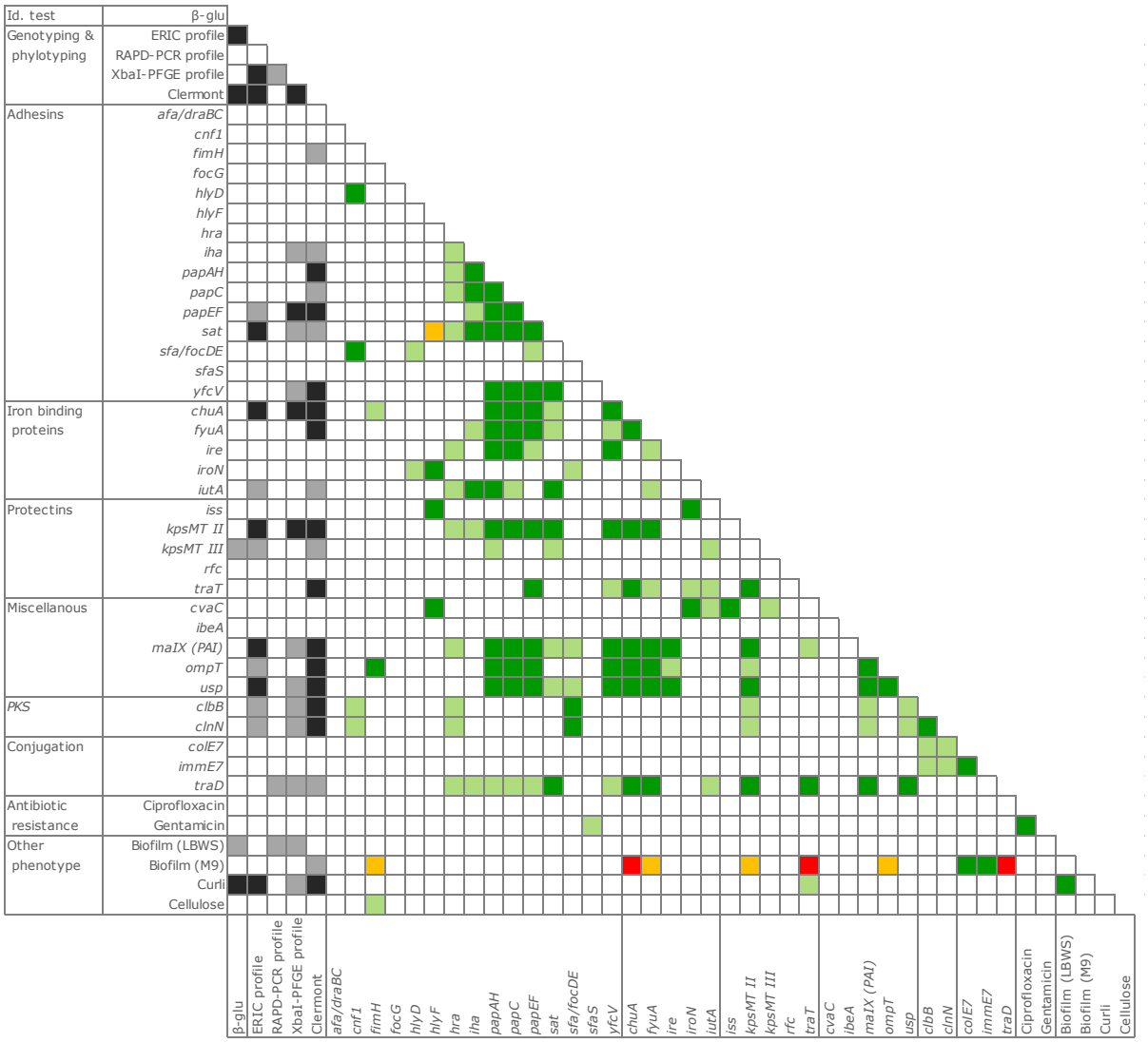

Table 2.14 *Genetic determinants and phenotypic properties evaluated by Cramér'sVmetrics. Association with genotype/phylotype clusters, grey; positive co-occurrence, green; negative co-occurrence, red. Dark and light shades indicate the levels of statistical significance ($p < 0.01$ and $p < 0.05$, respectively).*

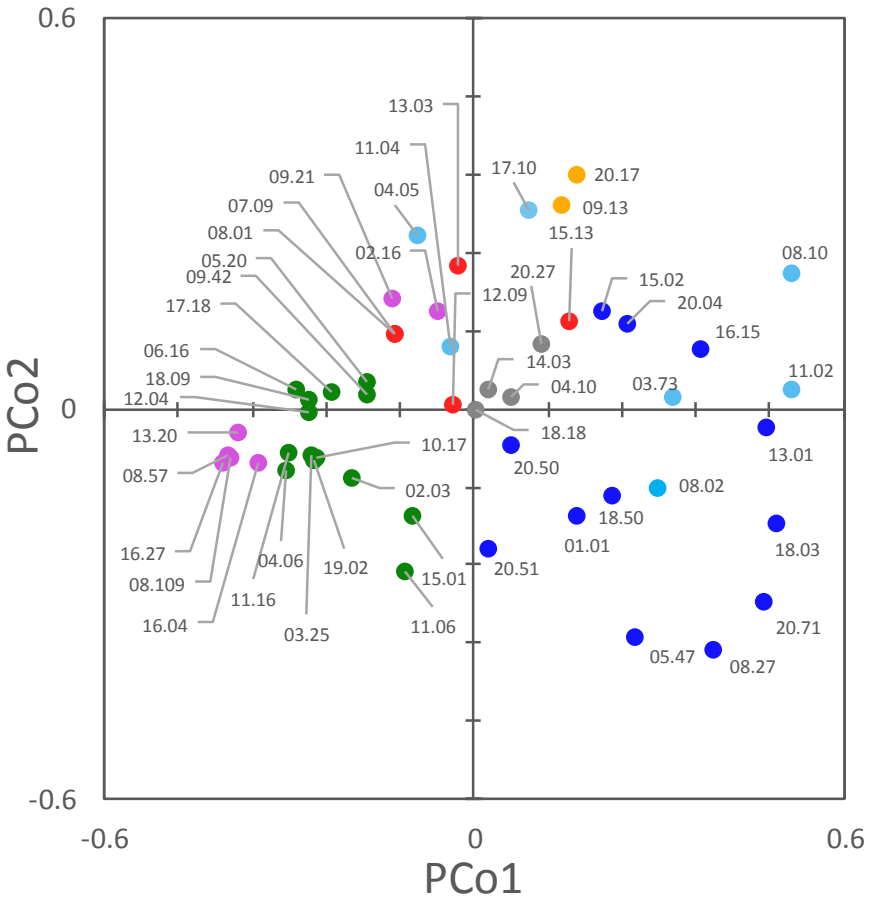

Figure 2.19 *2D Principal Coordinate Analysis (PCoA) visualization of E. coli strains, computed from the Dice's distance matrix of genetic and phenotypic determinants. Strains are colored based on their phylogroup: A, cyan; B1, blue; B2, green; C, yellow; D, red; E, grey; F, pink.*

2.5 Conclusions

A characterization of *E. coli* and NECE isolated from fecal samples of healthy subjects was performed. Several strains of *E. coli* had the potential to cause extraintestinal infections because of the presence of genes associated with adhesins, siderophores, and toxins. The recurrence of strains sharing a pattern of virulence genes similar to that of potentially pathogenic strains may pose a health threat. Conversely, exploration of NECE isolates from healthy subjects is still at its infancy, and deeper *in vivo* studies are necessary to understand the capability to became virulent induce infections.

A main outcome of this study shows the great sensitivity of the *E. coli* isolates to antibiotics, which are susceptible to the common antimicrobial therapy used for Gram-negative bacteria, such as amoxicillin/clavulanate. This suggests that the *E. coli* populations inhabiting the gut of healthy subjects and of patients are more likely differentiated on the basis of antibiotic resistance than virulence factors, emphasizing the importance of the prudent use of antibiotics in the general population. On the other hand, several NECE are resistant to amoxicillin plus clavulanic acid, but are sensitive to all the other tested antibiotics.

Interestingly, all the isolates obtained from a single sample, *E. coli* and NECE, were resistant to this couple of drugs, whereas in the others NECE belonging to diverse species were resistant, and *E. coli* not. As a whole, enterobacteriaceae from healthy subjects are still sensitive to most of the antibiotics, confirming that the problem of antibiotic resistance is restricted frequent and inappropriate use the antibiotics, and to the hospital setting where antibiotic pressure is highest.

3.0 *Clostridium ramosum* and obesity: isolation from mice and genotyping

3.1 INTRODUCTION

3.1.1 The role of intestinal microbiota in obesity

Obesity is a main world public health problem that affects more than 1.9 billion adults, with the 39% of adults being obese and overweighed (WHO, 2015). Obesity it is often associated with dyslipidemia and hypertension, impaired glucose homeostasis, and together all these conditions are known as metabolic syndrome (Alberti et al., 2009). Both adipogenic environment and genetic factors are the cause of obesity. Several exogenous factors may influence energy homeostasis, whereas genetic factors contribute only to a lesser extent to the metabolic syndrome (Wells, 2012). In western countries, the main determinants of obesity are the consumption of energy-dense foods in combination with the reduced physical activity and low energy requirement (Wells, 2012).

In the last decade, a lot of researches focused on the study of gut microbiota as an element that directly affects the health or disease status. It has been proposed by several studies that the composition of gut microbiota may play a crucial role in the onset of obesity (Baothman et al., 2016).

Generally, the human gut microbiota is composed by bacteria belonging to 5 major phyla: *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria,* and *Verrucomicrobia* (Qin et al., 2010). *Bacteroidetes* and *Firmicutes* represent approx. the 90% of the total bacterial community (Qin et al., 2010; Tang et al., 2017). Obese patients have a lower diversity and richness in gut bacterial composition than lean subjects (Ley et al., 2006; Graessler et al., 2013).

Both human (Ley et al., 2006; Furet et al., 2010; Kong et al., 2013) and mice studies (Ley et al., 2006; Turnbaugh et al., 2009) consistently indicated that the ratio between *Firmicutes* and *Bacteroidetes* was higher in obese than in lean subjects. A recent meta-analysis on overweight humans did not confirm the lower proportion of *Bacteroidetes*, and for this reason, it may not be universally true that differences in the gut microbiota composition reflect the

increases in body mass index (BMI) (Ley et al., 2006). Nevertheless, the gut microbiota can influence body weight (Ley et al., 2006; Karlsson et al., 2013). When fecal bacteria from obese woman were given to lean germ-free mice (GF), animals started to accumulate body fat and to develop metabolic problems usually associated with obesity (Tremaroli et al., 2015).

To clarify the role of gut microbiota in obesity development, several mechanisms have been proposed. However, despite the fact that a lot of evidences have been reported, there are still gaps in the knowledge on the true role of gut microbiota in obesity development (Tehrani et al., 2012; Wang et al., 2015). One possible mechanism is related to energy regulation and to the ability of bacteria to ferment undigestible dietary polysaccharides (dietary fibers), yielding short chain fatty acids (SCFA) (Khan et al., 2016). Once absorbed, SCFA can induce lipogenesis and increase the storage of triglyceride through molecular pathways. SCFA activate two proteins involved in the lipogenesis: the carbohydrate responsive element-binding protein (*ChREBP*) and the sterol regulatory element-binding transcription factor 1 (*SREBP1*). SCFA also suppress the fasting induced adipocyte factor (*FIAF*), that inhibits lipoprotein lipase (*LPL*) and induce triglycerides accumulation in host adipocytes (Khan et al., 2016). Moreover, the gut microbiota can decrease liver fatty acid oxidation, with the suppression of adenosine monophosphate kinase (*AMPk*) (Lopez et al., 2017). *AMPk*is a cellular energy marker in the liver and in the muscle and its inhibition increases body fat accumulation (Hardie et al., 2008). Gut microbiota may contribute to the onset of obesity by triggering systemic inflammation (Jana et al., 2017). Lipo-polysaccharides (LPS) and other gram-negative bacteria cell component are responsible of this effect due to its bind with *TLR4* (Cani et al., 2007). *TLRs* are well characterized immune transmembrane protein capable to upregulate inflammatory cytokines and chemokines, and activate intracellular signaling pathways to regulate the nature, magnitude, and duration of inflammatory response (Frost et al., 2002; Cani et al., 2007;). *TLRs* activation, triggered by LPS binding, induces the production of several pro-inflammatory, antiviral, and anti-bacterial cytokines (Soares et al., 2010).

3.1.2 Animal models as tools to investigate the role of gut bacteria in obesity development

Murine models have been widely used in biomedical research thanks to substantial similarities in anatomy, physiology, and genetics with the human biology. The knowledge of mouse genetics and the availability of numerous genetically modified mice models facilitate functional studies. Mouse models have been widely used also for their low maintenance cost,

high reproductive rates, and short life cycle. In the last decade, mouse models are being increasingly used in gut microbiota research, in particular for investigating the role and function of the gut microbiota and its relationship with diseases.

Mouse models have been widely used in biomedical studies because mice and humans are quite similar in physiology and anatomical structures. In both species the gastrointestinal tract is composed of organs are anatomically similar even if there are some notable differences which might be the reason of the diverging diets, body sizes, and metabolic necessities.

Murine models in gut microbiota research allow functional research on host-microbe interactions, in order to assess causality in disease-associated alterations in gut microbiota composition.

In the last years, the impact of diet on the gut microbiota composition has received an increasing attention in research. In particular, many studies focused on the impact of the 'Western' diet (high in simple carbohydrates and animal fats) on the incidence of metabolic disorders, such as obesity and diabetes, and the implications of gut microbiota (Wu et al., 2011; Zhang et al., 2012). Mouse models with diet-induced obesity or with genetic modifications are used for investigating the role that the diet or the genetic background on obesity development may have (Hariri and Thibault, 2010; Lutz and Woods 2012). Obesity in mice is usually assessed by measuring the gain in body weight or in body fat.

Figure 3.1 *ob/ob mouse compared to a lean mouse.*

Most of the studies use GF mice and knockout gene mice strains. To investigate pathologies like obesity and type 2 diabetes, *ob/ob* mouse strain is widely used. *ob/ob* mice have a homozygous spontaneous mutation for leptin gene, then leptin-deficient mice gain weight

rapidly and can reach three times the normal weight of wild-type controls. *ob/ob* mice exhibit hyperphagia, a diabetes-like syndrome of hyperglycemia, glucose intolerance, elevated plasma insulin, subfertility, impaired wound healing, and an increase in hormone production from both pituitary and adrenal glands. Furthermore, they are hypometabolic and hypothermic, with an obesity characterized by the increase of both adipocyte number and size (Figure 3.1) (Garris et al., 2004).

Polygenic mouse models are more representative of the polygenic obesity situation in humans (Lutz and Woods, 2012). Multiple genes are involved in the onset of obesity and obesity-associated type 2 diabetes in humans. The *New Zealand Obese* (NZO) mice represent a model of polygenic obesity and male sex-limited type 2 diabetes. They easily develop severe obesity even when fed with standard diet containing 4.5% fat. NZO mice are characterized by anti-insulin receptor antibodies, defection in leptin-transporters and hypertension (Jurgens et al., 2006).

Mice models commonly used to study high fed diet (HFD), induced obesity, and diabetes are the obesity-prone strain *C57BL/6J* and the strain *C3H*, that develops moderate obesity in response to HFD intervention (Fleissner et al.,2010; Poggi et al., 2007). The substrains *C3H/HeJ* and *C3H/HeOuJ* were created from *C3H* mouse. The former carries a mutation in the *TLR4* gene that leads to a defect in LPS sensitivity (Suganami et al., 2007). The latter, that does not carry the mutation present in *C3H/HeJ*, is an appropriate model to investigate the interaction among intestinal bacteria, obesity, and low-grade inflammation.

Bacteroidetes and *Firmicutes* are the two major phyla that colonize both human and mice gut microbiota (Eckburg et al., 2005; Ley et al., 2005; Ley et al., 2006). However, the 85% of bacterial genera found in the mouse gut are not present in human (Ley et al., 2005). Colonization of germ-free (GF) mice with human fecal samples can contribute to converge the mouse model into a more human-like model in which bacteria that determine obesity development can be studied properly (Turnbaugh et al., 2009).

The human gut microbiota harbors approximately 500 to 1000 bacterial species, with high complexity and inter-individual differences. For this reason, it is not that easy to investigate on host-microbe and microbe-microbe interactions. Gnotobiotic mice colonized with a defined number of bacterial strains can be used to investigate species-specific effects (Samuel and Gordon, 2006; Mahowald et al., 2009; Caesar et al, 2012). The use of humanized gnotobiotic mice resulting from the inoculation of human gut microbiota into germ-free mice, is a powerful research tool. An example of humanized gnotobiotic mice is the *SIHUMI "simplified and defined human intestinal microbiota"* model, in which seven bacterial strains belonging to the

species *Anaerostipes caccae, Bacteroides thetaiotaomicron, Bifidobacterium longum, Blautia producta, Clostridium ramosum, Escherichia coli* and *Lactobacillus plantarum* are inoculated in germ-free mice (Becker et al., 2011). The humanized gnotobiotic mouse model is widely employed in many studies because it allows perturbations in a 'human-like system' and it is considered the gold standard for confirming host-microbe and microbe-microbe interactions (Faith et al., 2011; Goodman et al., 2011; McNulty et al., 2011; Smith et al., 2013a).

3.1.3 *Clostridium ramosum*

For historical reasons, the genus divided *Clostridium* includes a large number of different bacteria whose only common features are the obligately anaerobic growth, Gram-positive type cell wall, the absence of sulphate reduction, and the ability to form endospores (Ludwig et al., 2009; Rainey et al., 2009; Parte, 2014). This resulted in a single genus encompassing more than 200 species with enormously different properties, including proteolytic and cellulolytic bacteria, and some pathogens (Parte, 2014). In 1994, Collins and colleagues divided *Clostridium* genus into 19 clusters with the use of 16S rRNA gene sequences. They roughly represented family-level taxa and each cluster included several proposed genera (Collins et al., 1994). In the last 20 years, many former divided *Clostridium* spp. have been reassigned to new genera, some moving to novel families or orders and even to novel classes such as Erysipelotrichia and Negativicutes (Ludwig et al., 2009; Marchandin et al., 2010). This is the case of *C. ramosum* that was reassigned to Erysipelotrichia class as *Erysipelatoclostridium ramosum*. However, most of these organisms still retained the '*Clostridium*' name (Rainey et al., 2009; Parte, 2014), resulting in a taxonomic and nomenclature conundrum, where the *Clostridium* genus designation did not necessarily indicate a close relationship to the type species.

Clostridium ramosum is an obligate anaerobic spore-forming bacillus, non-motile, indigenous to the human intestinal tract. It was firstly described by Veillon and Zuber in 1898, after isolation from a patient with appendicitis and pulmonary gangrene (Tally et al., 1974). Originally it was named *Bacillus ramosum*, then it was renamed *C. ramosum* in 1971 after being found to be a spore-forming anaerobe (Tally et al., 1974; Holdeman et al., 1977; Muakakassa et al., 1983). Within the clostridia there is a subgroup of organisms known as the "RIC group", from the initial letters of its species' names, encompassing *C. ramosum, C. innocuum,* and *C. clostridioforme*. This group has gram-stain variability, spores that are absent or difficult to see, and atypical clostridial colony morphology (Figure 3.2) (Alexander et al.,

1995). *C. ramosum* is a gut bacterium, it is isolated rarely as a pathogen and is generally considered non-pathogenic (Leal et al., 2008; Kannoet al., 2009; Becker et al., 2011).

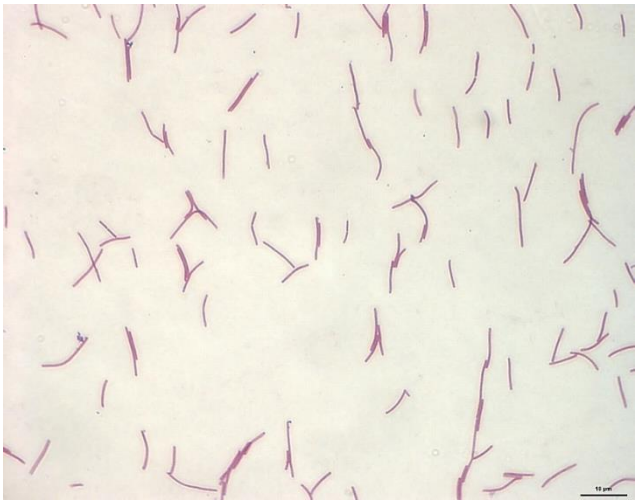

Figure 3.2 *C. ramosum DSM 1402, type strain.*

3.1.3.1 *Clostridium ramosum* implications in obesity research

In obese humans and mice, the concentration of intestinal *Firmicutes* has been reported to be higher compared to a lean status, with a reduction of intestinal *Bacteroidetes* (Ley et al., 2005, Ley et al., 2006). However, these findings are not consistent with other studies, where a significant variation in the *Firmicutes/ Bacteroidetes* ratio in obese subjects was not detected (Duncan et al., 2008, Schwiertz et al., 2010). A higher proportion of *Firmicutes* partially due to the proliferation of *Erysipelotrichia* in obese mice has been found (Fleissner et al., 2010). An increase of intestinal *Erysipelotrichia* was detected also in microbiota of obese human subjects and in obese mice fed with Western diet (Turnbaugh et al., 2008; Turnbaugh et al., 2009; Ferrer et al., 2013). A link between the presence of intestinal *Clostridium ramosum*, a member of the class *Erysipelotrichia*, and the metabolic syndrome symptoms in a type 2 diabetic women was found (Karlsson et al., 2013). The bloom of *Erysipelotrichia*, and in particular of *C. ramosum*, suggested that these bacteria may have a role in obesity development, even if the mechanisms behind evidence are not clear.

Gnotobiotic mice with a simplified human intestinal microbiota (SIHUMI) subjected to high fat diet (HFD) for four weeks presented an increased abundance of *C. ramosum* (Becker et al., 2011; Woting et al., 2014). Cholic acid boosted intestinal concentration of *Erysipelotrichi*, and likely enhanced the growth of *C. ramosum* in response to bile acids produced during HFD (Islam et al., 2011; Woting et al., 2014). After a four weeks HFD,

SIHUMI and monoassociated mice with *C. ramosum* (Cra) showed marked obesity symptoms compared to control mice (SIHUMI w/o *C. ramosum*). A significantly higher body weight gain in SIHUMI and Cra mice was detected, with an increased gene expression of glucose transporter 2 (Glut2) in jejunal mucosa and of fatty acid translocase (CD36) in ileal mucosa in the obese SIHUMI and Cra mice compared with the less obese SIHUMIw/oCra. These findings suggested that *C. ramosum* boosts intestinal glucose and lipid absorption when present in gut's mice (Woting et al., 2014).

C. ramosum seems involved in host peripheral serotonin (5-hydroxytryptamine: 5-HT) secretion and differentiation of colonic intestinal stem progenitors induction toward the secretory 5-HT-producing lineage (Mandić et al., 2018). In an *in vitro* model, elevated 5-HT levels regulate the expression of major proteins involved in intestinal fatty acid absorption. The same proteins were upregulated in monoassociated mice with *C. ramosum*, suggesting that the presence of this bacterium in the gut promotes 5-HT secretion and thereby could facilitates intestinal lipid absorption and development of obesity (Mandić et al., 2018). Gut microbiota could be the link to understand the interdependence between nutrition, serotonin signaling, and its effects on metabolic diseases such as obesity.

3.2 AIM OF THE PROJECT

From January to July 2018 I worked as a visiting student at the German Institute of Human Nutrition Institute (DIfE) in Potsdam, Germany, and in particular at the GAMI (Gastrointestinal Microbiology) Department under the supervision of Professor Michael Blaut and Dr. Anni Woting. The research at GAMI focused on *C. ramosum* and its putative role in obesity development. The presence of *C. ramosum* in mice strains prone to obesity and/or in mice subjected to a high-fat diet (HFD) was investigated. Aim of the project was to assess the presence of *C. ramosum* in feces or gut content of different mice included in research projects related to obesity and metabolic syndrome.

The main tasks of the research were:

- Isolation on PRGCP plates of *C. ramosum* from feces or gut content of obese mice of both sexes, different ages, belonging to *NZO, ob/ob, C3H/HeOuJ,* and *C57BL/6J* strains.
- Molecular genotyping of the isolates by RAPD-PCR and PFGE, the latter in collaboration with Dr. Karsten Tedin and Lisa Niemann, at Department of Veterinary Medicine at the Freie Universitàt Berlin.
- Sequencing of 16S rRNA genes of the different *C. ramosum* biotypes.
- Metabolic characterization of *C. ramosum* biotypes by API 20A kit and comparison with the type strain *C. ramosum* DSM 1402.
- Assessment of antibiotic sensitivity by disk diffusion assay.

3.3 MATERIAL AND METHODS

3.3.1 Mice involved in *C. ramosum* screening

The presence of *C. ramosum* in colonic and caecum content was assessed in samples obtained fom 31 C57BL/6J mice subject to high-fat diet (HFD). In particular, 8 of were fed with an intervention diet with fats accounting for the 45% of total nutrients (45% HFD); 7 were fed an intervention diet with fats accounting for the 60% of total nutrients (60% HFD); 16 mice were controls fed a low-fat diet (LFD) (Table 2). The presence of *C. ramosum* was investigated also in fecal samples of 13 C57BL/6J, 14 NZO, 20 *ob/ob*, and 14 C3H/HeOuJ mice subjected to LFD (Table 1).

ID	Strain	Sex	Weeks	Diet	Sample origin
1A	C57BL/6J	MALE	21	HFD 45%	colon+caecum
2A	C57BL/6J	MALE	21	HFD 45%	colon+caecum
3A	C57BL/6J	MALE	20	HFD 45%	colon+caecum
4A	C57BL/6J	MALE	21	HFD 45%	colon+caecum
5A	C57BL/6J	MALE	46	HFD 45%	colon+caecum
6A	C57BL/6J	MALE	49	HFD 45%	colon+caecum
7A	C57BL/6J	MALE	49	HFD 45%	colon+caecum
8A	C57BL/6J	MALE	81	HFD 45%	colon+caecum
9A	C57BL/6J	MALE	21	LFD	colon+caecum
10A	C57BL/6J	MALE	21	LFD	colon+caecum
11A	C57BL/6J	MALE	21	LFD	colon+caecum
12A	C57BL/6J	MALE	21	LFD	colon+caecum
13A	C57BL/6J	MALE	98	LFD	colon+caecum
14A	C57BL/6J	MALE	78	LFD	colon+caecum
15A	C57BL/6J	MALE	98	LFD	colon+caecum
16A	C57BL/6J	MALE	98	LFD	colon+caecum
17A	C57BL/6J	MALE	20	HFD 60%	colon+caecum
18A	C57BL/6J	MALE	21	HFD 60%	colon+caecum
19A	C57BL/6J	MALE	21	HFD 60%	colon+caecum
20A	C57BL/6J	MALE	21	HFD 60%	colon+caecum

ID	Strain	Sex	Weeks	Diet	Sample origin
21A	C57BL/6J	MALE	82	HFD 60%	colon+caecum
22A	C57BL/6J	MALE	98	HFD 60%	colon+caecum
23A	C57BL/6J	MALE	98	HFD 60%	colon+caecum
24A	C57BL/6J	MALE	20	LFD	colon+caecum
25A	C57BL/6J	MALE	21	LFD	colon+caecum
26A	C57BL/6J	MALE	21	LFD	colon+caecum
27A	C57BL/6J	MALE	21	LFD	colon+caecum
28A	C57BL/6J	MALE	102	LFD	colon+caecum
29A	C57BL/6J	MALE	99	LFD	colon+caecum
30A	C57BL/6J	MALE	82	LFD	colon+caecum
31A	C57BL/6J	MALE	28	LFD	colon+caecum
1_BL6	C57BL/6J	MALE	18	LFD	fecal
2_BL6	C57BL/6J	MALE	57	LFD	fecal
3_BL6	C57BL/6J	MALE	35	LFD	fecal
4_BL6	C57BL/6J	MALE	7	LFD	fecal
5_BL6	C57BL/6J	MALE	7	LFD	fecal
6_BL6	C57BL/6J	MALE	6	LFD	fecal
7_BL6	C57BL/6J	MALE	6	LFD	fecal
8_BL6	C57BL/6J	FEMALE	18	LFD	fecal
9_BL6	C57BL/6J	FEMALE	18	LFD	fecal
10_BL6	C57BL/6J	FEMALE	57	LFD	fecal
11_BL6	C57BL/6J	FEMALE	6	LFD	fecal
12_BL6	C57BL/6J	FEMALE	6	LFD	fecal
13_BL6	C57BL/6J	FEMALE	6	LFD	fecal
1_NZO	NZO	MALE	57	LFD	fecal
2_NZO	NZO	MALE	41	LFD	fecal
3_NZO	NZO	MALE	28	LFD	fecal
4_NZO	NZO	MALE	23	LFD	fecal
5_NZO	NZO	MALE	17	LFD	fecal
6_NZO	NZO	MALE	17	LFD	fecal
7_NZO	NZO	FEMALE	57	LFD	fecal
8_NZO	NZO	FEMALE	29	LFD	fecal
9_NZO	NZO	FEMALE	21	LFD	fecal
10_NZO	NZO	FEMALE	20	LFD	fecal
11_NZO	NZO	FEMALE	5	LFD	fecal
12_NZO	NZO	FEMALE	4	LFD	fecal
13_NZO	NZO	FEMALE	6	LFD	fecal
14_NZO	NZO	FEMALE	6	LFD	fecal
1_ob/ob	ob/ob	FEMALE	21	LFD	fecal
2_ob/ob	ob/ob	FEMALE	18	LFD	fecal
3_ob/ob	ob/ob	FEMALE	15	LFD	fecal
4_ob/ob	ob/ob	MALE	25	LFD	fecal
5_ob/ob	ob/ob	MALE	15	LFD	fecal
6_ob/ob	ob/ob	MALE	21	LFD	fecal

ID	Strain	Sex	Weeks	Diet	Sample origin
7_ob/ob	ob/ob	FEMALE	9	LFD	fecal
8_ob/ob	ob/ob	FEMALE	9	LFD	fecal
9_ob/ob	ob/ob	FEMALE	6	LFD	fecal
10_ob/ob	ob/ob	FEMALE	6	LFD	fecal
11_ob/ob	ob/ob	FEMALE	6	LFD	fecal
12_ob/ob	ob/ob	FEMALE	6	LFD	fecal
13_ob/ob	ob/ob	FEMALE	4	LFD	fecal
14_ob/ob	ob/ob	MALE	7	LFD	fecal
15_ob/ob	ob/ob	MALE	6	LFD	fecal
16_ob/ob	ob/ob	MALE	6	LFD	fecal
17_ob/ob	ob/ob	MALE	6	LFD	fecal
18_ob/ob	ob/ob	MALE	6	LFD	fecal
18_ob/ob	ob/ob	MALE	6	LFD	fecal
20_ob/ob	ob/ob	MALE	4	LFD	fecal
63_C3H	C3H/HeOuJ	MALE	6	LFD	fecal
64_C3H	C3H/HeOuJ	MALE	6	LFD	fecal
65_C3H	C3H/HeOuJ	MALE	6	LFD	fecal
66_C3H	C3H/HeOuJ	MALE	6	LFD	fecal
71_C3H	C3H/HeOuJ	FEMALE	6	LFD	fecal
72_C3H	C3H/HeOuJ	FEMALE	6	LFD	fecal
73_C3H	C3H/HeOuJ	FEMALE	6	LFD	fecal
74_C3H	C3H/HeOuJ	FEMALE	6	LFD	fecal
75_C3H	C3H/HeOuJ	MALE	21	LFD	fecal
76_C3H	C3H/HeOuJ	MALE	18	LFD	fecal
77_C3H	C3H/HeOuJ	MALE	15	LFD	fecal
78_C3H	C3H/HeOuJ	MALE	25	LFD	fecal
79_C3H	C3H/HeOuJ	MALE	15	LFD	fecal
80_C3H	C3H/HeOuJ	MALE	12	LFD	fecal
81_C3H	C3H/HeOuJ	FEMALE	12	LFD	fecal
82_C3H	C3H/HeOuJ	FEMALE	20	LFD	fecal

Table 3.1: *List of the mice included in the C. ramosum screening.*

3.3.2 Enumeration of total anerobic bacteria and *C. ramosum* isolation

Colonic and caecal samples were collected in sterile containers and stored in an anaerobic jar with the gas pack (GasPak^{TM} EZ Anaerobe Container System, DB), in order to preserve anaerobic bacteria, and processed within 1 h.

For fecal sample collection, mice were put individually in empty autoclaved cages, and allowed to defecate normally. Two fecal pellets were stored into an empty 1.5 mL eppendorf

tube with a sterile toothpick. The epperndorf tubes es were stored in an anaerobic jar and processed within 1 h.

Processing of the samples was performed with anaerobic media and buffers in aerobiosis, caring of working as fastest as possible. Samples were weighted and diluted 1:10 in anaerobic PBS buffer (8.5 g/L NaCl; 0.6 g/L Na_2HPO4; 0.3 g/L KH_2PO_4; 0.1 g/L Peptone (Oxoid, Bacto Peptone); 0.5 g/L Cystein; 1000 µL/L Resazurin; pH 7.0; N_2/CO_2 (80/20, v/v)).

In a 96 multiwell plates 450 µL of PBS buffer added with a 2.5% agar solution was poured in each well necessary for further serial dilutions. Meanwhile, after 10 minutes of vortexing, samples were centrifuged at 300 *g* for 1 minute at 4 °C. then, 50 µL of the supernatant was diluted from 10^{-2} to 10^{-11} in the multiwell plate with anaerobic PBS buffer added with a 2,5% agar solution previously prepared.

Dilutions from 10^{-6} to 10^{-11} were plated on Columbia blood agar (bioMérieux, Nürtingen, Germany). Dilution from 10^{-2} to 10^{-7} were plated on PRGCP selective agar media for *C. ramosum* (Propionate-rifampicin-gentamicin-colimycin-polymyxin medium; Senda et al., 1985), that has the following composition: Trypticase peptone 10 g/L; Proteose peptone No. 3 3 g/L; Meat extract 5 g/L; Yeast extract 2 g/L; Lactose 7 g/L; NaCl 5 g/L; KH_2PO_4 2 g/L; Na_2HPO_4 5 g/L; L-cysteine HCl-H_2O 0.3 g/L; Sodium thioglycollate [$C_2H_3O_2SNa$] 0.2 g/L; Tween 80 0.25 g/L; Agar 15 g/L; Sodium propionate 15 mg/L; Bromocresol green 10 mg/L; Bromoscresol purple 5 mg/L; pH at 7.00; 1 ml/L rifampicin 30 µg/ml; 1 ml/L amphotericin B 10 µg/ml; 1 ml/L gentamicin 30 µg/ml; 10 ml/L colistin sodium methanesulfonate 48 µg/ml; 1,95 ml/L polymyxin B 80 units/ml).

Agar plates were incubated at 37°C under strictly anoxic conditions into anaerobic workstation (DW Scientific - Modular Atmosphere Controlled System Anaerobic Workstation; Malmesbury, Wiltshire, UK) for 48 h.

3.3.3 Identification of *C. ramosum* colonies

3.3.3.1 Stereomicroscope identification

Single colonies isolated on PRGCP are observed under the stereomicroscope (Carl Zeiss Stemi 2000 Stereo Microscope) in order to exclude other Clostridia capable to grow on PRGCP selective medium. On PRGCP, *C. ramosum* colonies are recognized as translucent, greyish, with blue spot in the center.

3.3.3.2 Gram staining

Gram staining is a common differential staining procedure used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative bacteria by coloring these cells in violet and red/pink respectively.

Gram positive bacteria stain violet because of the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Converserly, Gram negative bacteria stain red/pink, which is attributed to a thinner peptidoglycan wall, that does not retain the crystal violet during the decoloring process.

Colonies isolated on PRGCP and putatively ascribed to *C. ramosum* were subjected to Gram staining and spore staining following this protocol (Gram, 1884):

- Put a drop of culture bacterial suspension on a microscope slide;
- Air dry the drop and then heat-fix by passing the slide over a bunsen flame two or three times.
- Cover the heat-fixed smear with crystal violet solution.
- Let stand for 3 minutes.
- Wash off crystal violet gently with distilled water for 10 seconds.
- Shake off excess water.
- Cover the smear with Gram's iodine solution for 3 min.
- Carefully decolorize with acetone/ethanol (50:50 v:v) for 10 seconds.
- Wash with 0.1% basic fuchsin solution for 10 seconds.
- Rinse with water and air dry.

3.3.3.3 Endospore staining

Endospore staining is a differential staining technique where the spore is stained in a manner so that it can be distinguished from the vegetative part of the cell.

In the Schaeffer-Fulton endospore staining method, a primary stain-malachite green is forced into the spore by steaming the bacterial emulsion. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells may be decolourized with water. Safranin is then applied to counterstain any cells that have been decolorized. At the end of the staining process, vegetative cells will be pink, and endospores will be dark green.

Colonies isolated on PRGCP and putatively ascribed to *C. ramosum* were subjected to Schaeffer-Fulton endospore staining technique following this protocol (Schaeffer and Fulton, 1933):

- Put a drop of culture bacterial suspension on a microscope slide with a drop of 10%-formaldehyde
- Air dry the drop for 1 h
- Overlay slide completely with 5% (wt/vol) aqueous malachite green solution
- Boil 20 sec and leave for further 30 sec air dry
- Rinse with distilled water
- Counterstain with safranin 2.5% (wt/vol) alcoholic solution for 1 min.
- Rinse with water and air dry.

3.3.3.4 Optical microscope observation

Stained slides of selected colonies after Gram and endospore staining were observed with Eclipse E600 microscope (Nikon, Düsseldorf, Germany) and Lucia software version 4.5.1 was used for taken pictures.

3.3.4 Sample storage

Selected strains putativeli ascribed to *C. ramosum* were maintained in YH-BHI (Yeast Hemine Brain Heart Infusion Broth; BHI 37 g/L; yeast extract 5 g/L; cysteine cysteine HCl-H2O 0,5 g/L; Hemine 10 mL/L; Resazurine 0,10 mL/L) liquid medium following the anoxic techniques of Hungate (1969) and Bryant (1972). For cryopreservation, 1 mL of grown culture was mixed with 0.3 mL of glycerol suspensions 30 %, v/v), then stored at −80 °C.

3.3.5 RAPD-PCR genotyping and taxonomic attribution

The *C. ramosum* putative strains were subjected to RAPD-PCR clustering and compared with the type strain *C. ramosum* DSM 1402. Genomic DNA was extracted with RTP Bacteria DNA Mini Kit 250 (Stratec Molecular; Berlin, Germany) following the protocol "Isolation of DNA from bacteria pellets ($1 \cdot 10^9$ bacteria cells). The RAPD-PCR amplification was performed in 50 µL of a mixture containing: Dream Taq Buffer 10X (Invitrogen w/o Mg), MgCl2 (50mM), 100 µM of M13-RAPD primer (5'-GAGGGTGGCGGTTCT-3'), 0.1 µM of each dNTP, 1 U Taq polymerase (x), and 50 ng of gDNA from the isolates.

The thermocycle was the following: 94 °C for 3 min; 45 cycles at 94 °C for 1 min, 34 °C for 1 min, and 72 °C for 2 min; and final elongation at 72°C for 7 min. The PCR products were separated in a 25 × 25 cm 2% (w/v) agarose gel electrophoresis for 1 h at 120 V in TAE buffer. RAPD-PCR fingerprints were digitally captured with the gel documentation imager G:box (Syngene, UK), then analyzed with Gene Directory 2.0 software (Syngene, UK), which calculated similarities and derived a dendrogram with an unweighted pair group method with arithmetic means (UPGMA).

3.3.6 16S rRNA sequencing

To attribute each biotype to the species, the 16S rRNA genes were amplified, sequenced, and compared with Genbank database by the use of BLASTn algorithm (www.ncbi.nlm.nih.gov/blast). Amplification was performed with 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') Universal primers (Miller et al., 2013) in 50 µL of reaction volume containing Dream Taq Buffer 10X (Invitrogen w/o Mg), $MgCl_2$ (50mM), 0.1 µM of each dNTP, 1 U Taq polymerase, and 50 ng of gDNA from the isolates.

PCR amplification was performed following thermocycler: heat lid at 110 °C, 4 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; an additional final 10 min cycle at 72 °C. 8 µL of PCR products were mixed with 1.6 µL of EZ-Vision® DNA dye (VWR Chemicals) and loaded into 1% agarose gel for the electrophoretic run at 80V for 40 minutes to check the presence of the amplicone. Quantification of the PCR product was performed using the Nanodrop. Purification of the amplicone was performed using the innuPREPDOUBLEpure Kit (Analytik Jena; Jena, Germany), following the protocol "purification and concentration of PCR products up to 50 µL". The PCR product at the concentration of 10 ng/ µL was send to Eurofins genomics for partial sequencing the 16S rRNA gene.

3.3.7 PFGE - Pulsed Field Gel Electrophoresis

C. ramosum biotypes previously identified by RAPD-PCR were genotyped by PFGE using the protocol developed by Dr. Lothar Wieler (Director Institute of Microbiology and Epizootics, Centre for Infection Medicine, Freie Universität, Berlin). The protocol is based on the PulseNet protocol for *C. botulinum*, with some specific changes for *Clostridium* genus (https://www.cdc.gov/pulsenet/pdf/c-botulinum-protocol-508c.pdf).

The strains were in Hungate tubes of YE-BHI medium overnight at 37°C under anoxic conditions. 150 µL of the overnight culture were centrifuged for 10 min at 16200 *g* at 4° C. The pellet was suspended in 150 µL of suspension buffer (10 mM Tris pH 7.2; 20 mM NaCl; 50 mM EDTA). Bacterial suspension was incubated at 37° for 10 minutes. 40 µL of lysozyme (40 mg/mL) and 4 µL of mutanolysin (5U/µL) were added to the cell suspension. The suspension was incubated in the in Thermomixer comfort (Eppendorf) at 37°C for 10 min. 150 µL of 1.1% melted agarose (SeaKem® Gold Agarose, Lonza) was added and the mixture was poured into plug molds. After solidification at 4 °C, the five plugs obtained were transferred into 2 mL eppendorf containing 500 mL Lysis buffer (10 mM Tris, pH 7,2; 50 mM EDTA, pH 8.0; 50 mM NaCl; 0.2% Deoxycholat; 0.5% Lauroylsarkosin). The sample was placed in the Thermomixer at 37 °C for 2 h with gentle shaking. The plugs were removed from the lysis buffer and placed into a new 2 mL Eppendorf containing 483 µL of proteinase K buffer and 17 µL of proteinase K. The sample was incubated for 30 min at 55°C with gentle shaking in the thermomixer. The plugs were washed three times with wash buffer (10 mM Tris pH 7.6; 0.1 mM EDTA) at room temperature for 30 minutes, then stored in this wash buffer at 4 °C.

The digestion of genomic DNA was performed with the restriction enzyme SmaI (CCC↓GGG) (Thermo Fisher Scientific) at the final concentration of 10 u/µl for 4 h at 30°C. The restriction fragments were separated in 1.1% agarose in 0.5 X TBE buffer using Chef-DR III Pulsed Field Electrophoresis System Biorad. The electrophoresis was carried out for 21 h at 14 °C, 6V/cm, with initial and final switching times of 0.5 s and 40 s, with an included angle of 120°. DNA of *S. aureus* NCTC 8325 digested with SmaI was used as reference.

3.3.8 Metabolic characterization

Metabolic characterization of the biotypes was performed with the API 20A (Biomerieux) according to the manufacturer's instructions:

- Grow the strains for 18-24 h in anaerobic conditions on blood agar plates.
- Using a swab, harvest all the cells growth on blood agar.
- Emulsify the cells into an ampules containing 4 mL of API 20 A Medium (Trypticase 5 g, Yeast extract 5 g, Sodium chloride 2.5 g, L-tryptophane 0.2 g, L-cystine 0.4 g, Hemin of porcine origin 0.005 g, Vitamin K 0.01 g, Sodium sulfite 0.1 g, Demineralized water,pH 7) rotating the swab and rubbing it against the side of the ampule. The final turbidity should be ≥ 3 McFarland units. Use immediately the suspension.
- Prepare an incubation box using the lid of the API 20 chamber and distribute about 5 ml of distilled water into the honeycombed wells of the tray to create a humid atmosphere.

- Record the strain references on the elongated flap of the tray.
- Remove an API 20 A strip from the packaging and place it in the incubation tray.
- Using a sterile pipette, inoculate the strip with the cell suspension.
- Place the lid on the tray and incubate for 24 hours (± 2 hours) at 36 °C ± 2 °C in anaerobiosis.

3.3.9 Kirby-Bauer Disk Diffusion Susceptibility Test Protocol

Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, according to Jan Hudzicki, American Society for Microbiology (2009), was performed on the biotypers of *C. ramosum* in order to test the susceptibility to 8 different antibiotics on blood agar plates (bioMérieux, Nürtingen, Germany), as recommended by the Clinical and Laboratory Standards Institute guidelines. The following antibiotic disks (Biomerieux) were tested: amikacin (AN) (30 µg), ciprofloxacin (CIP) (5 µg), erythromycin (E) (15 µg), imipenem (IPM) (10 µg), Kanamycin (K) (30 µg), penicillin G (P) (10 µg), piperacillin (PRL) (75 µg), vancomycin (VA) (30 µg).

- Use a sterile inoculating loop or needle, touch four or five isolated colonies of the organism to be tested.
- Suspend the organism in 2 mL of sterile saline.
- Vortex the saline tube to create a smooth suspension.
- Adjust the turbidity of this suspension to 0.5 McFarland standard units.
- Dip a sterile swab into the inoculum tube.
- Rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. The swab should not be dripping wet.
- Inoculate the dried surface of a Blood agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculum 4. Rim the plate with the swab to pick up any excess liquid.
- Allow the plate to sit at room temperature at least 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step.
- Place the appropriate antimicrobial-impregnated disks on the surface of the agar, using either forceps to dispense each antimicrobial disk one at a time, or a multidisk dispenser to dispense multiple disks at one time.

- Disks should not be placed closer than 24 mm (center to center). Each disk must be pressed down with forceps to ensure complete contact with the agar surface or irregular zone shapes may occur.
- Invert the plates and place them in a 37°C in anaerobic condition for 24 hours.
- Following incubation, measure the growth inhibition zone sizes around the antibiotic disk using a ruler; include the diameter of the disk in the measurement.
- For result interpretation use the published CLSI (Clinical and Laboratory Standards Institute) guidelines, about the susceptibility or resistance of the organism to each drug tested.
- For each drug, indicate on the recording sheet whether the zone size is susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart (Figure 3.3).

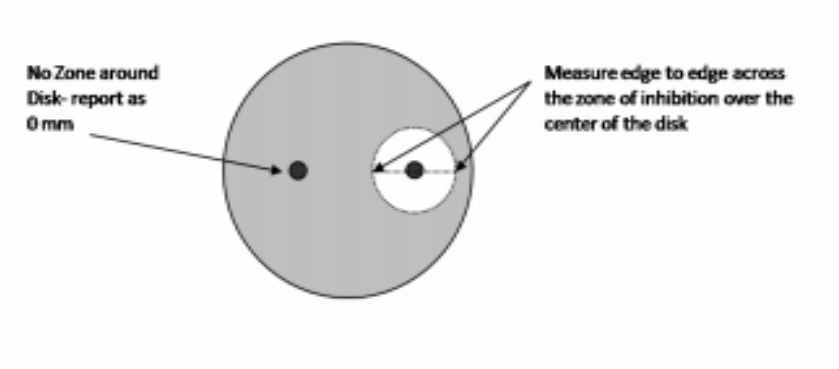

Figure 3.3 *Interpretation of the Kirby-Bauer Disk Diffusion Susceptibility Test.*

3.3.10 Statistical Analysis

Dice's coefficient was utilized to gauge the distance between *C. ramosum* biotypes, based on the sets of binary data, i.e., the presence/absence of genes or phenotypic properties. Distance matrices between strains were utilized to compute and display UPGMA dendrograms.

Hierarchical cluster based on the results of API 20A test and Kirby-Bauer Disk Diffusion Susceptibility Test of selected strains were carried out by Heatmapper tool (www2.heatmapper.ca/expression/) with average linkage criteria and Euclidean distance.

3.4 RESULTS AND DISCUSSION

3.4.1 Total concentration of anaerobic bacteria

From the mice involved in the research projects, samples of intestinal content from colon and caecum, and faces were collected and plated on blood agar in order to determine the concentration of total anaerobic bacteria. The average concentration of anaerobic bacteria among all samples were $4.9 \cdot 10^9$ CFU/g, with no statistical differences between the relative concentrations of the mice strains involved (Figure 3.4).

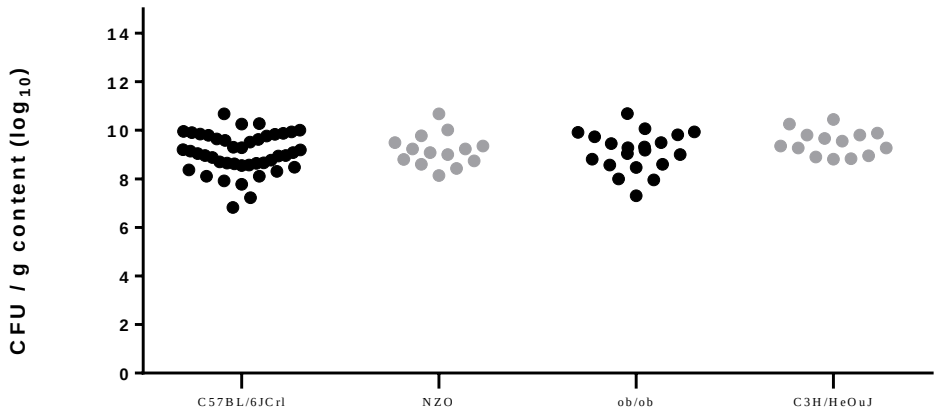

Figure 3.4 *Anaerobic bacteria concentration, estimated on blood agar, of samples from the different mice lineages. ANOVA test was performed, no statistical differences were observed.*

3.4.2 Isolation of *C. ramosum* on PRGCP selective agar media

From the selective agar media PRGCP it was possible to isolate *C. ramosum* colonies only for three mice out of 92. In particular, *C. ramosum* was isolated from both ceacal and colonic

content of a male, intermediate age, *C57BL/6J* mice subjected to a 45% HFD with the average concentration of $2.6 \cdot 10^4$ CFU/g. The strain was recorded as 3_COL.

A second strain was isolated from the fecal samples of a female, intermediate age, *NZO* subjected to LFD with the concentration of $1{,}1 \cdot 10^4$ CFU/mg (9_NZO). A third strain of *C. ramosum* from a male, old *C57BL/6J* mice subjected to LFD, at the concentration of $4{,}0 \cdot 10^3$ CFU/mg (3_BL6) (Figure 3.5).

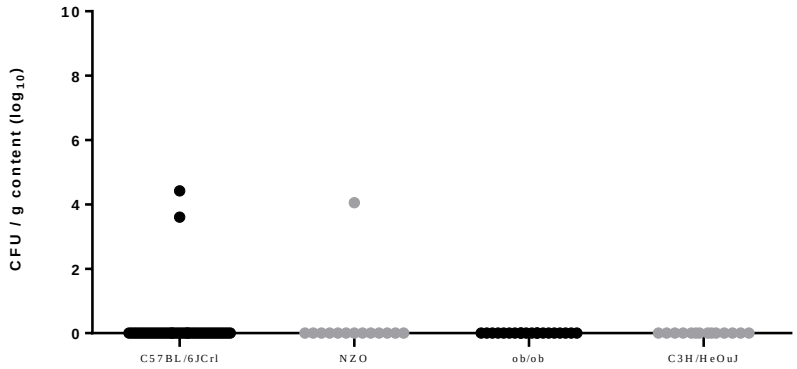

Figure 3.5: *Concentration of C. ramosum on PRGCP, estimated on blood agar, in samples from the different mice lineages.*

Against all odds, albeit the group of the animals were subjected to high-fat diet, were obese, or affected by type 2 diabetes, the isolation of *C. ramosum* was not so frequent as hypothesized. *C. ramosum* is a strict anaerobic bacterium, and for this reason colonic and caecal samples were rapidly collected in anaerobic jars after the animal killing, then provided rapidly to the laboratory where anoxic practices were respected. The same method was applied for fecal samples collection. The presence of anaerobic bacteria in the fecal samples at mean charge of $4.9 \cdot 10^9$ CFU/g, suggested that the anaerobic population was properly preserved.

3.4.3 Optical microscope observation

The *C. ramosum* strains were observed under the optical microscope and compared to the type strain *C. ramosum* DSM 1402. 3_COL, 3_BL6, and 9_NZO were similar to *C. ramosum* DSM 1402 type strain: thin, long Gram-negative/Gram-variable rods according to literature (Lavigne et al., 2003). Spore staining of 3_COL, 3_BL6, 9_NZO, and *C. ramosum* DSM 1402 type stran did not reveal spores (Figure 3.6).

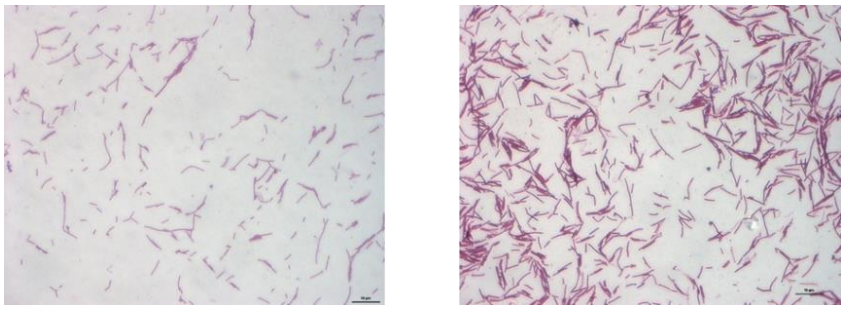

Figure 3.6 *Gram stain (left) and spore stain (right) of 3_COL sample.*

3.4.4 RAPD-PCR genotyping and taxonomic attribution

The three selected strains were analyzed through RAPD-PCR fingerprinting in order to confirm the presence of different biotypes, that were taxonomically characterized by partial sequencing of 16S rRNA genes to confirm the attribution to the species *C. ramosum*. The strains presented a strain-specific RAPD-PCR pattern (Figure 3.7; Figure 3.8) and the 16S rRNA genes presented a 99% identity to *C. ramosum.*

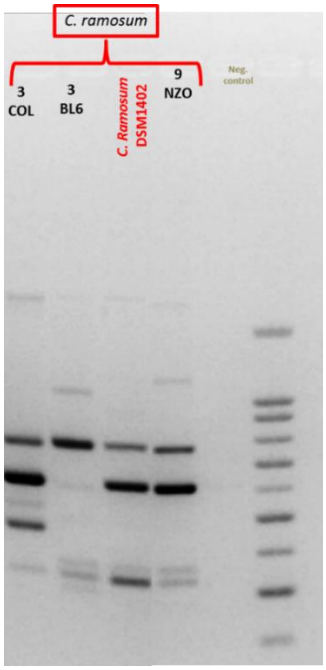

Figure 3.7: *RAPD-PCR fingerprint of the C. ramosum strains.*

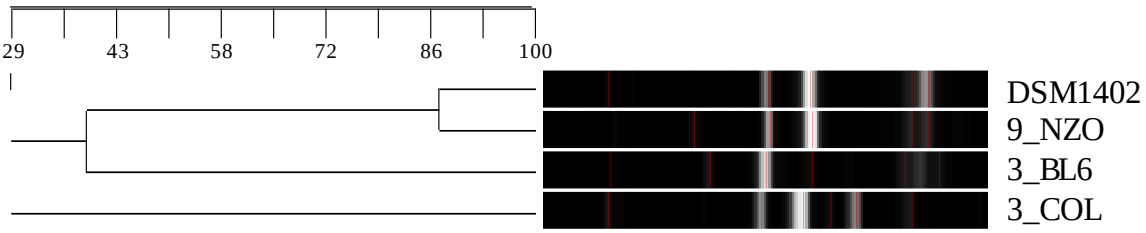

Figure 3.8: *RAPD-PCR pattern of C. ramosum strains: a UPGMA dendrogram derived from Dice's coefficients, calculated on the basis of the band profiles.*

3.4.5 PFGE

The *C. ramosum* strains 3_COL, 3_BL6, 9_NZO were typed by PFGE according to a protocol developed by Prof. Dr. Lothar Wieler (Director Institute of Microbiology and Epizootics, Centre for Infection Medicine, Freie Universität, Berlin), with some changes specific for *Clostridium* genus, based on the PulseNet protocol for *Clostridium botulinum*.

C. ramosum strains 9NZO, 3COL, and 3_BL6 presented unique and different PFGE fingerprinting band patterns, different from that of the type strain (Figure 3.8; Figure 3.10).

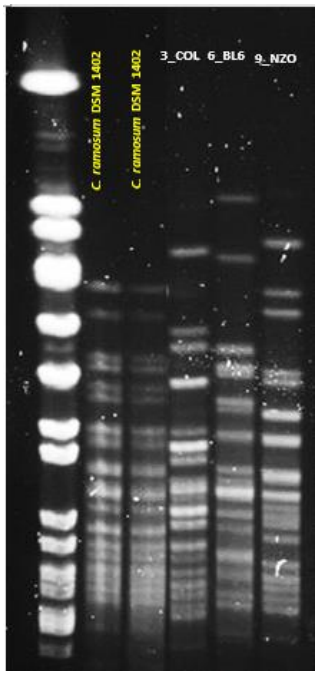

Figure 3.9 *PFGE of of C. ramosum type strain DSM 1402 and of C. ramosum strains 3_COL, 6_BL6 and 9_NZO. S. aureus NCTC 8325 has benn used as a molecular size marker*.

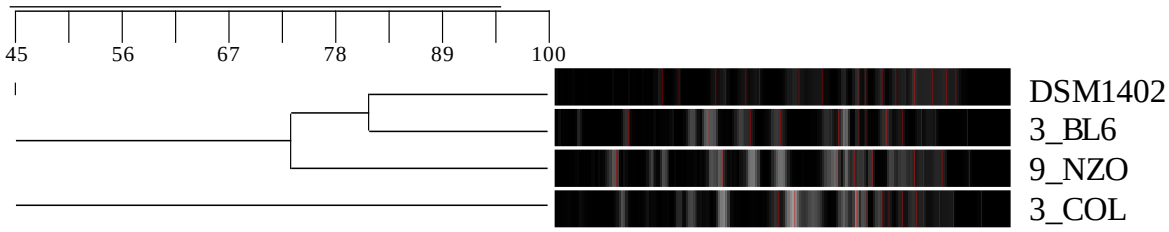

Figure 3.10 *XbaI-PFGE pattern of C. ramosum strains: a UPGMA dendrogram derived from Dice's coefficients, calculated based on their band profiles.*

The three endogenous strains of *C. ramosum* presented strain-specific RAPD-PCR and PFGE patterns. The combination of the outcome of both techniques suggested that *C. ramosum* 3_COL that presents minor similarity with the type strain and the other two isolates. On the other hand, 3_BL6 and 9_NZO have a high degree of similarity with the type strain *C. ramosum* DSM 1402.

3.4.6 Phenotypic characteristics of the isolated *C. ramosum* strains

API 20A was performed on the *C. ramosum* strains isolated from mice in order to find metabolic differences between them and the type strain *C. ramosum* DSM 1402. The type strain was able to grow on D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D- cellobiose, D-mannose, D-raffinose, D-trehalose, and on esculin ferric citrate. *C. ramosum* 9_NZO, 3_COL, and 3_BL6 were able to grow on the same componds as *C. ramosum* type strain, but 3_COL and 3_BL6grew also on L-rhamnose.

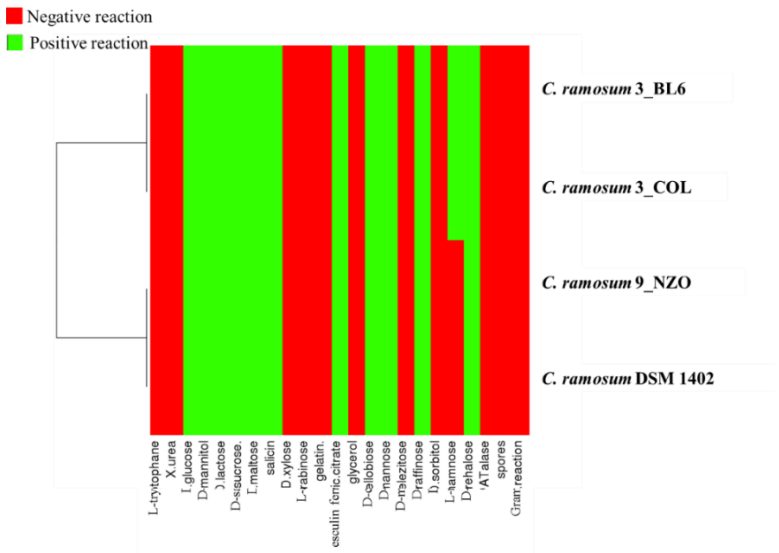

Table 3.2 *API 20A results obtained with the use of Heatmapper tool (www2.heatmapper.ca/expression/) with average linkage criteria and Euclidean distance.*

The hierarchical cluster based on average linkage criteria and Euclidean distances created with Heatmapper tool (www2.heatmapper.ca/expression/) revealed the presence of two distinct clusters: one encompassing the endogenous strains *C. ramosum* 3_BL6 and 3_COL that can hydrolize L-rhamnose, and the other including the endogenous *C. ramosum* 9_NZO and the type strain *C. ramosum* DSM 1402 (Table 3.2).

3.4.7 Antibiotic resistance screening

C. ramosum DSM 1402 and *C. ramosum* 9_NZO, 3_COL, and 3_BL6 were tested for antimicrobial susceptibility. All the new isolates were resistant to amikacin, ciprofloxacin, kanamycin, and penicillin G. Moreover, *C. ramosum* type strain and the other three endogenous strains shared an intermediate behaviour towards erythromycin. The hierarchical cluster obtained with Heatmapper tool based on average linkage criteria and Euclidean distances recognized two clusters not corresponding to the API20A ones. *C. ramosum* 9_NZO and 3_COL shared the whole pattern of antibiotic response, apart from Piperacillin that resulted completely active against the first, whereas required a higher concentration to be active against the second, due to its intermediate behaviour.

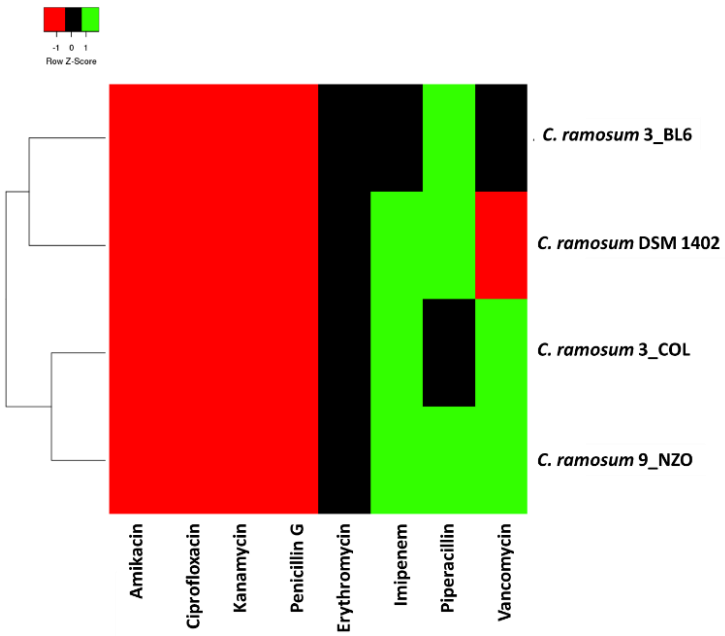

Table 3.3 *Atibiotic resistance pattern obtained with the use of Heatmapper tool (www2.heatmapper.ca/expression/), with average linkage criteria and Euclidean distance.*

The second cluster encompassed *C. ramosum* type strain and 3_BL6, albeit they differed for Imipenem and Vancomycin response. The type strain was susceptible to Imipenem, while *C. ramosum* 3_BL6 showed an intermediate sensitivity. On the other hand, *C. ramosum* type strain was resistant toVancomycin, while 3_COL strain showed an intermediate response (Table 3.3)

3.5 CONCLUSIONS

C. ramosum has been claimed as a contributot to obesity development in germ-free mice subjected to high-fat diet by modulating serotonin availability in the intestinal epithelium (Mandic et al., 2018). For this reason, at Gastrointestinal Microbiology (GAMI) Department at the German Institute of Human Nutrition (DIfE) investigated the presence of endogenous *C. ramosum* strains in obesity prone mice strains in diverse research projects. A total of 92 mice from different research projects internal at DIfE were analysed in order to isolate *C. ramosus* and associate it to obesity. Isolation of *C. ramosum* from intestinal samples of a number of obese mice was quite rare, suggesting that this species probably is not a common inhabitant of their gut microbiota.

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5.0 REPORT ON THE ACTIVITIES

PhD SCHOOL OF AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIOTECHNOLOGIES

XXXII CYCLE

REPORT ON THE ACTIVITIES

PhD student: Lucia Righini

Tutor: Prof. Maddalena Rossi

I° year:

PARTICIPATION TO THE SCHOOL COURSES:

Date: 12/10/2017, 14:30-16:30 - 13/10/2017 10:00-12:00
Title/subject: "Biotechnology of fermented non-alcoholic beverages" prof. Maria Gullo

Date: 6/10/2017 15:00-17:00
Title/subject: "Animal models" prof. Gian Carlo Manicardi

Date: 6/10/2017 9:00-11:00
Title/subject: "Model plants II" prof. Justyna Anna Milc

Date: 3/10/2017 11:00-13:00 , 14:00 – 16:00
Title/subject: "Genetic improvement of food yeasts: current state, perspectives and limits" and "Sex-determination systems drive genetic diversity in yeasts" prof. Lisa Solieri

Date: 29/09/2017 9:00-11:00
Title/subject: "Model plants I" prof. Justyna Anna Milc

Date: 19/09/2017 11:00-13:00 , 14:00 – 16:00
Title/subject: "The model organism Saccharomyces cerevisiae: mitochondrial inheritance as case study" prof. Lisa Solieri

Date: 14/07/2017, 9:30-11:30
Title/subject: "Microbial collection" prof. Luciana de Vero

Date: 21/06/2017, 14:30-17:30 - 23/06/2017, 14:30-17:00 - 28/06/2017, 14:30-17:00

Title/subject: “Applications of multivariate analysis in the agri-food context” prof. Alessandro Ulrici

PARTICIPATION TO SYMPOSIA

Date: 3-7/07/2017
Location: Trieste
Title: ICGEB-ICTP-APCTP Workshop on “Systems Biology and Molecular Economy of Microbial Communities”

Date: 20-22/09/2017
Location: Bolzano
Title: XXII Workshop on “the Developments in the Italian PhD Research on Food Science Technology and Biotechnology”

II° year:

PARTICIPATION TO THE SCHOOL COURSES:

Date: 03/10/2018, 11:00-16:0
Title/subject: “Sex-determination systems drive genetic diversity in food yeasts” and “Application of Lactic acid bacteria in dairy food science: starter cultures and probiotic potential” prof. Lisa Solieri

Date: 20/09/2018 9:00-11:00 and 01/10/2018 14:00-16:00
Title/subject: “Food bioactive compounds"; prof. Davide Tagliazucchi

PERIOD OF STUDY ABROAD:

Where: German Institute of Human Nutrition (DIfE) Potsdam-Rehbrücke (Germany)
How long: six months, 15/01/2018 – 13/07/2018

III° year:

PARTICIPATION TO THE SCHOOL COURSES:

Date: 18/09/2019, 9:00-13:00
Title/subject: “Microbial Biotechnologies for Biorefineries”; dr. Alberto Amaretti

Date: 07/06/2019, 11:00-13:00
Title/subject: "Introduction to Mendeley: Theory and practice" dr. Kameni Leugoue

Date: 06/06/2019, 10:00-12:00

Title/subject: "Method set-up for the identification and quantification of food contaminants"; dr. Giuseppe Montevecchi

Date: 14, 15, 28 and 29 January 2019
Title/subject: "Scientific English"; prof. Adrian Wallwork

PUBLICATIONS:

"Profiling of Protein Degraders in Cultures of Human Gut Microbiota"
Amaretti A., Gozzoli C., Simone M., Raimondi S., Righini L., Pérez-Brocal V., García-López R., Moya A., Rossi M.
Front Microbiol. 10 (2019): 2614. 2019 Nov. doi: 10.3389/fmicb.2019.02614

"Antibiotic Resistance, Virulence Factors, Phenotyping, and Genotyping of *E. coli* Isolated from the Feces of Healthy Subjects"
Raimondi S., Righini L., Candeliere F., Musmeci E., Bonvicini F., Gentilomi G., Starčič Erjavec M., Amaretti A., Rossi M.
Microorganisms. 10;7(8). 2019 Aug. doi: 10.3390/microorganisms7080251.

"Longitudinal Survey of Fungi in the Human Gut: ITS Profiling, Phenotyping, and Colonization"
Raimondi S., Amaretti A., Gozzoli C., Simone M., Righini L., Candeliere F., Brun P., Ardizzoni A., Colombari B., Paulone S., Castagliuolo I., Cavalieri D., Blasi E., Rossi M., Peppoloni S.
Front Microbiol. 10;10:1575 2019 Jul. doi: 10.3389/fmicb.2019.01575.