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Applications of bacteriophages for the control of pathogenic *Escherichia coli*

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Nicola MANGIERI
R12028

Tutor: **Dr. Claudia PICOZZI**
Ph.D. Dean: **Prof. Ella PAGLIARINI**

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1 ABSTRACT

Some *Escherichia coli* are characterized by virulence factors that can cause disease in humans. With the spread of antibiotic resistance genes in *E. coli* as well, the researchers are studying different solutions. One of the most promising alternatives to control pathogenic bacteria is the application of bacteriophages.

The main purpose of this PhD work was to control pathogenic *E. coli* through the application of bacteriophages.

Twenty phages were isolated from feces, sewage, and bedding material from livestock. The viral particles have been shown not to carry genes that encode for Shiga-toxins and intimin and have been therefore used against Shiga toxin-producing *E. coli* (STEC). No STEC showed resistance to all phages, but some strains revealed weak sensitivity. Among the most effective phages and based on their different RAPD (Random Amplification of Polymorphic DNA), three (which were used to make the "cocktail"), were used at different multiplicity of infection (MOI = 0,1, 1, and 10). Significant differences ($p < 0,05$) were reported in the mean values of optical density compared with the control. The best performance was obtained with the highest MOI. Additionally, the phage cocktail was tested on fresh cucumbers. The results showed a reduction in pathogenic *E. coli* of 1,97–2,01 log CFU/g at 25ºC and of 1,16–2,01 log CFU/g at 4ºC during 24 h.

Bacteriophages alone or in a cocktail were used to prevent biofilm formation at 4 different MOI (1, 2, 10, 100). The crystal violet assay showed a 43,64% reduction in biofilm formation of the analyzed strains compared to the control. The most effective was a cocktail composed by six phages. This phage cocktail was also used in trials to remove already formed biofilm. The results showed no significant differences between the control and samples ($p<0,05$). The isolated phages were also used to control bacteria related to urinary tract infections (UTIs), after being screened for the presence of inducible prophage (17,43% have a prophage). The results indicated that at least one phage was

effective against 262 out of 270 bacteria. Sequencing and TEM images showed that the bacteriophages used belonged to the *Myoviridae* family; no pathogenesis or lysogenesis related genes were found in phage genomes.

Three STEC strains were studied for prophage release, by real time qPCR, after a stress related to cheese making process: addition of NaCl at 1, 1,5 and 2% w/v, lactic acid at 0,5, 1,5 and 3% (v/v), anaerobic condition, pasteurization, UV, and after exposure to ciprofloxacin, nalidixic acid and norfloxacin. Induction of the prophages showed that the addition of NaCl at 1,5 and 2% significantly increased the phages release compared to the control, while lactic acid addition at the three concentrations tested had a significant repressing effect on phage release.

In conclusion, the positive results obtained in this work in the control of pathogenic *E. coli*, prevention of biofilm formation, together with the genetic characteristic of phages, suggest that the isolated phages could be used to improve food safety.

1.1 Riassunto

Alcuni *Escherichia coli* sono caratterizzati dalla presenza di fattori di virulenza che possono causare malattie nell'uomo. Con la diffusione dei geni di antibiotico resistenza anche in *E. coli*, i ricercatori stanno cercando soluzioni differenti. Una delle alternative più promettenti per il controllo dei batteri patogeni è l'applicazione dei batteriofagi.

L'obbiettivo principale di questo lavoro di dottorato è stato quello di controllare *E. coli* patogeni attraverso l'applicazione di batteriofagi.

Venti batteriofagi sono stati isolati da feci, liquami e materiale da lettiera del bestiame. I virus non hanno mostrato la presenza di geni codificanti per le tossine Shiga e l'intimina e sono stati usati per il controllo degli *E. coli* verotossici (STEC). Nessuno STEC mostrava resistenza a tutti i fagi ma alcuni ceppi hanno mostrato solo debole sensibilità. Tra i fagi più efficaci e in base al loro differente profilo genetico RAPD (Random Amplification of Polymorphic DNA), tre fagi (che sono usati utilizzati per formare il "cocktail"), sono stati applicati a differenti molteplicità di infezione (MOI = 0,1, 1 e 10). Differenze significative ($p<0,05$) sono state riportate nei valori medi di densità ottica comparati con il controllo. Il miglior risultato è stato ottenuto con la MOI più alta. Inoltre, il cocktail fagico è stato testato su cetrioli freschi. I risultati mostravano una riduzione degli *E. coli* patogeni di 1,97–2,01 log CFU/g a 25ºC e di 1,16–2,01 log CFU/g a 4ºC dopo 24 ore.

I batteriofagi utilizzati singolarmente o in un cocktail a 4 diverse MOI (1, 2, 10, 100) sono stati applicati per prevenire la formazione di biofilm. L'esperimento crystal violetto mostrava una riduzione della formazione di biofilm del 43,64% dei ceppi analizzati rispetto al controllo. Il cocktail fagico è stato utilizzato anche in esperimenti per la rimozione di biofilm già formato. I risultati non mostravano una differenza significativa tra il controllo e i campioni trattati ($p<0,05$).

I fagi isolati sono stati utilizzati per il controllo dei batteri implicati nelle infezioni del tratto urinario (UTIs), dopo essere stati scrinati per la presenza

di batteriofagi temperati inducibili (il 17,43% mostrava la presenza di profagi). I risultati indicavano che almeno un fago era in grado di lisare 262 su 270 batteri testati. Il sequenziamento e le immagini ottenute con il TEM mostravano che i batteriofagi appartenevano alla famiglia dei *Myoviridae*; nessun gene legato alla patogenesi o alla lisogenia è stato trovato nei genomi fagici.

Tre ceppi STEC sono stati studiati per il rilascio dei profagi attraverso la real time qPCR, dopo uno stress legato alla produzione di formaggio come l'aggiunta di NaCl (1, 1,5 e 2% w/v), acido lattico (0,5, 1,5 e 3% v/v), anaerobiosi, pastorizzazione, UV, e dopo l'esposizione alla ciprofloxacina, acido nalixidico e norfloxacina. L'induzione dei profagi mostrava che l'aggiunta di NaCl al 1,5% e 2% aumentava significativamente il rilascio dei fagi rispetto al controllo, mentre l'aggiunta di acido lattico alle tre concentrazioni testate ha avuto un effetto significativamente repressivo nel rilascio dei profagi.

Concludendo, i risultati positivi ottenuti in questo lavoro nel controllo degli *E. coli* patogeni, nella prevenzione della formazione di biofilm, insieme alle caratteristiche genetiche dei fagi, suggeriscono che i batteriofagi isolati potrebbero essere utilizzati per implementare la sicurezza alimentare.

2. INTRODUCTION and LITERATURE REVIEW

2.1 *Escherichia coli*

Escherichia coli is Gram-negative, lactose-fermenting, facultatively anaerobic, rod shaped bacterium, about 1 μm in diameter by 2 μm in length belonging to the large family of *Enterobacteriaceae. E. coli* can be able to swim thanks to a set of (on average of four) rotating helical flagellar filaments (Berg, 2003), it can be attracted or repelled by monitoring the substrate through the guidance of chemoreceptors (Ravichandar *et al.,* 2017). It can grow at temperature between 15°C and 45ºC with an optimum of 37 ºC; and at pH between 5 and 9 with an optimum of 7. The main carbon source is D-glucose, but fermentation of a variety of other sources is also possible: L-Arabinose, Lactose, Maltose, D-Mannose, D-sorbitol and D-Xylose (Brenner *et al*., 2005). The main fermentation products are lactate, acetate, succinate, formate (subsequently converted to H_2 and CO_2) and ethanol (Willey *et al.,* 2014). *E. coli* is present in the human gastrointestinal tract mostly as a common and harmless member of the microbiota, but some strains can have a variety of virulence factors, causing a wide range of disease in humans (Croxen *et al.,* 2013). Most of these factors derives from mobile genetic elements, such as transposons, insertion sequences, bacteriophages and plasmids, which can be integrated in the chromosome or replicate within the cell (Kaper *et al*., 2004). Some combinations of virulence factors generate specific pathotypes that could be divided in intestinal or extraintestinal *E. coli*, colonizing various sites in human body (fig. 1). Intestinal *E. coli* can be divided in: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) which include Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC) (Yang *et al.,* 2017). Extraintestinal *E. coli*

(ExPEC) can be subdivided in: uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC).

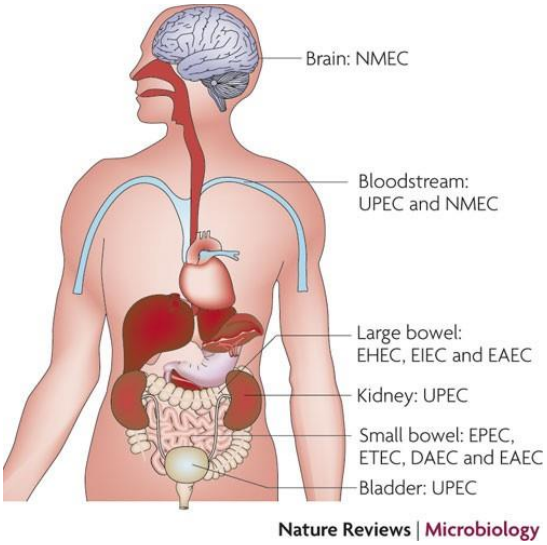

Figure 1. Sites of colonization in the human body of pathogenic E. coli (Croxen and Finlay, 2010).

2.1.1 Intestinal Pathogenic *E. coli*

2.1.1.1 Enterotoxigenic *E. coli* (ETEC)

These pathogenic bacteria are the main cause of traveller's diarrhoea and childhood diarrhoea in developing countries and in semitropical areas such as Latin America, South Asia and Africa. Food is the main source of contamination, but genetic risk factors are also involved (de la Cabada and Dupont, 2011). ETEC strains secrete two types of enterotoxins: heat-stable enterotoxins (STs), heat-labile enterotoxin (LT) or a combination. ST toxin is a low-molecular weight peptide that binds to guanylyl cyclase receptors in the small intestine, increasing intracellular levels of cyclic GMP, altering Na^+ adsorption, which causes the watery diarrhoea (Qadri *et al.,* 2005). LT toxin is an 86 kDa protein similar to cholera enterotoxin in function and

structures, both being AB_5-toxin. The ETEC strains release the LT toxin and by deregulating host adenylate cyclase they produce imbalance in the adsorption capacity of the intestinal cells causing diarrhoea; moreover, it improves the adhesion to the epithelial cells of the intestine (Wang *et al.,* 2012).

2.1.1.2 Enteropathogenic *E. coli* (EPEC)

EPEC strains are the primary pathogens involved in fatal diarrhoea in children (Croxen and Finlay, 2010); in recent years, it has become more widespread in developing than in developed countries (Croxen *et al.,* 2013). The main symptoms are watery diarrhoea, abdominal pain, nausea, vomiting, and fever (Yang *et al*., 2017). EPEC is a foodborne pathogen and humans are the main reservoir. These bacteria cause attaching and effacing (A/E) lesions in the intestinal mucosa. The lesions are characterized by bacterial intimate attachment to the enterocyte membrane and by effacement in the brush microvilli of the cell (Blanco *et al.,* 2006). The virulence genes responsible for A/E lesions are based on a chromosomal pathogenicity island called locus of enterocyte effacement (LEE), that includes *eae* (encoding intimin) gene (Blanco *et al.,* 2006).

2.1.1.3 Enterohaemorrhagic *E. coli* (EHEC) and Shiga toxin producing *E. coli* (STEC)

Enterohaemorrhagic *E. coli* (EHEC) are associated with haemorrhagic colitis (HC) and usually cause A/E lesions but also produce Shiga toxin (Stx; also known as verotoxin, VT) which can cause kidney damage resulting in haemolytic uremic syndrome (HUS). Strains that produce Stx are also referred to as STEC (Shiga-toxigenic *E. coli*) or VTEC (verotoxigenic *E. coli*) (Kaper *et al.,* 2004). In the 19th century, a toxin was discovered in *Shigella*

dysenteriae (initially called *Bacillus dysenteriae*) by Kiyoshi Shiga (Trofa *et al*., 1999). In 1983, a homologous toxin was detected in enterohaemorrhagic strains of *E. coli* (EHEC) and named Stx1 (Shiga-like toxin) to differentiate it from its counterpart in *Shigella dysenteriae* (Karmali *at al.,* 1983). Shiga toxin is an AB_5 protein composed by two subunits. As a first step, the B-subunit binds to the target glycolipid receptor (Gb3) and allows the toxin to enter the cells. In cells, the A-subunit is moved from Golgi apparatus and endoplasmic reticulum to the cytosol where it modifies the rRNA, inhibiting protein biosynthesis (Pezeshkian *et al.,* 2016). STEC infections are commonly associated with diarrhoea, gastrointestinal disease and in some cases can lead to severe infections such as haemorrhagic colitis and haemolytic uremic syndrome (HUS), characterized by acute renal failure, thrombocytopenia, microangiopathic haemolytic anaemia, neurological complications and mortality rate of 5% (Juillot and Römer, 2014). According to EFSA (2019), 8161 cases of STEC infections in humans were reported in Europe in 2018, up from the previous year (5958 cases in 2017). O26 (36,5%) was the most commonly reported serogroup among HUS cases followed by O157 (28,3%), O145 (7,6%), O80 (6,7%) and O111 (4,0%), while 4% of the involved isolates were untypable. Considering the general infections in humans caused by STEC, over 50% is represented by O157 and O26 serogroups. However, the relative proportion of O157 to non-O157 serogroups decreased. Cattle and other ruminants are considered the main reservoir of STEC and human infections generally result from the ingestion of contaminated food such as meat, milk and water (Gyles, 2007). Plant-based foods such as unpasteurized cider and apple juice, lettuce, cantaloupes, alfalfa sprouts and radish sprouts if contaminated with cattle manure can still be sources of infections in humans (Gyles, 2007).

In 2011, an unusual enteroaggregative O104:H4 STEC strain caused a large outbreak of gastroenteritis with 3816 cases and 22% of them developing into HUS, causing 54 deaths. The infections were due to the consumption of contaminated sprouts (Frank *et al*., 2011). After this outbreak in Germany, the

European Commission approved a new food safety criterion requiring the absence of STEC O157, O26, O111, O103, O145 and O104:H4 in 25 g of sprouted seeds (Regulation (EC) No 209/2013). STEC strains can also be regarded for antimicrobial resistance as reported by Mora *et al*. (2005) who tested 26 antimicrobial agents against 141 O157:H7 strains and 581 non-O157 strains. In two groups, 41% of the strains were resistant to at least one compound. In particular, 12% of non-O157 serotypes showed resistance to at least five antimicrobial agents.

2.1.1.4 Enteroinvasive *E. coli* (EIEC)

EIEC strains are closely related to *Shigella* spp, responsible for a disease called Shigellosis. This infection can cause invasive inflammatory colitis, occasionally dysentery and watery diarrhoea that is indistinguishable from infection caused by other pathogenic *E. coli* (Kaper *et al.,* 2004). In the colon, the EIEC cross the microfold cells (M cells) by transcytosis to the underlying submucosa, then bacterial cells replicate causing an inflammatory response and destruction of the intestinal epithelial barrier (Croxen and Finlay, 2010). This infection is more frequent in countries that have poor general sanitation, because the main route of transmission is the oral-fecal route. In developed countries, EIEC is mainly associated with travel-related infection (Pasqua *et al.,* 2017).

2.1.2 Extraintestinal *E. coli*

2.1.2.1 Uropathogenic *E. coli* (UPEC)

UPEC strains are the major causative agent of Urinary trait infection (UTIs) with several pathogenicity elements involved as fimbriae, pili, capsule, flagella, toxins, iron scavenger receptor and lipopolysaccharide acting in the urinary tract (Karam *et al.,* 2019). UTI is a very common disease: it has been estimated that 150 million people are affected by this infection every year

(Flores-Mireles *et al.,* 2015) and 40% of women develop UTI at least once in their life (Micali *et al*., 2014). The main symptoms are hematuria, dysuria, unpleasant odour and abdominal pain (Foxman, 2014). UTI is evidenced by a presence of over 10^5 UFC/ml in the urine and can be clinically subdivided in uncomplicated and complicated UTI, by the presence of structural or neurological urinary trait abnormalities (Zacché and Giarenis, 2016). *E. coli* is the main microorganism associated with this infection, being approximately 65-75% of community-acquired UTIs (Flores-Mireles *et al*., 2015); the other Gram-negative bacteria are *Klebsiella* spp, *Pseudomonas aeruginosa*, and *Proteus* spp, and the Gram-positive: *Streptococcus agalactiae* and *Staphylococcus saprophyticus* (Foxman, 2014). UPEC strains can be found in the intestine, periurethral area, vaginal cavity and urinary tract; the main route of transmission between individuals occurs via person-to-person contact, including sexual relations and fecal-oral route (Foxman, 2014). The infection begins with the colonization of the intestine by EPEC strains and, due to the presence of virulence factors encoded in LEE regions, it can colonize the periurethral area and descend from the urethra to the bladder where colonization occurs due the formation of pod-like bulges, containing bacteria enclosed in a polysaccharide-rich matrix surrounded by a shell of uroplakin (Anderson *et al.,* 2003). To control UPEC strains in human, antibiotics are widely used but increased antibiotic resistance in Gram-negative bacteria complicates the treatment of both complicated and uncomplicated UTIs (Nicolle, 2011).

2.1.2.2 Neonatal meningitis-associated *E. coli* (NMEC)

NMEC strains are the main responsible of a rare but devastating disease: neonatal meningitis, which has a 33% mortality rate and severe neurological complications in many of the survivors (Stoll *et al.,* 2011). The characteristics of these pathogens compared to commensal *E. coli* are not easily defined using phenotypic and genotyping methods; NMEC strains are known to be

able to survive in the blood, cross the brain barrier, and infect the meninges of infants, causing meningitis (Wijetunge *et al.,* 2015). The reservoir of this pathogen remains not completely clear. Tivendale and colleagues demonstrated that an avian-pathogenic *E. coli* was able to cause meningitis in a rat model of human disease (Tivendale *et al.,* 2010), this makes poultry a plausible reservoir of extraintestinal *E. coli* and a zoonotic risk for the transmission to humans (Ewers *et al.,* 2009; Meena *et al*., 2020).

2.1.2.3 Sepsis-causing *E. coli* (SEPEC)

Bacterial sepsis is a condition characterized by the presence of microorganisms in the bloodstream and if bacteria multiply, it progresses to septicaemia, resulting in infection of several organs with dysfunction, decreased perfusion, and hypotension (Smith *et al.,* 2007). The bacterium most frequently associated with this condition is *E. coli.* The origin could be linked to an infection in various sites of the body such as kidneys, bowel, skin or lungs (Smith *et al.,* 2007). Sepsis and meningitis are the main cause of neonatal deaths in developing countries (Vergnano *et al.,* 2005). SEPEC strains have several disease-associated virulence factor genes such as those that code for toxins: α-hemolysin (*hlyA*), cytolethal distending toxins (*cdt-I* to *cdt-V*) and cytotoxic necrotizing factor 1 (*cnf1*) (Čurová *et al*., 2014).

2.2 Bacteriophages and their applications

Bacteriophage or simply phage was first described by Frederick Twort and Félix d'Hérelle in 1915 and 1917, respectively. Phages are parasitic organisms that specifically target bacteria, being considered the most abundant and ubiquitous biological entity on earth (Dy *et al.,* 2014). D'Hérelle was the first to use bacteriophage as antimicrobial to treat dysentery in soldiers during World War I (Hausler, 2008). The first applications were enthusiastically received, but after the discovery of antibiotics and their wide application during World War ll, phage therapy was relegated to countries such as Soviet Union and Eastern Europe (Abedon *et al.,* 2011).

2.2.1 Bacteriophage biology

Bacteriophages have a simple structure. They consist of a protein capsid which often has icosahedral shape, containing nucleic acids. The phage genome could be double-stranded (ds) or single-stranded (ss) DNA or single-stranded RNA (Goodridge *et al*., 2003). The phage tail, which may be contractile, is connected to tail fibers, which contain the receptors for the attachment to bacteria; however, not all phages have a tail (Hanlon, 2007). The phage tail can recognize specific cell-surface-receptors such as lipopolysaccharide (LPS), membrane proteins (such as porins), pili, or flagella, making phages specific to their host (Dy *et al.,* 2014). Bacteriophages can be divided in virulent and temperate according to the type of life cycle: lytic or lysogenic, respectively (fig. 2). In the lytic cycle, the bacteriophage injects the DNA into the bacterial host and replicates, taking control of the host's molecular machinery. Then, phages lyse the host with the production of two types of protein: holines and lysins. The first works to perforate the

bacterial cytoplasmic membrane and synergize with the lysins, giving access to bacterial peptidoglycan; the lysins instead destroy the bacterial cell wall (Cisek *et al.,* 2017; Kakasis and Panitsa, 2019).

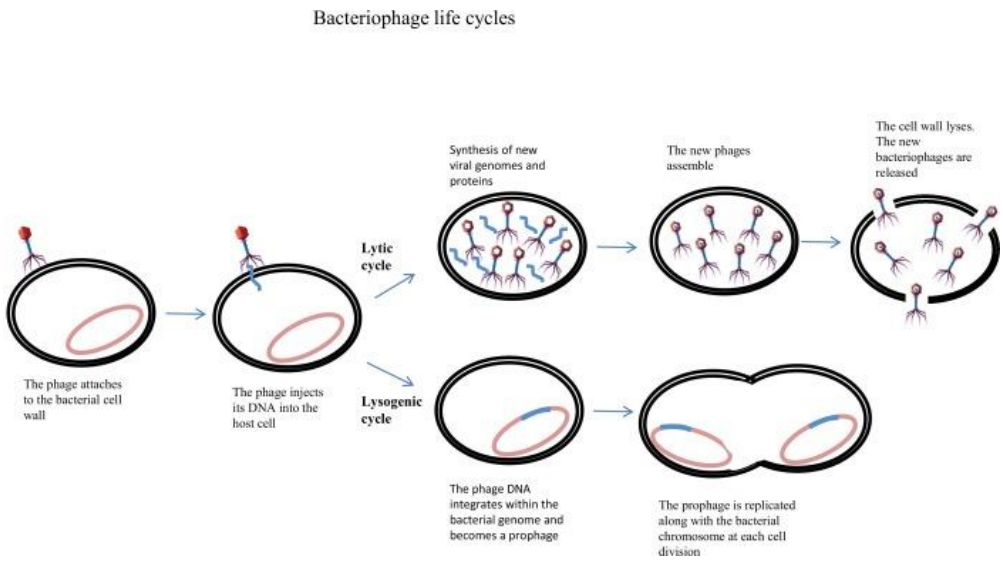

Figure 2. Life cycle of bacteriophage (Kakasis and Panitsa, 2019).

In the lysogenic cycle the first part is identical; but then the phage DNA integrates into the bacterial genome and replicates along with generations until an environmental stress induces the passage to a lytic cycle, killing the bacterial host (Lin *et al.,* 2017).

The division between lysogenic or lytic cycle is a key point in the application of phages as antimicrobials. Indeed, bacteriophages characterized by lysogenic cycle, called temperate phages, are involved in gene transmission among bacteria representing a serious risk for the diffusion of virulence or antibiotic genes (Cisek *et al.,* 2017). For example, the horizontal transmission of Shiga toxin genes, which is the principal virulence factor in STEC strains, is under the control of phages. Temperate *stx* phages after stress or spontaneously are free to infect new bacteria leading the emergence of STEC pathogens (Rahaman *et al.,* 2018).

2.2.2 Bacteriophage applications

In recent years, the application of bacteriophage to control pathogenic bacteria, first used almost a century ago, is undergoing a new renaissance driven by the emergence of multidrug resistant bacteria (Gordillo Altamirano and Barr*,* 2019). In 2016, the United Nations General Assembly discussed the problem of antibiotic resistance, considering it the greatest global risk, and the use of bacteriophage as a suitable candidate for the control of pathogenic bacteria (Lin *et al.,* 2017). Thus, the application of bacteriophage and their derivatives, among alternatives to antibiotics, seems to have the potential to be more successful (Czaplewski *et al.,* 2016). An advantage is that it could be used in small doses because phages are able to replicate in presence of their host. Furthermore, it is possible to apply engineered modification to bacteriophages in order to improve the efficacy spectrum and a rapid target elimination (Czaplewski *et al.,* 2016). It could be possible to apply bacteriophage derivatives like endolysin that hydrolyses the peptidoglycan of Gram-positive bacteria and by the addition of some others component, like Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), which improves membrane permeability, can also be used for Gram-negative pathogens (Walmagh *et al.,* 2012), or through modification of endolysin derived peptide against multidrug-resistant bacteria (Peng and Yuan, 2018).
Recently, the application of phages to control pathogenic bacteria is being displayed in different fields such as medicine, food safety, veterinary, phytopathogens, surface disinfection and much more (Ofir and Sorek, 2018). Food products can be treated with bacteriophage suspensions to control the number of viable target bacteria. There are currently many phage products on the USA, New Zealand and Australia markets formulated against several food pathogens, such as *L. monocytogenes, Salmonella* spp. and *E. coli* O157:H7. In 2016, EFSA issued a positive opinion on the safety of Listex™ P100, a preparation containing phage P100, to be applied to ready-to-eat

products such as deli meats, frankfurters, soft cheeses, etc. for the control of *L. monocytogenes.* This preparation was not considered a risk to human health due to the lack of toxicity in rats and the strictly lytic cycle of phage, being unable to transduce bacterial DNA (EFSA, 2016). Bacteriophage is considered as natural, environmental-friendly (since most preparation contain only phage isolated from environment without the addition of chemicals), and low-cost technology to safeguard food from bacterial contamination, but is addressed by many consumers concerns (Moye *et al.,* 2018).

In general, according to the common daily ingestion of bacteriophages normally present in water and food, these viral particles are considered safe by Food and Drug Administration (US Food and Drug Administration, 2006). The general application of bacteriophages to control pathogenic and spoilage bacteria in food was reviewed by EFSA in 2009, resulting in a positive evaluation (EFSA, 2009). However, such application has not yet been authorized in the European Union.

2.3 References

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., Kutter, E. M., 2011. Phage treatment of human infections. Bacteriophage, 1(2), 66-85. https://doi.org/10.4161/bact.1.2.15845

Anderson, G. G., Palermo, J. J., Schilling, J. D., Roth, R., Heuser, J., Hultgren, S. J., 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science, 301(5629), 105-107. doi: 10.1126/science.1084550

Berg, H. C., 2003. The rotary motor of bacterial flagella. Annu. Rev. Biochem. 72, 19–54. DOI: 10.1146/annurev.biochem.72.121801.161737

Blanco, M., Blanco, J. E., Dahbi, G., Mora, A., Alonso, M. P., Varela, G., Pilar, M., Schelotto, F., Blanco, J., 2006. Typing of intimin (*eae*) genes from enteropathogenic *Escherichia coli* (EPEC) isolated from children with diarrhoea in Montevideo, Uruguay: identification of two novel intimin variants (μB and ξR/β2B). Journal of medical microbiology, 55(9), 1165-1174. https://doi.org/10.1099/jmm.0.46518-0

Brenner, D. J., Krieg, N. R., Staley, J. T., Garrity, G. M., Brenner, D. J., Vos, P. De, Noel, R., 2005. Bergey's Manual of Systematic Bacteriology (2nd ed.). https://doi.org/10.1007/0-387-28022-7

Cisek, A. A., Dąbrowska, I., Gregorczyk, K. P., Wyżewski, Z., 2017. Phage therapy in bacterial infections treatment: one hundred years after the discovery of bacteriophages. Current microbiology, 74(2), 277-283. DOI 10.1007/s00284-016-1166-x

Croxen, M. A., Finlay, B., 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat. Rev. Microbiol. 8, 26–38. https://doi.org/10.1038/nrmicro2265

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., Finlay, B. B., 2013. Recent Advances in Understanding Enteric Pathogenic *Escherichia coli*. Clinical Microbiology Reviews, 26(4), 822–880. https://doi.org/10.1128/cmr.00022-13

Curová, K., Kmet'ová, M., Vargová, R., Lovayová, V., Siegfried, L., 2014. Toxins of extraintestinal *Escherichia coli* isolated from blood culture. Clin Microbiol. 2014; 3:1–4, doi: 10.4172/2327-5073.1000171

Czaplewski, L., Bax, R., Clokie, M., Dawson, M., Fairhead, H., Fischetti, V. A., Foster, S., Gilmore, B. F., Hancock, R. E. W., Harper, D., Henderson, I. R., Hilpert, K., Jones, B. V., Kadioglu, A., Knowles, D., Ólafsdóttir, S., Payne, D., Projan, S., Shaunak, S., Silverman, J., Thomas, C. M., Trust, T. T., Warn, P., Rex, J.H., 2016. Alternatives to antibiotics-a pipeline portfolio review. Lancet Infect. Dis. 16, 239–251. doi: 10.1016/s1473-3099(15)00466-1

De la Cabada Bauche, J., DuPont, H. L., 2011. New developments in traveler's diarrhea. Gastroenterology & hepatology, 7(2), 88-95.

Dy, R. L., Richter, C., Salmond, G. P., Fineran, P.C., 2014. Remarkable mechanisms in microbes to resist phage infections. Annual review of virology, 1, 307-331. https://doi.org/10.1146/annurev-virology-031413-085500

European Food Safety Authority (EFSA), 2009. Scientific opinion of the Panel on Biological Hazards on a request from European Commission on the use the use and mode of action of bacteriophages in food production. EFSA Journal 2009 1076, 1-26. DOI: 10.2903/j.efsa.2009.1076

EFSA and ECDC, 2019. The European Union One Health 2018 Zoonoses Report. EFSA Journal, 17(12), 5926. https://doi.org/10.2903/j.efsa.2019.5926

European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA), 2016. Multi-country outbreak of Shiga toxin-producing *Escherichia coli* infection associated with haemolytic uraemic syndrome. Stockholm: ECDC. Available from: http://ecdc.europa. eu/en/publications/Publications/RRA-Escherichia-coli-O26- Romania-Italy-April2016.pdf

Ewers, C., Antão, E. M., Diehl, I., Philipp, H. C., Wieler, L. H., 2009. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Applied and environmental microbiology, *75*(1), 184-192. doi: 10.1128/AEM.01324-08

Flores-Mireles, A. L., Walker, J. N., Caparon, M., Hultgren, S. J., 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat. Rev. Microbiol. 13, 269–284. doi: 10.1038/nrmicro3432

Frank, C., Werber, D., Cramer, J. P., Askar, M., Faber, M., an der Heiden, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M.J., Follin, P., Müller, L., King, L.A., Rosner, B., Buchholz, U., Stark, K., Krause, G., 2011. Epidemic Profile of Shiga-Toxin–Producing *Escherichia coli* O104:H4 Outbreak in Germany. New England Journal of Medicine, 365(19), 1771–1780. https://doi.org/10.1056/nejmoa1106483

Foxman, B., 2014. Urinary Tract Infection Syndromes Occurrence, Recurrence, Bacteriology, Risk Factors, and Disease Burden. Infect Dis Clin N Am 28 (2014) 1–13 http://dx.doi.org/10.1016/j.idc.2013.09.003

Goodridge, L., Gallaccio, A., Griffiths, M. W., 2003. Morphological, host range, and genetic characterization of two coliphages. Applied and environmental microbiology, 69(9), 5364-5371. doi: 10.1128/AEM.69.9.5364-5371.2003

Gordillo Altamirano, F. L., Barr, J. J., 2019. Phage therapy in the post antibiotic era. Clin. Microbiol. Rev. 32, e00066–e00018. doi: 10.1128/CMR.00066-18

Gyles, C. L., 2007. Shiga toxin-producing *Escherichia coli*: An overview. Journal of Animal Science, 85 (suppl_13), E45–E62. https://doi.org/10.2527/jas.2006-508

Hanlon, G. W., 2007. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. International Journal of Antimicrobial Agents, 30(2), 118–128. https://doi.org/10.1016/j.ijantimicag.2007.04.006

Hausler, T., 2008. ‘‘Viruses vs. Superbugs’’. MacMillan, New York.

Juillot S., Römer W., 2014. Shiga toxins. S. Morabito (Ed.), Pathogenic *Escherichia coli*: Molecular and Cellular Microbiology, 978-1-908230-37-9, Caister Academic Press, Wymondham, pp 79-101.

Kakasis, A., Panitsa, G., 2019. Bacteriophage therapy as an alternative treatment for human infections. A comprehensive review. International journal of antimicrobial agents, 53(1), 16-21. https://doi.org/10.1016/j.ijantimicag.2018.09.004

Kaper, J. B., Nataro, J. P., Mobley, H. L., 2004. Pathogenic *Escherichia coli*. Nature reviews microbiology, 2(2), 123-140. doi:10.1038/nrmicro818

Karam, M. R. A., Habibi, M., Bouzari, S., 2019. Urinary tract infection: Pathogenicity, antibiotic resistance and development of effective vaccines against Uropathogenic *Escherichia coli*. Molecular immunology, 108, 56-67. https://doi.org/10.1016/j.molimm.2019.02.007

Karmali, M., Petric, M., Steele, B., Lim, C., 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. The Lancet, 321(8325), 619-620. doi: 10.1016/s0140-6736(83)91795-6.

Lin, D. M., Koskella, B., Lin, H. C., 2017. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World journal of gastrointestinal pharmacology and therapeutics, 8(3), 162–173. https://doi.org/10.4292/wjgpt.v8.i3.162

Meena, P. R., Tejavath, K. K., Singh, A. P., 2020. Poultry-origin Extraintestinal *E. coli* strains carrying the Traits Associated with Urinary Tract Infection, Sepsis, Meningitis, and Avian Colibacillosis in India. Journal of Applied Microbiology. doi: 10.1111/jam.14905

Micali, S., Isgro, G., Bianchi, G., Miceli, N., Calapai, G., Navarra, M., 2014. Cranberry and recurrent cystitis: more than marketing? Crit. Rev. Food Sci. Nutr. 54, 1063–1075. doi: 10.1080/10408398.2011.625574

Mora, A., Blanco, J. E., Blanco, M., Alonso, M. P., Dhabi, G., Echeita, A., Gonzáles, E. A., Bernández, M. I., Blanco, J., 2005. Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in Spain. Research in Microbiology, 156(7), 793–806. https://doi.org/10.1016/j.resmic.2005.03.006

Moye, Z. D., Woolston, J., Sulakvelidze, A., 2018. Bacteriophage applications for food production and processing. Viruses, 10(4), 1–22. https://doi.org/10.3390/v10040205

Nicolle, L.E., 2011. Update in adult urinary tract infection. Curr. Infect. Dis. Rep. 2011; 13:552-560. doi: 10.1007/s11908-011-0212-x.

Ofir, G., Sorek, R., 2018. Contemporary phage biology: from classic models to new insights. Cell, 172(6), 1260-1270. https://doi.org/10.1016/j.cell.2017.10.045

Pasqua, M., Michelacci, V., Di Martino, M.L., Tozzoli, R., Grossi, M., Colonna, B., Morabito, S., Prosseda, G., 2017. The Intriguing Evolutionary Journey of Enteroinvasive *E. coli* (EIEC) toward Pathogenicity.
Front. Microbiol. 8:2390. doi: 10.3389/fmicb.2017.02390

Peng, Q., Yuan, Y., 2018. Characterization of a novel phage infecting the pathogenic multidrug-resistant *Bacillus cereus* and functional analysis of its endolysin. Applied microbiology and biotechnology, 102(18), 7901-7912.

Pezeshkian, W., Hansen, A. G., Johannes, L., Khandelia, H., Shillcock, J. C., Kumar, P. S., Ipsen, J. H., 2016. Membrane invagination induced by Shiga toxin B-subunit: from molecular structure to tube formation. Soft matter, 12(23), 5164-5171. doi: 10.1039/C6SM00464D

Qadri, F., Svennerholm, A.-M., Faruque, A. S. G., Sack, R. B., 2005. Enterotoxigenic *Escherichia coli* in Developing Countries: Epidemiology, Microbiology, Clinical Features, Treatment, and Prevention. Clinical Microbiology Reviews, 18(3), 465–483. https://doi.org/10.1128/cmr.18.3.465-483.2005

Rahman, M., Nabi, A., Asadulghani, M., Faruque, S. M., Islam. M. A., 2018. Toxigenic properties and stx phage characterization of *Escherichia coli* O157 isolated from animal sources in a developing country setting. BMC Microbiol. 18, 98. https://doi.org/10.1186/s12866-018-1235-3

Ravichandar, J. D., Bower, A. G., Julius, A. A., Collins, C. H., 2017. Transcriptional control of motility enables directional movement of *Escherichia coli* in a signal gradient. Sci. Rep. 7**,** 8959. https://doi.org/10.1038/s41598-017-08870-6

Smith, J. L., 2007. Extraintestinal pathogenic *Escherichia coli*. Foodborne Pathogen Disease. 4(2):134-63. doi: 10.1089/fpd.2007.0087.

Stoll, B.J., Hansen, N.I., Sanchez, P.J., Faix, R.G., Poindexter, B.B., Van Meurs, K.P., Bizzarro, M.J., Goldberg R.N., Frantz, I.D., Hale, E.C., Shankaran, S., Kennedy, K., Carlo, W.A., Watterberg, K.L., Bell, E.F., Walsh, M.C., Schibler, K., Laptook, A.R., Shane, A.L., Schrag, S. J., Das, A., Higgins, R.D., 2011. Early onset neonatal sepsis: the burden of group B streptococcal and *E. coli* disease continues. Pediatrics. 127(5):817–26. DOI: https://doi.org/10.1542/peds.2010-2217

Tivendale, K. A., Logue, C. M., Kariyawasam, S., Jordan, D., Hussein, A., Li, G., Wannemuehler, Y., Nolan, L. K., 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. Infection and immunity, 78(8), 3412-3419. doi: 10.1128/IAI.00347-10

Trofa, A. F., Ueno-Olsen, H., Oiwa, R., Yoshikawa, M., 1999. Dr. Kiyoshi Shiga: Discoverer of the *Dysentery Bacillus*. Clinical Infectious Diseases, 29(5), 1303–1306. https://doi.org/10.1086/313437

US Food and Drug Administration, 2006. Food Additives Permitted for Direct Addition to Food for Human Consumption; Bacteriophage Preparation. Retrieved from https://www.govinfo.gov/content/pkg/FR-2006-08-18/pdf/E6-13621.pdf

Vergnano, S., Sharland, M., Kazembe, P., Mwansambo, C., Heath, P. T., 2005. Neonatal sepsis: an international perspective. Archives of Disease in Childhood-Fetal and Neonatal Edition, 90(3), F220-FF224. http://dx.doi.org/10.1136/adc.2002.022863

Walmagh, M., Briers, Y., Dos Santos, S. B., Azeredo, J., Lavigne, R., 2012. Characterization of modular bacteriophage endolysins from *Myoviridae* phages OBP, 201φ2-1 and PVP-SE1. PLoS One, 7(5), e36991. https://doi.org/10.1371/journal.pone.0036991

Wang, X., Gao, X., Hardwidge, P.R., 2012. Heat-labile enterotoxin-induced activation of NF-kappaB and MAPK pathways in intestinal epithelial cells impacts enterotoxigenic *Escherichia coli* (ETEC) adherence. Cell Microbiol., 14:1231–1241. doi: 10.1111/j.1462-5822.2012.01793.x

Wijetunge, D. S. S., Gongati, S., DebRoy, C., Kim, K. S., Couraud, P. O., Romero, I. A., Weksler, B, Kariyawasam, S., 2015. Characterizing the pathotype of neonatal meningitis causing *Escherichia coli* (NMEC). BMC microbiology, 15(1), 211. https://doi.org/10.1186/s12866-015-0547-9

Willey M., J., M. Sherwood, L., Christopher, J. W., 2014. Prescott's Microbiology (9th ed.). McGraw-Hill, New York.

Yang, S.C., Lin, C.H., Aljuffali, I.A., Fang, J.Y., 2017. Current pathogenic *Escherichia coli* foodborne outbreak cases and therapy development. Arch Microbiol 199, 811–825. https://doi.org/10.1007/s00203-017-1393-y

Zacchè, M. M., Giarenis, I., 2016. Therapies in early development for the treatment of urinary tract inflammation. Expert Opinion on Investigational Drugs, 25(5), 531-540. https://doi.org/10.1517/13543784.2016.1161024

3. AIMS and OBJECTIVES

The general aims of this project were the isolation and characterization of new bacteriophages that can be used to control pathogenic *E. coli* and the study of the role of prophages released by *E. coli* in horizontal gene transmission.
To achieve these aims, several goals were pursued:

- The isolation of new bacteriophages from cattle farms in Lombardy and their molecular characterization; the application of phages for the control of Shiga toxin-producing *E. coli* (STEC) in plate test, in liquid media and in a challenge test on fresh cucumbers.

- The prevention of biofilm formation by applying the most effective bacteriophages alone or in cocktails and the reduction of already formed biofilm, both formed by STEC strains, with application of phage cocktails.

- The control of a large collection of Uropathogenic *E. coli* through the application of bacteriophages and the sequencing of phage whole genomes, in order to ensure the safety of their application.

- The study of the release of temperate bacteriophages by STEC strains under different stressors related to cheese making process and to different antibiotics.

4. RESULTS CHAPTER(s):

4.1 Evaluation of a Potential Bacteriophage Cocktail for the Control of Shiga-Toxin Producing *Escherichia coli* in Food

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4.1.1 Abstract

Shiga-toxin producing *Escherichia coli* (STEC) are important foodborne pathogens involved in gastrointestinal diseases. Furthermore, the recurrent use of antibiotics to treat different bacterial infections in animals has increased the spread of antibiotic-resistant bacteria, including *E. coli*, in foods of animal origin. The use of bacteriophages for the control of these microorganisms is therefore regarded as a valid alternative, especially considering the numerous advantages (high specificity, self-replicating, self-limiting, harmless to humans, animals, and plants). This study aimed to isolate bacteriophages active on STEC strains and to set up a suspension of viral particles that can be potentially used to control STEC food contamination. Thirty-one STEC of different serogroups (O26; O157; O111; O113; O145; O23, O76, O86, O91, O103, O104, O121, O128, and O139) were investigated for their antibiotic resistance profile and sensitivity to phage attack. Ten percent of strains exhibited a high multi-resistance profile, whereas ampicillin was the most effective antibiotic by inhibiting 65% of tested bacteria. On the other side, a total of 20 phages were isolated from feces, sewage, and bedding material of cattle. The viral particles proved not to carry genes codifying Shiga-toxins and intimin. No STEC was resistant to all phages, although some strains revealed weak sensitivity by forming turbid plaques. Three different bacteriophages (forming the “cocktail”) were selected considering their different RAPD (Random Amplification of Polymorphic DNA) profiles and the absence of virulence-encoding genes and antibiotic-resistance genes. The lytic ability against STEC strains was investigated at different multiplicity of infection (MOI = 0,1, 1, and 10). Significant differences ($p < 0,05$) among mean values of optical density were observed by comparing results of experiments at different MOI and controls. An effective reduction of bacterial population was obtained in 81% of cases, with top performance when the highest MOI was applied. The efficacy of the phage cocktail was tested on fresh cucumbers. Results showed a reduction in pathogenic *E. coli*

by 1,97–2,01 log CFU/g at 25°C and by 1,16–2,01 log CFU/g at 4°C during 24 h, suggesting that the formulated cocktail could have the potential to be used in bio controlling STEC different serogroups.

4.1.2 Introduction

Certain strains of *Escherichia coli*, a bacterium that is naturally resident in the human gut, can cause gastrointestinal diseases, bloody diarrhea that can develop in complex illnesses as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (Kaper *et al*., 2004). These strains, characterized by the production of Shiga toxins and often abbreviated as STEC (Shiga toxin-producing *E. coli*), have ruminants as major reservoir. The most common route of transmission to humans is via undercooked contaminated meats or fresh dairy products from raw milk (Karmali *et al*., 2010). The last European Union One Health 2018 Zoonoses Report (EFSA and ECDC, 2019) indicated STEC infections in humans as the third most commonly reported zoonosis in the EU with a notification rate increased by 39,0% compared with 2017. Serotype O157:H7 is still the most common one related to human illness, but non-O157 strains, and in particular O26, O103, and O91, are increasing in importance (Croxen *et al*., 2013; EFSA and ECDC, 2019). With these data in mind, it is easy to understand the importance to improve techniques for the control of STEC for food safety and consumer protection. New approaches such as radiation, high pressure, pulsed electric field, and ultrasound are quite expensive and sometimes can not be applied to fresh and ready-to eat products. Instead, the use of bacteriophage for reducing microbial pathogens in food is well established (Moye *et al*., 2018) and in 2009 the European Food Safety Authority (EFSA) reported that bacteriophages can be very effective in the elimination of pathogens from meat, milk, and dairy products (EFSA, 2009). The benefit of using bacteriophages as biocontrol instruments far outweigh the drawbacks. In fact, phages are highly active and specific; harmless to humans, animals and plants; mostly able to resist to food processing stressors; self-replicating and self-limiting. Furthermore, bacteriophages are abundant in food indicating that phages can be found in the same environment of their bacterial host and daily ingested by humans and animals providing evidence of no harmful effects (McCallin *et al*., 2013).

Finally, they do not affect texture, taste, smell, and color of food and they have proved to extend shelf life and in some cases, they showed to lyse the host cells even at temperatures as low as 1°C (Greer, 1988).

According to literature, the principal efforts of using bacteriophages against STEC strains have been directed mainly toward serogroup O157 (O'Flynn *et al.*, 2004; Abuladze *et al*.,2008; Sharma *et al*., 2009; Viazis *et al*., 2011; Hudson *et al*., 2015; Snyder *et al*., 2016). However, given the increase in the finding of non-O157 in food, different authors have focused their efforts also toward other serogroups (Tomat *et al*., 2013; Tolen *et al*., 2018; Liao *et al.*, 2019).

The aim of this research study is to obtain a phage suspension that can be used against the major number of STEC strains as possible (O157 and non-O157). Ideally, this preparation could be implemented to different stages of production, from disinfection of equipment and contact surfaces (biosanitation) to treatment of raw products and RTE foods (biocontrol).

4.1.3 Materials and methods

4.1.3.1 Bacterial strains and growth conditions

Escherichia coli strains provided from different institutes (ATCC; Istituto di Ispezione degli Alimenti di Origine Animale, Milan, Italy; Collaborative Centre for Reference and Research on *Escherichia* (WHO); Statens Serum Institut (SSI), Denmark; Istituto Superiore di Sanità, Rome, Italy) or collected in previous studies (Picozzi *et al*., 2017) were used in this work (Table 1). Strains were isolated from human stool, raw goat milk and milking filters. Bacterial cultures were grown aerobically in Luria-Bertani (LB) broth medium (5 g L^{-1}NaCl, 5 g L^{-1}yeast extract, 10 g L^{-1}Tryptone) at 37 °C. LB agar plates were prepared with LB broth supplemented with 1,5% or 0,5% Agar (EMD Chemicals, San Diego, CA) for plating bacteria or phage plaque testing respectively.

4.1.3.2 Antibiotic resistance assay

The resistance of STEC strains to antimicrobial compounds was tested by disk diffusion susceptibility test (Matuschek *et al*., 2014). STEC strains were cultivated in LB broth at 37°C until they reached a concentration of about 5×10^8 cells/mL. Cultures were streaked onto the surface of a LB agar plate, using a sterile cotton swab in three different directions. Sterile paper disks (6 mm in diameter) were applied onto the surface of the plate and spotted with six different antibiotics: ampicillin (AMP 10 µg), chloramphenicol (CHF 30 µg), ciprofloxacin (CIP 5 µg), nalidixic acid (NAL 30 µg), norfloxacin (NOR 10 µg) (Sigma-Aldrich, St. Louis, USA) and amoxicillin-clavulanic acid (AMC 20 µg) (So. Se. Pharm Srl, Pomezia, Italy). For each isolate, the Multiple Antibiotic Resistance (MAR) index, defined as a/b, where a represents the number of antibiotics to which the isolate was resistant, and b represents the number of antibiotics to which the isolate was exposed, was calculated (Krumperman,

1983). Intermediate test results (partial sensitivity) were considered as negative (sensitive). Since chloramphenicol was dissolved in 50% (v/v) ethanol, a disk containing only 50% (v/v) ethanol and no antibiotic was also added as a negative control, together with a disk with sterile water. After incubation overnight at 37°C the diameters of the inhibition zones (mm) were measured and then interpreted as susceptible, intermediate or resistant according to the EUCAST clinical breakpoints (EUCAST, 2019).

4.1.3.3 Phage isolation and purification

In order to isolate bacteriophages, twenty-two samples were collected from three breeding farms in the area of Milan. Approximately 100 g of feces, bedding material or sewage from cattle and sheep were sampled in sterile 200 mL cup and stored at 4°C until processing. Bacteriophages were isolated as previously described with slightly modification (Megha *et al*., 2017). Briefly, 8 g of each sample were mixed with 1 mL of LB broth and 1 mL of a culture of indicator *E. coli* strain (CNCTC 6896 or CNCTC 6246) in exponential phase (OD_{600}= 0,2-0,3). The suspension was incubated overnight at 37°C with shaking (120 rpm) and then centrifuged at 13000 *g* for 10 min at 4°C (Rotina 380 R, Hettich, Tuttlingen, Germany). The supernatant was filtered through a 0,45 µm pore size cellulose acetate syringe filter (Sartorius, Göttingen, Germany).

The crude filtrate was analyzed for the presence of phages *via* spot-test. Five mL of LB soft agar (0,5%) supplemented with $CaCl_2$ 0,01 M were mixed with 100 µL of exponential-phase culture *E. coli* strains CNCTC 6896 or CNCTC 6246 and poured on LB bottom agar (1,5%) to create a double layer. Then, 10 µL of each filtrate were spotted onto the agar surface and plates were incubated overnight at 37°C. A clear zone of bacterial lysis denoted the presence of phages.

The supernatants containing phages were then decimally diluted in LB broth, 100 µL of which were mixed with 100 µL of exponential-phase of *E. coli* indicator culture and incubated at 37°C for 15 min. After incubation, mixtures were suspended in 5 mL of melted LB soft agar (0,5% w/w agar) supplemented with 0,01 M $CaCl_2$ and poured onto LB bottom agar (1,5% w/w agar). Plates were then incubated overnight at 37°C. Well-separated plaques were picked up with a sterile Pasteur-pipette, transferred to a tube containing 100 µL of exponential-phase of indicator *E. coli* culture along with 10 mL of LB broth supplemented with $CaCl_2$ and incubated overnight at 37°C. Afterwards, samples were centrifuged at 6700 *g* for 10 min and filtered through a 0,45 µm pore size cellulose acetate syringe filter (Sartorius). The filtered suspensions were stored at 4°C. The phage titer of each viral suspension was assessed as stated above and the number of plaque forming units (PFU/mL) was calculated.

4.1.3.4 Host range analysis

The host range of each phage was determined through a spot assay as described above, using exponential-phase STEC strains listed in Table 1. Briefly, 10 µL aliquot of each phage suspension were spotted onto each bacterial overlay and incubated overnight at 37°C. Plaque formation was evaluated according to lysis intensity. The experiment was performed in triplicate. Results were used to formulate a mixed viral suspension containing three different bacteriophages, named “cocktail”. The concentration of viral particles in the mixture was the same for each phage (about 10^7 PFU/mL) and it was prepared in order to obtain the expected MOI.

4.1.3.5 Bacterial cell lysis assay

The lytic effect of the phage cocktail on STEC strains was assessed through the measurement of optical density (OD) at 600 nm (7315

Spectrophotometer, Jenway, Stone, UK). Phage cocktails were added to LB broth supplemented with $CaCl_2$ containing exponential-phase STEC strains (ca 7,5 x 10^8 cells/mL) to different Multiplicity of Infection (MOI): 0,1, 1 and 10. The suspensions were incubated at 37°C and OD_{600nm} was measured at 0 and every 60 minutes over 6 hours. A positive control sample was carried out by inoculating each bacterial strain without adding any phage cocktail. A negative control sample consisting of inoculated phage cocktail without any bacteria was also included in each assay. In order to normalize and compare the results obtained in various experiments, the value of the "area under the curve" (auc) of optical density formed by the growing of bacteria in six hours was determined. This value integrated the carrying capacity, the growth rate and the contribution of initial population in a single data (Sprouffske and Wagner, 2016).

The data were analyzed using R Core Team (2017), software packages "Growthcurver" and "ggplot2" for graphic elaboration. The ANOVA of the data was elaborated with Statgraphics Centurion (v. 18, Statistical Graphics Corp., Herndon, VA, USA); the Tukey's HSD test wad used to compare the sample means in order to evaluate significant differences.

4.1.3.6 Bacteriophage DNA extraction

To obtain high-titer phage stock solutions, 50 mL of lysate were precipitated by adding 10% (w/v) of polyethylene glycol 6000 (PEG) (Merck, Darmstadt, Germany) in 0,5 M NaCl (final concentration) at 4°C for at least 4 hours. Thereafter, samples were centrifuged at 8000 *g* for 20 min, to allow phage precipitation. The precipitate was resuspended in 400 µL of Sodium-Magnesium (SM) buffer (0,05 M Tris-HCl buffer, pH 7,5, 0,1 M NaCl, 0,008 M MgSO4, and 0,01% gelatin) by shaking at 120 rpm for 4 hours at 25°C. Then, 20 µL of EDTA solution (0,5 M; pH 8,0), 50 µL of SDS 10% (w/v) and 5 µL of proteinase K (Sigma-Aldrich, St. Louis, USA, final concentration 50 µg/mL) were added and phage lysates were incubated at 37°C for 1 hour. Finally,

standard phenol-chloroform DNA purification with ethanol precipitation was carried out to obtain purified phage DNA (Sambrook and Russell, 2001). Samples were stored at -20°C until use.

4.1.3.7 Assessment of the presence of toxin genes and RAPD fingerprinting

For the isolated phages the presence of genes encoding Shiga-like toxins (*stx1, stx 2, stx2f*) and intimin (*eae*) was assessed by polymerase chain reaction (PCR), according to EU-RL VTEC_Method_01_Rev 0 (2013) protocol. DNA from a temperate phage of O26 STEC strain F1-1 was used as a positive control. Multiplex-PCR reactions were set up in a 25 µl final volume containing: 10x buffer with MgCl2, dNTPs 0,2 mM, 25 pmol of each primer (Paton and Paton, 1998; Scheutz *et al*., 2012), 1 U of Taq polymerase (Biotechrabbit, Hennigsdorf, Germany) and 1 µl of template DNA. The thermal profile consisted in 35 PCR cycles (1 min of denaturation at 95 °C; 2 min of annealing at 65 °C for the first 10 cycles, decrementing to 60 °C by cycle 15; 1,5 min of elongation at 72 °C, incrementing to 2,5 min from cycle 25 to 35). Random amplification of polymorphic DNA (RAPD) was carried out with M13 primer (5'-GAGGGTGGCGGTTCT-3') (Huey and Hall, 1989) at a final concentration of 0,5 mM with the same PCR reaction mix reported before. Thermal parameters for denaturing, annealing, and extension temperatures were 94°C for 2 min, 94°C for 20 s 35 °C for 20 s for 40 cycles and a final elongation at 72°C for 2 min. The PCR products were subjected to electrophoresis in 1% agarose gel in 1X TAE buffer (Tris-acetate 40 mM, ETDA 1 mM, pH 8) added with 0,4 µg/mL of ethidium bromide with a 100 bp DNA ladder (BiotechRabbit, Henningsdorf, Germany) at a voltage of 80 V for 1,5 hour prior to visualization with UV transilluminator (GELDOC XR-System, Bio-Rad Laboratories, Hercules, USA).

4.1.3.8 Efficacy of the phage cocktail against STEC in fresh cucumber

In order to evaluate the capability of the phage cocktail to reduce STEC contamination in fresh produce, a challenge test was set up slightly modifying the protocol of Snyder *et al*. (Snyder *et al*., 2016). Fresh cucumbers were purchased from a local market and thoroughly washed to remove any soil trace. Cucumbers were sliced and cut in pieces of approximately 3 g and both sides were treated with a UV lamp for 1 hour to reduce the background microbiota. The pieces were then divided in three batches of about 10 g each and placed in Petri dishes. Two were spotted with 10 µl of a pathogenic *E. coli* culture in exponential phase (0,2) at a concentration of 1×10^6 CFU/ml. Then, one batch was dipped for 2 min in a beaker containing 50 mL of the three phages (FM10, DP16 and DP19) at the same titer (1×10^7 PFU/mL). The other batch was dipped in the same solution without phages. All the pieces were allowed to dry for 1 h in a biosafety cabinet, transferred in sterile plastic box, and stored at 4°C and 25°C. Bacterial counts were carried out at the beginning (t_0), after 6 (t_6) and 24 hours (t_{24}). The cucumber pieces were diluted in Tryptone Salt (1 g L^{-1} Tryptone, 9 g L^{-1}NaCl) and homogenized in Stomacher® (400 Circulator, Seward, Worthing, England) for 2 minutes. Appropriate dilutions of the samples were then plated in TBX agar plates (Sharlau, Sentmenat, Spain). Three different experiments were run. The non-contaminated batch was analyzed to evaluate the bacterial count after UV treatment using the same protocol but measured only at t_0.

4.1.4 Results

4.1.4.1 Antibiotic resistance assay

Six antibiotics were tested, namely amoxicillin/clavulanic acid, ciprofloxacin, norfloxacin, chloramphenicol, ampicillin and nalidixic acid. Each of the thirty-one STEC strains, submitted to disk diffusion susceptibility test, showed sensitivity to at least one of the antimicrobial compounds investigated. Data were interpreted according to parameters proposed by EUCAST (2019). Strains 228GS (O145), ED238 (O121) and PO128 (O128) showed the widest resistance, being inhibited by only one antibiotic out of six (MAR=0,83). On the other hand, 6182-50 (O113) and F95-3 (O26) were sensitive to all six compounds (MAR=0,00) (Table 1). Ampicillin, an antibiotic used in human medicine for the treatment of coliform infections, was the most effective antimicrobial agent, showing inhibition on 20 STEC strains out of 31 (65%), while nalidixic acid showed the lowest efficacy by inhibiting only 10 strains out of 31 (32%) (Table 1). The disk containing a solution of 50% (v/v) ethanol did not produce any inhibition, showing that the observed efficacy of the chloramphenicol solution was not due to the presence of the alcohol. No correlation among serogroup and antibiotic resistance was demonstrated.

Table 1. STEC strain used in this work with information relative to antibiotic resistance according to EUCAST (2019). AMC = Amoxicillin/clavulanic acid; AMP = Ampicillin; CHL = Chloramphenicol; CIP = Ciprofloxacin; NAL = Nalidixic acid; NOR = Norfloxacin. a: American Type Culture Collection; b: Istituto di Ispezione degli Alimenti di Origine Animale (Milan, Italy); c: Picozzi et al., 2016; d: Collaborative Centre for Reference and Research on Escherichia (WHO) (Orskov et al., 1977) e: Statens Serum Institut (SSI) in Denmark; f: Istituto Superiore di Sanità (Rome, Italy).

Strain	Serogroup	Antibiotic resistance	MAR index
ATCC35150[a]	O157	AMC, CHL, AMP	0,50
393[b]	O26	CIP, NOR, CHL, NAL	0,67
15R[b]	O76	AMC, NAL	0,34
214CH[c]	O157	CIP, AMP, NAL	0,50
228GS[c]	O145	AMC, CIP, NOR CHL, NAL	0,83
229RACH[c]	O111	AMC, AMP, NAL	0,50
239RA[c]	O26	CHL, NAL	0,34
243RACH[c]	O26	AMC, CIP, CHL, NAL	0,67
243ROI-A[c]	O26	NOR, NAL	0,34
33C[b]	O23	AMP, NAL	0,34
380USA[b]	O157	NOR, CHL, NAL	0,50
6182-50[d]	O113	-	0
62 19/L[b]	O157	NOR, CHL, AMP, NAL	0,67
C679-12[e]	O104	AMC, NOR, CHL	0,50
ED13[f]	O157	CIP, CHL, AMP, NAL	0,67
ED142[f]	O111	CIP, NOR, AMP	0,50
ED161[f]	O86	AMC, CIP, NOR	0,50
ED172[f]	O103	CIP, NOR	0,34
ED173[f]	O145	CIP, NOR, AMP	0,50
ED226[f]	O113	AMC, CIP, NOR, NAL	0,67
ED33[f]	O139	AMC, CIP	0,34
ED56[f]	O26	CIP, NOR, CHL, NAL	0,67
ED76[f]	O91	CIP, NOR, CHL, AMP	0,67
ED82[f]	O111	AMC, NOR, AMP, NAL	0,67
ED238[f]	O121	CIP, NOR CHL, AMP, NAL	0,83
F1-1[c]	O26	NAL	0,17
F10-4[c]	O26	CIP, NAL	0,34
F11-4[c]	O26	AMC, CIP, NAL	0,50
F95[c]	O26	AMC, NAL	0,34
F95-3[c]	O26	-	0
PO128c	O128	AMC, CIP, NOR CHL, NAL	0,83

4.1.4.2 Isolation of bacteriophage and host range determination

A total of 20 *E. coli* bacteriophages were collected; in particular, 15 phages were isolated from 15 bovine feces samples, 2 phages from 2 bovine bedding material samples and 3 phages from 3 sewage samples. FM3, FM6, FM8, FM10, DP13, DP14, DP15, DP16, DP17, DP18, DP19 and DP20 phages were detected and purified by using the indicator strain *E. coli* CNCTC 6896 whereas FM1, LF2, LF4, FM5, LF7, FM9, FM11 and LF12 phages with the indicator strain CNCTC 6246, respectively. No active viral particles could be recovered from 2 samples coming from ovine matrices. During the isolation process, plaques with different morphology were collected from plates at highest dilutions assuming that phages present at elevated titers would be more likely to display a lytic biological lifestyle.

Spot tests were performed to assess the ability of the isolated viral particles to infect and lyse thirty-one STEC strains previously collected from different sources. Strain sensitivity to each phage was evaluated by observing the type of clarification zone onto double layer LB agar plates: the formation of clear plaques was interpreted as high sensitivity to the phage, while that of turbid plaques as low sensitivity (Figure 3).

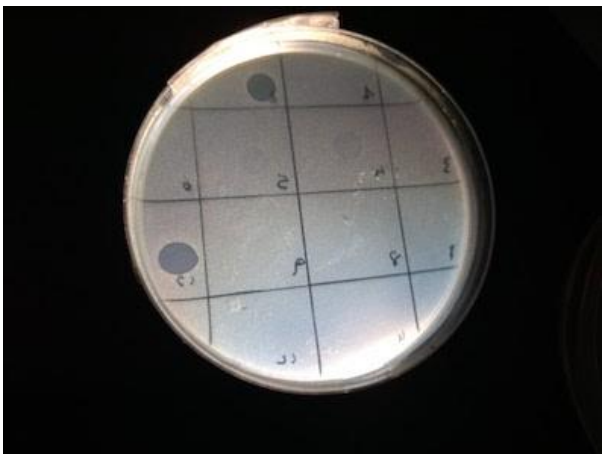

Figure 3. Image of spot test for the bacterial strain 33C (serogroup O23): phages LF2 (square 2) and FM10 (square 10) produced clear lysis plaques; phages FM3 (square 3), LF4 (square 4), and FM5 (square 5) generated turbid plaques; phages FM1 (square 1), FM6 (square 6), LF7 (square 7), FM8 (square 8), FM9 (square 9), FM11 (square 11), and LF12 (square 12) did not show any lysis.

FM10, isolated from bovine feces, was the bacteriophage with the broadest host range, being able to infect all the 31 STEC strains. Eleven phages (55%) showed to be active on more than 70% of examined strains. Among these, the most promising ones were LF2, FM9, DP13, DP15 and DP20 (Figure 4). Therefore, a viral suspension containing a controlled mixture of these bacteriophages with different host ranges could potentially be effective at inhibiting all the tested STEC strains. On the other side, phages FM1, FM5, FM11 and LF12 exhibited narrow spectra of activity, infecting 8 to 10 strains and suggesting that they could not be the optimal choice for a cocktail formulation.

As concern strains, *E. coli* F95-3 (O26) and 380USA (O157) showed to be most resistant ones being sensitive to only 5 phages out of 20.

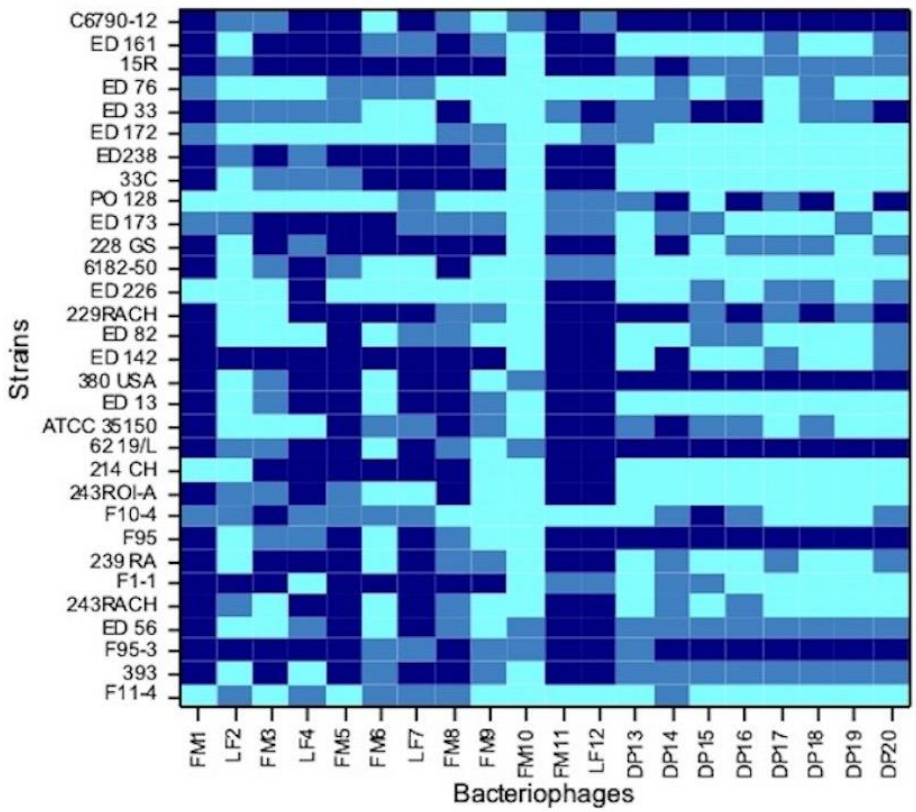

Figure 4. Heat-map showing the host range of each isolated phages. Dark blue, no sensitive strain; medium blue, strain with low sensitivity; light blue, strain with high sensitivity.

4.1.4.3 Assessment of bacterial inactivation kinetics

Basing on the data obtained from the host range assay, a cocktail containing phages FM10, DP16 and DP19 at the same titer (PFU/mL), was formulated. The effect of three different MOI's was investigated (0,1, 1 and 10) by monitoring the optical density of mixed suspensions through hourly measurements.

In order to compare the activity of the phage cocktail on each STEC strain throughout the incubation period, the value of the area under the curve (auc)

was calculated by fitting the experimental OD points with software packages cited in Materials and Methods section. It can be observed that, in general, a higher MOI corresponds to a more effective cell growth reduction (Table 2), represented by a lower value of auc showed by the development of bacteria over six hours (Figure 5). The auc mean value of positive controls (2,91) was significantly higher ($p < 0,05$) than the auc mean value of the experiments with the addition of phage cocktail; finally, with MOI at 0,1 the auc mean value was 2,12, with MOI at 1 was 1,78, and with MOI at 10 was 1,40. Moreover, the auc mean value of the experiments with MOI at 0.1 was different ($p < 0,05$) from that one obtained with MOI at 10. Also, the distribution of the auc values varied considering the different MOI used. In particular, while results of positive controls are clustered in a limited area, data of the other groups were widely distributed due to the fact that some strains are resistant to the phage cocktail and, in these cases, the auc values are similar to values obtained from controls (Table 2).

Table 2. Values of area under the curve (auc) obtained by adding the phage cocktail to each strain with different Multiplicity of Infection (MOI).

Strain	MOI 0,1	MOI 1	MOI 10	control
ATCC35150	0,18	0,14	0,13	3,05
393	2,61	2,34	0,76	2,69
15R	1,40	0,84	0,00	2,35
214CH	2,59	2,18	1,17	2,64
221RACH	3,09	2,45	2,2	3
228GS	0,17	2,02	0,00	3,04
239RA	3,81	2,49	2,37	3,78
243RACH	2,43	2,64	1,65	2,42
243RoI-A	3,08	2,04	1,51	2,97
33C	0,97	0,56	0,73	3,27
380USA	2,8	2,71	2,8	2,79
6182-50	3,58	2,35	0,00	3,8
62 19/L	3,62	2,41	3,13	3,53
C679-12	0,32	0,00	0,00	2,35
ED13	0,22	0,18	0,00	2,65
ED142	2,45	2,14	1,44	2,44
ED161	0,75	2,14	0,21	2,26
ED172	2,2	1,94	2,50	3,28
ED173	3,25	3,18	3,25	3,14
ED226	3,37	2,74	2,29	3,33
ED238	2,87	2,6	2,74	2,86
ED33	1,88	1,76	0,16	2,45
ED56	3,08	2,64	3,12	3,45
ED76	1,94	1,46	2,39	3,7
ED82	2,95	2,14	0,00	2,95
F1-1	0,21	0,1	1,69	2,23
F10-4	2,24	0,92	2,13	2,63
F11-4	2,56	0,74	0,00	3,08
F95	2,45	2,43	2,49	2,39
F95-3	2,57	2,62	2,22	2,63
PO128	0,00	0,43	0,34	2,85
mean	2,12b	1,78a,b	1,40a	2,91c

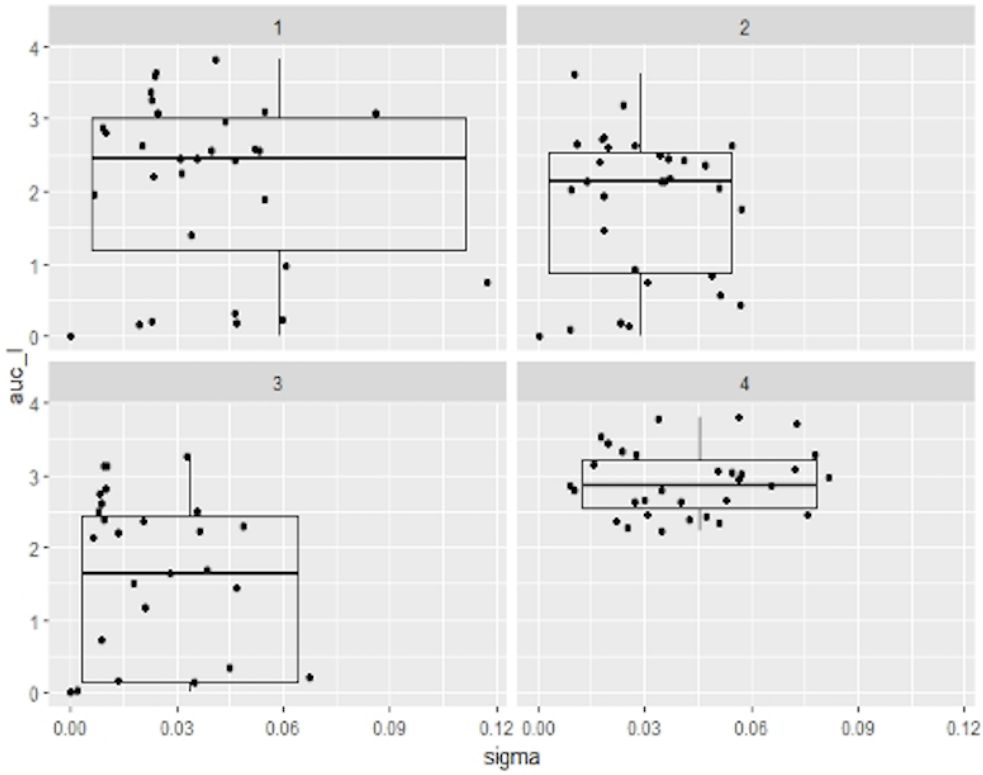

Figure 5. Box-plots representing the distribution describing the of area under the curve (auc) values onto x axe and the sigma value (the residual sum of squares from the non-linear regression model) onto y axe showed by mixed suspensions of the phage cocktail with each STEC strain, at three different multiplicity of infection (MOI) and relevant sigma value. 1, MOI 0,1; 2, MOI 1 3, MOI 10; 4, positive control.

The value of sigma (i.e. the residual sum of squares from the nonlinear regression model) revealed the goodness of the fit with parameters of the logistic equation for the observed data (Sprouffske K., 2016). In our case, a sigma value ≤ 0,12 can be deemed as a good value of fitting. Considering indeed that the expected cell growth of strains was that described by the positive controls, the addition of phage cocktail significantly changed the behavior of the suspensions with a lower value of sigma close to 0 for the highest MOI (Figure 5).

Nevertheless, six out of 31 STEC strains proved to be resistant to the phage cocktail (Table 2). In some cases, such as for strains ED82, ED226, 214CH,

ED142, ED33 and 243RACH, a MOI of 10 was necessary to observe substantial reduction of bacterial cell concentration. Otherwise, including some of those in first category, the application of a high MOI reduced the lytic effect of the cocktail.

4.1.4.4 Assessment of the presence of toxin genes and RAPD analysis

Since bacteriophages play a major role in horizontal gene transmission, the DNA extracted from each phage was examined by PCR for the presence of specific STEC virulence factors such as genes encoding Shiga toxins and intimin. No amplification was obtained from any sample, while the positive control exhibited amplification signals at the expected sizes, demonstrating that none of the twenty phages carried these genes. A RAPD analysis was performed to highlight potential similarity among phage isolates. In order to recognize if viral DNAs were contaminated with bacterial DNA of the host, amplifications were carried out also on DNA extracted from the two indicator strains. In supplementary figure 1 results from the amplification on the three selected phages are reported showing different and reproducible fingerprints. Viral DNA samples revealed different patterns in comparison to their respective propagation bacteria, so the observed differences are likely attributed to dissimilarity among phages. Moreover, the DNAs from the three selected phages were subjected to a complete genome sequencing (personal communication of prof. David Pride, University of San Diego, U.S.A.) that confirmed the absence of virulence-encoding genes and antibiotic-resistance genes.

4.1.4.5 Preliminary testing on inoculated fresh produce

A first trial to evaluate the efficacy of the phage cocktail to control STEC in fresh produce was done using cucumber as model system. Samples after washing and UV treatment did not show a residual bacterial count (< 10

CFU/g). Fresh cucumber slices were artificially contaminated with approximately ˜10^3 CFU/g of the sensitive strain. Treatment with the phage cocktail led to a reduction of bacterial counts of 1,97 and 2,01 log CFU/g at 25°C and of 1,16 and 2,01 log CFU/g at 4°C, after 6 and 24h respectively (Table 3).

Table 3. Average of ATCC35150 microbial count of three replicates at three time, at two different temperature express in log CFU/g.

	25°C		**4**°C	
ATCC35150	**Control**	**Cocktail**	**Control**	**Cocktail**
t0	2,88 (±0,24)		2,88(±0.24)	
t6	4,06(±0,84)	2,09(±0,45)	3,11(±0,17)	1,95(±0,41)
t24	9,01(±0,04)	7,00(±0,44)	3,26(±0,52)	1,25(±1,09)

4.1.5 Discussion

Twenty phages active on STEC strains were isolated from cattle feces, bedding material and sewage, indirectly confirming that bovine gut is a natural reservoir for these pathogens and often the main route of contamination for raw materials and dairy products, especially when prepared in inadequate hygienic conditions. The relative high number of virions isolated in this study from a small collection of samples corroborates these substrates as a consistent source of *E. coli* bacteriophages. A preliminary characterization pointed out viral populations showing different plaque morphologies and host ranges. The formation of turbid plaques in few strains could be due to the presence of resistance phenomena such as abortive infection mechanisms (Dy *et al*., 2014), which prevent the spread of progeny virions and thus protect clone cells from infection.

Although phage FM10 proved to be able to lyse all the STEC strains used in these study, we decided to use a cocktail of different bacteriophages to be more effective and reduce the emergence of phage resistance (Goodridge and Abedon, 2003). The choice of using three phages seemed, in accordance with literature (O'Flynn *et al*., 2004; Bai *et al.*, 2019; Yin *et al.*, 2019), a good compromise considering the possibility of phage recombination and the generation of new host specificity, and even the high production costs. Besides, according to the "Red Queen hypothesis" ("It takes all the running you can do, to keep in the same place"), as bacteria develop phage defense mechanisms for their survival, phages continuously adapt to these altered host systems in order to avoid a complete destruction (Lythgoe and Read, 1998).

The lytic ability of our phage cocktail has been assessed at different MOIs. As expected, in 61% of cases the higher MOI allowed an effective reduction of the bacterial population. A lower performance was observed in experiments with few strains (380USA, 6219/L, ED56 and F95-3), probably because no phage, present in the mixed suspension, had been capable to form clear lysis

plaques on them in the previous screening tests. The behavior of the phage cocktail on ED173 and F95 strains is difficult to interpret since they did not show any lowering of the auc values although they revealed sensitivity to the attack by phage F10 when alone. STEC strains are mostly lysogenic and, therefore, they continuously synthesize repressor proteins to maintain its lifestyle which can inhibit further infection. Moreover, the current MOI drives the decision made by the phage when its DNA is injected into the host cell (Blotnick *et al.*, 2018); in this work we have not evaluated which intracellular events may have occurred. However, it has been confirmed that the choice of bacteriophages forming clear lysis plaques, probably going in virulent cycle, is preferable to those that generate turbid plaques. The phage cocktail used in this work allowed a 2 log reduction of *E. coli* cells after 24h incubation both at 4 and 25°C making it a promising tool for the biocontrol of STEC on fresh produce.

No correlation was observed between host range and serogroup or antibiotic resistance spectrum. Antibiotics are not allowed for food applications but the presence of antibiotic resistant pathogens on these substrates are considered a risk for public health. However, control of antibiotic resistant pathogens is a global challenge, especially considering the difficulty in developing new classes of antimicrobials. The high resistance evidenced in our strains to nalidixic acid (a quinolone antibacterial agent for oral administration) and ciprofloxacin (a second-generation fluoroquinolone), both used to treat *E. coli* infections, reinforces the hypothesis that the use of these molecules against STEC strains might be ineffective. Antibiotic and phage resistance are provided by different mechanisms suggesting that the formulation of a phage cocktail active on different STEC strains that can be used on crops can help in prevent foodborne disease and the subsequent treatment of patients with inefficient antibiotics. Furthermore, a number of bacteriophage cocktails have been already granted as Generally Recognized as Safe (GRAS) by the FDA and already available on the market (e.g. SalmoFresh™, ListShield™ and PhagheGuard S™) (Moye *et al*., 2018). Our phage formulation showed to

inhibit strains 228G and PO128 that exhibited resistance to different antibiotics and to reduce significantly all other tested STEC strains.

4.1.6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.1.7 Author Contributions

NM and CP planned the experiments. NM and RC did the experiments and analyses. NM and RF processed the data. NM and CP wrote the manuscript. RF and CP revised the manuscript.

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4.1.10 References

Abuladze, T., Li, M., Menetrez, M. Y., Dean, T., Senecal, A., and Sulakvelidze, A. (2008). Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 74, 6230–6238. doi:10.1128/AEM.01465-08.

Bai, J., Jeon, B., and Ryu, S. (2019). Effective inhibition of *Salmonella Typhimurium* in fresh produce by a phage cocktail targeting multiple host receptors. Food Microbiol. 77, 52–60. doi:10.1016/j.fm.2018.08.011.

Blotnick, J. A., Vargas-Garcia, C. A., Dennehy, J. J., Zurakowski, R., and Singh, A. (2018). The effect of multiplicity of infection on the temperateness of a bacteriophage: Implications for viral fitness. in 2017 IEEE 56th Annual Conference on Decision and Control, CDC 2017 (Institute of Electrical and Electronics Engineers Inc.), 1641–1645. doi:10.1109/CDC.2017.8263885.

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., and Finlay, B. B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin. Microbiol. Rev. 26, 822–880. doi:10.1128/CMR.00022-13.

Dy, R. L., Richter, C., Salmond, G. P. C., and Fineran, P. C. (2014). Remarkable Mechanisms in Microbes to Resist Phage Infections. Annu. Rev. Virol. doi:10.1146/annurev-virology-031413-085500.

EFSA (2009). The use and mode of action of bacteriophages in food production - Endorsed for public consultation 22 January 2009 - Public consultation 30 January – 6 March 2009. EFSA J. 7, 1–26. doi:10.2903/j.efsa.2009.1076.

EFSA and ECDC (2019). The European Union One Health 2018 Zoonoses Report. EFSA J. 17, 5926. doi:10.2903/j.efsa.2019.5926.

EUCAST (2019). The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. http://www.eucast.org.". 0–99. Available at: http://www.eucast.org.

Ferguson, S., Roberts, C., Handy, E., and Sharma, M. (2013). Lytic bacteriophages reduce *Escherichia coli* O157. Bacteriophage 3, e24323. doi:10.4161/bact.24323.

Goodridge, L., and Abedon, S. (2003). Bacteriophage biocontrol and bioprocessing: Application of phage therapy to industry. SIM News 53, 254–262. Available at: http://193.137.20.178/rede/oceanos/2desafio/16.pdf.

Greer, G. G. (1988). Effects of Phage Concentration, Bacterial Density, and Temperature on Phage Control of Beef Spoilage. J. Food Sci. 53, 1226–1227. doi:10.1111/j.1365-2621.1988.tb13570.x.

Hudson, J. A., Billington, C., Wilson, T., and On, S. L. W. (2015). Effect of phage and host concentration on the inactivation of *Escherichia coli* O157:H7 on cooked and raw beef. Food Sci. Technol. Int. 21, 104–109. doi:10.1177/1082013213513031.

Kaper, J. B., Nataro, J. P., and Mobley, H. L. T. (2004). Pathogenic Escherichia coli. Nat. Rev. Microbiol. 2, 123–140. doi:10.1038/nrmicro818.

Karmali, M. A., Gannon, V., and Sargeant, J. M. (2010). Verocytotoxin-producing *Escherichia coli* (VTEC). Vet. Microbiol. 140, 360–370. doi:10.1016/j.vetmic.2009.04.011.

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 46, 165–170. doi:10.1128/aem.46.1.165-170.1983.

Liao, Y.-T., Salvador, A., Harden, L. A., Liu, F., Lavenburg, V. M., Li, R. W., et al. (2019). Characterization of a Lytic Bacteriophage as an Antimicrobial Agent for Biocontrol of Shiga Toxin-Producing *Escherichia coli* O145 Strains. Antibiotics 8, 74. doi:10.3390/antibiotics8020074.

Lythgoe, K. A., and Read, A. F. (1998). Catching the Red Queen? The advice of the rose. Trends Ecol. Evol. 13, 473–474. doi:10.1016/S0169-5347(98)01486-4.

Matuschek, E., Brown, D. F. J., and Kahlmeter, G. (2014). Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 20, 255–266. doi:10.1111/1469-0691.12373.

McCallin, S., Alam Sarker, S., Barretto, C., Sultana, S., Berger, B., Huq, S., et al. (2013). Safety analysis of a Russian phage cocktail: From MetaGenomic analysis to oral application in healthy human subjects. Virology 443, 187–196. doi:10.1016/j.virol.2013.05.022.

Megha, P. U., Murugan, S., and Harikumar, P. S. (2017). Isolation and Characterization of Lytic Coliphages from Sewage Water. J. PURE Appl. Microbiol. 11, 559–565. doi:10.22207/JPAM.11.1.73.

Moye, Z. D., Woolston, J., and Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. Viruses 10, 1–22. doi:10.3390/v10040205.

O'Flynn, G., Ross, R. P., Fitzgerald, G. F., and Coffey, A. (2004). Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli*

O157:H7. Appl. Environ. Microbiol. 70, 3417–3424. doi:10.1128/AEM.70.6.3417-3424.2004.

Orskov, I., Orskov, F., Jann, B., and Jann, K. (1977). Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. 41, 667–710. doi:10.1128/mmbr.41.3.667-710.1977.

Paton, A. W., and Paton, J. C. (1998). Detection and characterization of shiga toxigenic escherichia coli by using multiplex PCR assays for *stx1, stx2, eaeA*, enterohemorrhagic *E. coli hlyA, rfb*(O111), and *rfb*(O157). J. Clin. Microbiol. doi:10.1128/jcm.36.2.598-602.1998.

Picozzi, C., Antoniani, D., Vigentini, I., and Foschino, R. (2016). Genotypic Characterization and Biofilm Formation of Shiga-toxin producing *Escherichia coli.* FEMS Microbiol Lett, 1–7. doi:10.1093/femsle/fnw291.

Scheutz, F., Teel, L. D., Beutin, L., Piérard, D., Buvens, G., Karch, H., et al. (2012). Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J. Clin. Microbiol. 50, 2951–2963. doi:10.1128/JCM.00860-12.

Sharma, M., Patel, J. R., Conway, W. S., Ferguson, S., and Sulakvelidze, A. (2009). Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and lettuce. J. Food Prot. 72, 1481–1485. doi:10.4315/0362-028X-72.7.1481.

Snyder, A. B., Perry, J. J., and Yousef, A. E. (2016). Developing and optimizing bacteriophage treatment to control enterohemorrhagic *Escherichia coli* on fresh produce. doi:10.1016/j.ijfoodmicro.2016.07.023.

Sprouffske, K., Wagner, A. (2016). Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC Bioinformatics 17, 172. https://doi.org/10.1186/s12859-016-1016-7

Sprouffske K. Using Growthcurver. https://cran.r-project.org/web/packages/growthcurver/vignettes/Growthcurver-vignette.html. Accessed 24 Mar 2016.

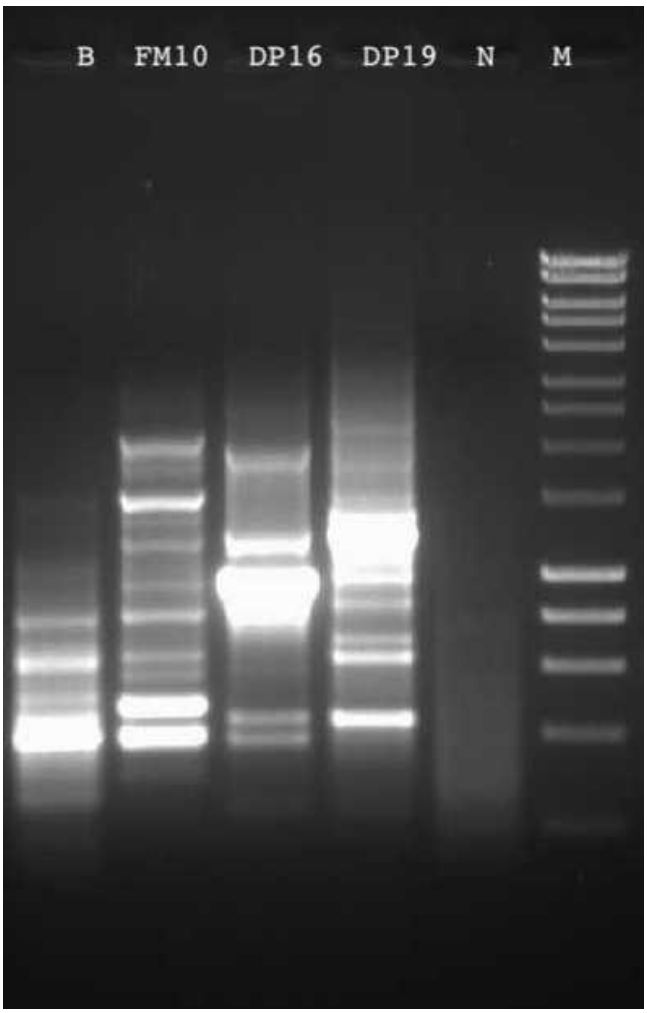
Tolen, T., Xie, Y., Hairgrove, T., Gill, J., and Taylor, T. (2018). Evaluation of Commercial Prototype Bacteriophage Intervention Designed for Reducing O157 and Non-O157 Shiga-Toxigenic *Escherichia coli* (STEC) on Beef Cattle Hide. Foods 7, 114. doi:10.3390/foods7070114.

Tomat, D., Mercanti, D., Balagué, C., and Quiberoni, A. (2013). Phage biocontrol of enteropathogenic and Shiga toxin-producing *Escherichia coli* during milk fermentation. Lett. Appl. Microbiol. 57, 3–10. doi:10.1111/lam.12074.

Viazis, S., Akhtar, M., Feirtag, J., and Diez-Gonzalez, F. (2011). Reduction of *Escherichia coli* O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and trans-cinnamaldehyde. Food Microbiol. doi:10.1016/j.fm.2010.09.009.

Yin, Y., Ni, P., Liu, D., Yang, S., Almeida, A., Guo, Q., et al. (2019). Bacteriophage potential against Vibrio parahaemolyticus biofilms. Food Control 98, 156–163. doi:10.1016/j.foodcont.2018.11.034.

Supplementary image 1. RAPD analysis on bacteriophages; B: bacterium in which the bacteriophages are propagated (CNCTC 6896); N: sample without DNA; M: all sizes marker (LeGene Biosciences, San Diego, USA).



4.2 Application of bacteriophage for preventing and removing biofilm formed by Shiga toxin-producing *Escherichia coli* (STEC)

4.2.1 Abstract

The capability of many bacteria to form biofilms can cause a serious risk in food industry, especially when bacteria are pathogenic to humans.
Thirty-one STEC strains were investigated for their ability to form biofilm. Among the best producers, four bacteria were chosen to be tested in phage control. Six bacteriophages (LF2, FM9, FM10, DP16, DP17 and DP19), used alone or in a cocktail at 4 different MOIs, were applied for preventing biofilm formation and a phage cocktail for removing biofilm already formed.
Results showed an average of reduction on biofilm formation of 43,46%, the MOI 100 was the best one with a reduction of 50,65%. Among the different phages, the cocktail composed by 6 bacteriophages was the most effective.
To the biofilm already formed by STEC strains, the six phages cocktail was applied, but no significative ($p<0,05$) reduction was reported compared to the control.

4.2.2 Short introduction

Escherichia coli is a Gram-negative bacterium belonging to the large family of *Enterobacteriaceae*. This bacterium is widely diffused in the microbiota of mammals including humans (Croxen *et al.*, 2013) and it is mostly considered to be harmless. However, some *E. coli* are characterized by the presence of virulence factors, related to the capability to adapt in new environment, that can cause disease in humans (Kaper *et al.*, 2004). In 1983, Karmali and colleagues, reported for the first time the association between the Hemolytic Uremic Syndrome (HUS) and a cytotoxin similar to the Shiga toxin produced by *E. coli* that has been found in patient feces (Karmali *et al*., 1983). Over time, Shiga toxin-producing *E. coli* (STEC) has been responsible for large outbreaks worldwide (Parsons *et al.*, 2016). In Europe, in 2018, STEC infection was the third zoonosis with 8161 confirmed cases; the trend had been increasing from 2014 to 2018 (EFSA, 2019). The most common serogroups were O157 and O26 which accounted for more than half of all cases, followed by O103, O91, O146, O145 and O128 (EFSA, 2019). Toxin production is not the only risk associated with these pathogenic bacteria. In fact, like other bacteria, *E. coli* can produce external polysaccharides (EPS) forming a matrix that allows adhesion to different surfaces, biological and not (Ferriol-González and Domingo-Calap, 2020). Bacteria within a biofilm represent a significant risk in food industry because they can be a persistent source of contamination that is difficult to remove (Van Houdt *et al*., 2010). In fact, accumulation of liquid media helps microorganisms and their decomposition products to form a biofilm layer, which, for example in case of heat exchangers, causes increased resistance in both liquid flow and heat transfer (Criado *et al*., 1994). Therefore, biofilm formation can be a relevant problem in brewing, dairy transformation, fresh produce, poultry and beef slaughtering industries (Chen *et al*., 2007; Frank *et al*., 2003; Jessene and Lammert, 2003; Somers *et al*., 2004). For example, STEC presence in meat processing plants is a relevant concern related to the

food process and also to public safety. Biofilm formation allows bacteria to survive for a long time and to be protected from biocides used in the food industry (Vogeleer *et al*., 2014). The main concern is the loss of efficacy of the biocide when bacteria form a complex structure named biofilm. The biofilm lifestyle of bacteria is naturally and largely spread throughout the environment; but the natural enemy of bacteria, the bacteriophage, has co-evolved to target bacteria, also, in this lifecycle (Hall-Stoodley *et al*., 2004). Biofilm communities are characterized by external polymeric substances (EPS) that limit physical contact between bacteria and bacteriophages; to overcome this barrier, phages are able to produce depolymerases to degrade EPS (Pires *et al*., 2016). However, bacteriophages have limitation in these strategies, such as the ability to remove the EPS layer which is affected by the metabolic state of bacteria. In fact, during the stationary phase the bacteria are more difficult to kill even by phages (Brüssow *et al*., 2004). Bacteriophages can be applied in several approaches to counter biofilm formation, such as treatment with genetically-modified phage, phage-derived enzymes, phage alone or in a cocktail or supplemented with antibiotics (fig. 6) (Ferriol-González and Domingo-Calap, 2020).

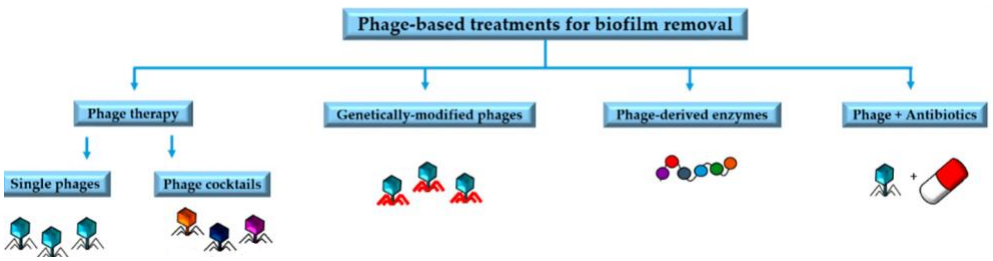


Figure 6. Phage-based treatments for biofilm removal (Ferriol-González and Domingo-Calap, 2020).

In this work, STEC strains were studied for their capability to produce biofilm. Among the best producers, four bacterial strains were chosen to be tested. Six single different bacteriophages or two different cocktails of three or six bacteriophages were used to control biofilm formed by bacteria.

4.2.3 Materials and methods

4.2.3.1 Bacterial culture

The bacteria used in this work are listed in table 4. The origin of these bacteria was described in table 1. The -20°C stock cultures were streaked on Chromocult Tryptone Bile X-Glucuronide (TBX) agar (Merck, Darmstadt, Germany) plates and incubated ad 37°C for 24 hours. Subsequently, a single colony was transferred into 10 mL of Luria Bertani (LB) broth (Alfa Aesar, Karlsruhe, Germany), incubated under the same conditions and used for the following experiments.

4.2.3.2 Bacteriophage propagation

Bacteriophages (LF2, FM9, FM10, DP16, DP17 and DP19 described in 4.1.4.2) were replicated through the double layer method described by Carey-Smith *et al.* (2006) and partially modified. Briefly, 100 µL of bacteriophage from a single plaque (described below) were mixed with 1 mL of bacterial culture of CNCTC6896 strain in exponential phase (OD_{600nm}=0,2-0,3). After ten minutes, 5 mL of LB soft agar (0,5%) and 40 µL of $CaCl_2$ 1 M were gently mixed and poured in LB agar (1,5%) plate. Following an overnight incubation at 37°C, 4 mL of SM buffer (100mM NaCl, 8mM $MgSO_4$, 50mM Tris-HCl, pH 7,5, 0,01% gelatin) were added to each plate. After one hour at room temperature, the buffer and the soft agar layer were collected in a 50 mL sterile tube. The suspension was then centrifuged at 4500 *g* for 10 minutes (Rotina 380 R, Hettich, Tuttlingen, Germany). The supernatant was transferred in a new tube; the centrifugation was repeated twice and then filtered through a 0,45 µm syringe filter (Minisart, Sartorius™), transferred in an ultracentrifugation tube (Quick-Seal® Round-Top Polypropylene Tube, Beckman Coulter®) and centrifuged a 100'000 *g* for 1 hour at 4 °C (Beckman Coulter L7-65, Ultracentrifuge). The supernatant was discarded and the pellet

resuspended in 3 mL of SM buffer. After overnight incubation at 4°C, the dissolved pellet was filtered through a 0,22 µm syringe filter (Minisart, Sartorius^{TM}) and stored at 4°C.

4.2.3.3 Biofilm formation assay

A crystal violet staining assay was performed in order to obtain a semi-quantitative determination of biofilm formation in STEC strains (Picozzi *et al*., 2016). Bacteria were grown overnight in a M9 salts medium (3,39% Na_2HPO_4, 1,5% KH_2PO_4, 0,25% NaCl and 0,5% NH_4Cl) supplemented with 0,5% glucose and in TSB (Tryptic Soy Broth) (Scharlab, Sentmenat, Spain) medium at 30°C and 37°C without shaking, in 96 wells polystyrene microtiter plates (Starlab, Hamburg, Germany). After incubation, a first reading (OD_{600nm}) was performed through a plate reader (PowerWave XS2, BioTek, Winooski, USA) using Gen5, to verify that all the controls were grown. To assess the number of attached cells, the supernatant of each well was discarded, washed twice with sterile water and then stained with 1% crystal violet for 20 min. Afterward, wells were washed again with sterile water and allowed to dry. To calculate the number of stained cells, the wells were poured with 200 µL of 95% ethanol by vigorous pipetting and the Optical Density (OD) at 600nm was measured again. The experiment was conducted in triplicate. Biofilm formation (BF) values were calculated according to the following equation as proposed by Naves *et al*. (2008): $BF=AB/CW$ where AB is the OD_{600nm} of stained attached bacteria and CW is the OD_{600nm} value of stained control wells containing only bacteria-free medium. BF values were classified into four categories according based on the amount of biofilm produced: strong (S): ≥ 6, moderate (M): 5,99 ≥ BF ≥ 4, Weak (W): 3,99 ≥ BF ≥ 2 and negative (N): < 2.

4.2.3.4 Biofilm prevention

Two bacteriophage cocktails and six bacteriophages used alone to prevent biofilm formation were tested. The bacteriophages were: LF2, FM9, FM10, DP16, DP17 and DP19. The isolation process was described in the previous chapter (4.1.3.3). Cocktail 1 consisted of FM10, DP16 and DP19. Cocktail 2 was composed by all the six phages. These bacteriophages were tested against four pathogenic *E. coli* strains: ED56, C679-12, ED226 and ED33 belonging to serogroups O26, O104, O113 and O139, respectively; and two *E. coli* strains used as control: CNCTC6246 (negative) and CNCTC25404 (positive) at four different Multiplicity of Infection (MOI): 1, 2, 10 and 100.
Bacterial culture (100 μl) was inoculated in LB broth in flat-bottomed 96 well-plate (Porvair Sciences Limited, UK); when at the exponential phase ($OD_{600} \cong 0,2$), 100 μl of bacteriophages suspended in SM buffer were added at different concentrations, according to the different MOI. No phage was added to the control. After 24 hours of incubation at 30°C, a first reading was performed to control that each bacterium had grown regularly. Then, the planktonic cells were removed and washed twice with distilled water. Cells attached to the surface were stained with crystal violet (1%) for 20 minutes. Afterwards wells were washed twice with distillate water and the stained cells were dissolved in 200 μl of 95% ethanol. Then, the cell concentration was measured at OD_{600nm} (PowerWave XS2, BioTek, Winooski, USA) using the software Gen5 and compared with the phages-free control. The experiment was conducted in triplicate.

4.2.3.5 Removal of the formed biofilm

For the removal of formed biofilm, a phage cocktail containing LF2, FM9, FM10, DP16, DP17 and DP19 was used. The different target biofilms consisted of four pathogenic *E. coli*: ED56, C679-12, ED226, ED33 belonging to O26, O104, O113 and O139 serogroups respectively, and one non-pathogenic and high biofilm producer *E. coli* strain (ATCC25404). Briefly, a pre-inoculum was prepared in LB broth; subsequently, 50 µl of bacterial culture was dispersed on membranes with a diameter of 25 mm and 0,4 µm pore size (Whatman™ Nuclepore Track-Etched Membranes) which were then placed on TSA plates. After incubation at 30°C for 24 hours, the membranes were transferred to a new plate. Then, 25 µl of phage cocktail at concentration of 1 x 10^8 PFU/ml, divided in 5 drops of 5 µl, were spotted on the surface of the membrane and incubated under the same conditions. As a control, 25 µl of SM buffer were spotted on membranes without adding phages solution. Then, the membranes were transferred to a tube containing 5 mL of Phosphate-Buffered Saline (PBS) (137 mM NaCl, 2,7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) and vigorously vortexed to remove the formed biofilm. Successively, the membranes were discarded, and the biofilm was homogenized for 90 sec (IKA T 10 basic ULTRA-TURRAX®). Samples were then diluted in PBS and plated on TSA plates. The plates were incubated for 16 hours at 37°C. The test was conducted in tree independent experiments. Colonies were counted and expressed as UFC/cm^2.

The analysis of variance was performed using the open-source software: R Core Team (R Core Team, 2017), packages: “agricolae”.

4.2.4 Results and discussion

4.2.4.1 Biofilm formation assay

In TSB medium, 15 out of 31 (48%) of investigated strains showed at least weak (W) biofilm formation at 30°C, while, 13 out of 31 formed at least W biofilm at 37°C, according to Naves *et al.* (2008). In M9 medium supplemented with 0,5% glucose a significantly fewer strains were able to form biofilm, namely 12,9% at 30°C and 3,2% at 37°C. 32% of investigated strains did not produce detectable biofilm under any of the conditions investigated (table 4). F95 and C679-12 showed Strong (S) biofilm production, at least in one condition performed. These results confirm that formation of biofilm by STEC strains on polystyrene surfaces is heterogeneous and strongly dependent on strain rather than serotype (Wang *et al.,* 2012).

Table 4. BF values of investigated STEC strains. Navy blue: Strong biofilm producers; Azure: moderate biofilm producers; light blue: Weak biofilm producers; No color: negative.

STRAIN	SEROGROUP	TSB 30°C	TSB 37°C	M9 30°C	M9 37°C
F11-4	O26	2,17	1,2	1,64	1,07
393	O26	2,37	2,25	1,26	1,06
F95-3	O26	1,44	1,37	1,41	1,1
ED 56	O26	2,37	2,28	1,28	1,16
243RACH	O26	1,68	1,01	1,36	1,08
F1-1	O26	1,37	0,93	1,43	1,09
239 RA	O26	1,48	1,06	1,27	1,06
F95	ND	6,77	4,83	**9,87**	**7,15**
F10-4	O26	1,96	1,29	1,51	1,63
243ROI-A	O26	2,6	1,7	1,56	1,14
214 CH	O157	1,62	1,26	1,16	0,94
6219/L	O157	2,19	2,38	3,02	1,23
ATCC35150	O157	1,59	1,12	2,09	1,1
ED 13	O157	1,14	0,92	1,49	0,81
380 USA	O157	2,58	3,65	1,36	0,96
ED 142	O111	1,71	2,45	1,7	1,16
ED 82	O111	1,27	0,77	1,29	0,95

229RACH	O111	1,12	1,22	1,32	1,25
ED 226	O113	4,3	3,6	1,44	1,68
6182-50	O113	2,4	2,31	2,5	1,62
228 GS	O145	2,9	1,38	1,22	1,08
ED 173	O145	2,9	1,98	1,33	1,33
PO 128	O128	1,36	1,02	1,25	1,32
33C	O23	3,91	3,95	1,32	1,44
ED238	O121	1,96	3,44	1,24	1,13
ED172	O103	3,83	1,87	1,62	1,29
ED33	O139	2,62	1,54	1,95	1,66
ED76	O91	4,51	3,29	1,21	1,22
15R	O76	1,97	1,1	1,3	1,26
ED161	O86	1,98	2,14	1,29	1,2
C679-12	O104	1,8	6,06	1,29	0,87

4.2.4.2 Effects of bacteriophages on biofilm prevention

In the food industry, biofilm formation can pose a safety hazard, especially when it forms on surfaces that come into contact with food. Furthermore, there is also the risk that the cells detach from the biofilm matrix and then end up on food, becoming a continuous source of contamination. Hence, the impact of biofilm on human health and the economic loss had enhanced efforts to control biofilm formation. In recent years, bacteriophages were applied as a tool to reduce biofilm formation in various pathogenic bacteria, such as *Pseudomonas aeruginosa* (Ahiwale *et al*. 2011; Hanlon *et al*., 2001; Knezevic *et al*. 2011; Pires *et al*. 2011), *Klebsiella pneumoniae* (Bedi *et al*., 2009), *E. coli* (Carson et al., 2010; Chibeu *et al.,* 2012; Zhang *et al.,* 2020), *Proteus mirabilis* (Carson *et al*., 2010) and *Staphylococcus epidermidis* (Curtin and Donlan, 2006). Thus, bacteriophages were used in this study to prevent the formation of biofilms produced by STEC strains. The bacteria used in the experiments were selected from those that showed biofilm formation in crystal violet assay and a in preliminary test on biofilm production on a membrane.
The biofilm prevention test was performed by comparing the attached cells of bacteria grown in presence of bacteriophage, used alone or in a cocktail, to

the control. Observing the OD_{600nm} values (table 5), it is possible to note that the mean value of the control is 0,306 while the value obtained after bacteriophage treatment at different MOI is 0,173, thus highlighting a reduction of attached cells of 43,46%. As regards the single MOI, each showed a different level of reduction. The least effective was MOI 2 with a reduction of 32,68%, after the MOI 1 and 10 with a reduction of 45,75% and 45,42%, respectively. MOI 100 appeared the most efficacious with a reduction of 50,65% (figure 7).

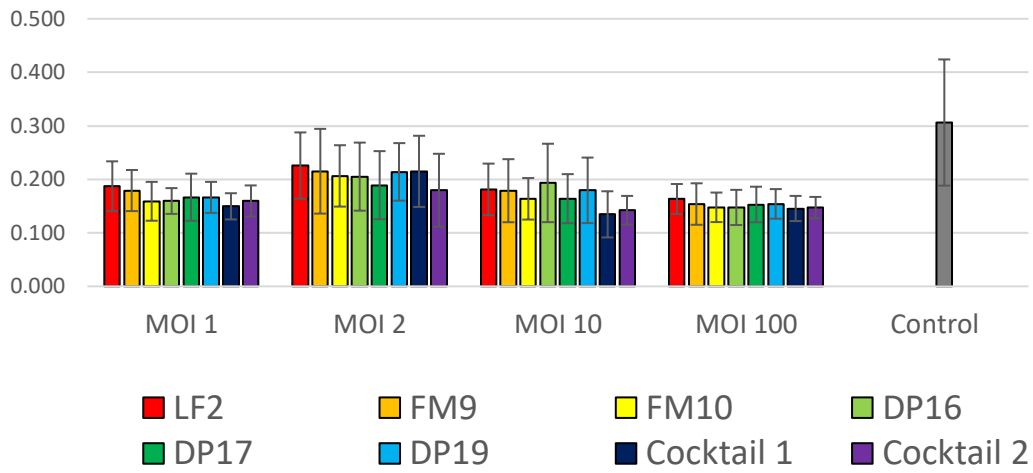


Figure 7. Biofilm production at different MOI compared the control

Table 5. Average of bacterial control and MOI in biofilm preventing.

Bacterial control average	Total MOI average
0,306	0,173

Table 6. Value of different MOI in biofilm preventing.

Phage	MOI 1	MOI 2	MOI 10	MOI 100	Average
LF2	0,187	0,226	0,181	0,163	0,189±0,03
FM9	0,179	0,215	0,179	0,154	0,182±0,03
FM10	0,159	0,206	0,164	0,148	0,169±0,03
DP16	0,159	0,205	0,193	0,147	0,176±0,03
DP17	0,167	0,189	0,164	0,153	0,168±0,02
DP19	0,166	0,214	0,179	0,154	0,178±0,03
Cocktail 1	0,15	0,215	0,135	0,145	0,161±0,04
Cocktail 2	0,159	0,18	0,142	0,147	0,157±0,02
Average	0,166 ±0,012	0,206 ±0,015	0,167 ±0,02	0,151 ±0,06	

Futhermore, the six bacteriophages tested showed an average reduction between 38,23% and 42,48%; the cocktails proved to be more effective with a reduction of 47,38% and 48,69% for cocktails composed of 3 and 6 phages respectively. Therefore, the cocktails did not show a significative difference compared to single phages (fig. 8). The reason may result from the same site of phage attachment on the bacterial surface that limit the simultaneous attack by phages.

As for the phages used alone, the most effective were DP17 and FM10 that showed a similar value (table 6) and a 45% reduction in the biofilm formed.

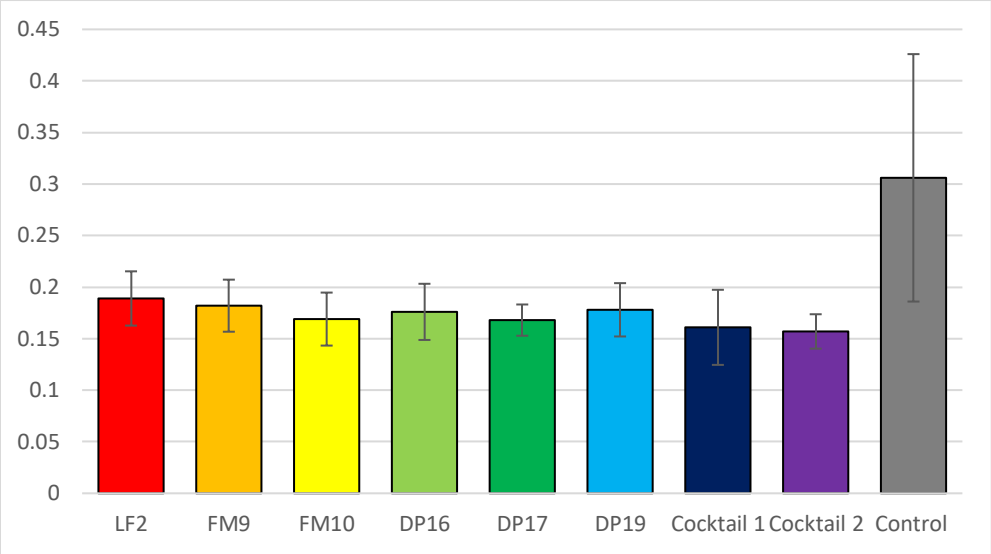


Figure 8. Average of different MOI compared to the control.

The biofilm prevention test was performed by mixing phages and bacteria in exponential phase in a 96 well-plate, and calculating the amount of biofilm formed through staining of the attached cells. The data showed that the bacteriophages can be useful in preventing the formation of biofilm. In fact, phages were able to reduce biofilm formation by 43,46% on average. Also, it needs to be considered that this value is the average of a group of selected biofilm producer bacteria. Each bacterial strain showed a particular behavior: ED33 (O139) was the most sensitive, while ED226 (0113) was the most resistant. Regarding the different bacteriophage applications, the cocktail 2, consisting of six different bacteriophages, was able to prevent biofilm formation by 48,69% compared to the control. The MOI 100 was the best for biofilm prevention, this characteristic has been previous demonstrated with the increase of the MOI, in particular, MOI 100 among 1, 10 and 100, there was a reduction in bacterial cells forming biofilm (Viazis *et al.,* 2011).

The nature of biofilm, the attachment to the surface and the risks involved in a continuous cell detachment from biofilm matrix could indicated that, if

possible, working on prevention is preferable (Simões *et al*., 2006). The main advantage of the application of bacteriophages is that the mechanisms involved in resistance are different from the ones coming from antimicrobials. Also, in presence of bacterial resistance, it is possible to change the phage preparation with other phages in a cocktail in a faster method comparing to the long and expensive development cycle of antibiotics (Sulakvelidze and Barrow, 2005; Viazis *et al.,* 2011).

4.2.4.3 Effects of bacteriophages on already formed biofilms

To remove the already formed biofilm, the six-phage cocktail, which was the most effective in prevention, was used against the biofilm formed in 24 hours by 4 different STEC strains belonging to different serogroups (O26, O104, O113, O139) and one high biofilm producer *E. coli* strain. This assay, used to test antimicrobial activity against static biofilm through cell measurement, allows to attribute the result to cell death rather than detachment (Merritt *et al.,* 2006). The results showed no significant differences ($P<0,05$) between the control and samples treated with the bacteriophage cocktail for all bacterial strains used as target (fig. 9). Kelly and colleagues (Kelly *et al*., 2012) found that biofilm formed by *Staphylococcus aureus* was reduced significantly only after 48 and 72 hours, not after 24 hours of exposure. Therefore, contact time could be the cause of the absence of significative reduction registered in this work. As reported in previous work, when the contact between bacterial cells producing biofilm and bacteriophages started from the beginning of the experiment it is possible to note a significative reduction, on contrary, no reduction was reported when the contact occurred after 24 hours, using the same actors; so, the old biofilm is hard to be removed and in general less favorable to phage diffusion (Abedon, 2016; Ferriol-González and Domingo-Calap, 2020).

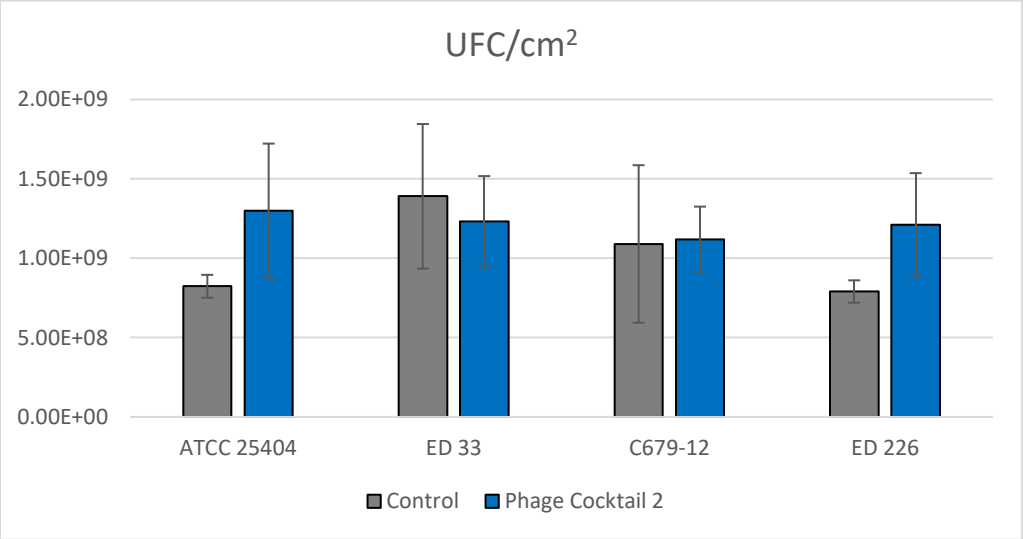


Figure 9. Effect of phage cocktail on already formed biofilm.

4.2.5 References

Abedon S. T., 2016. Bacteriophage exploitation of bacterial biofilms: phage preference for less mature targets? FEMS Microbiology Letters, Volume 363, Issue 3, fnv246, https://doi.org/10.1093/femsle/fnv246

Ahiwale, S., Tamboli, N., Thorat, K., Kulkarni, R., Ackermann, H., Kapadnis, B., 2011. In vitro management of hospital *Pseudomonas aeruginosa* biofilm using indigenous T7-like lytic phage. Current microbiology, 62(2), 335-340. doi: 10.1007/s00284-010-9710-6.

Bedi, M. S., Verma, V., Chhibber, S., 2009. Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of *Klebsiella pneumoniae* B5055. World Journal of Microbiology and Biotechnology, 25(7), 1145. doi: 10.1007/s11274-009-9991-8.

Brüssow, H., Canchaya, C., Hardt, W. D., 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol. Mol. Biol. Rev. 68(3):560-602. doi: 10.1128/MMBR.68.3.560-602.2004.

Carey-Smith, G. V., Billington, C., Cornelius, A. J., Hudson, J. A., Heinemann, J. A., 2006. Isolation and characterization of bacteriophages infecting *Salmonella* spp. FEMS microbiology letters, 258(2), 182-186. doi: 10.1111/j.1574-6968.2006.00217.x.

Carson, L., Gorman, S. P., Gilmore, B. F., 2010. The use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli*. FEMS Immunology & Medical Microbiology, 59(3), 447-455. doi: 10.1111/j.1574-695X.2010.00696.x

Chen, J., Rossman, M. L., Pawar, D. M., 2007. Attachment of enterohemorrhagic *Escherichia coli* to the surface of beef and a culture medium. LWT-Food Science and Technology, 40(2), 249-254. DOI: 10.1016/j.lwt.2005.10.011.

Chibeu, A., Lingohr, E. J., Masson, L., Manges, A., Harel, J., Ackermann, H. W., Kropinski A.M., Boerlin, P., 2012. Bacteriophages with the ability to degrade uropathogenic *Escherichia coli* biofilms. Viruses, 4(4), 471-487. doi: 10.3390/v4040471.

Criado, M. T., Suarez, B., Ferreiros, C. M., 1994. The importance of bacterial adhesion in the dairy industry. Food technology, Chicago. 48(2), 123-126.

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., Finlay, B. B., 2013. Recent Advances in Understanding Enteric Pathogenic *Escherichia coli*. Clinical Microbiology Reviews, 26(4), 822–880. https://doi.org/10.1128/cmr.00022-13.

Curtin, J. J., Donlan, R. M., 2006. Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. Antimicrobial agents and chemotherapy, 50(4), 1268-1275. doi: 10.1128/AAC.50.4.1268-1275.2006

EFSA, ECDC, 2019. The European Union One Health 2018 Zoonoses Report. EFSA Journal, 17(12), 5926. https://doi.org/10.2903/j.efsa.2019.5926.

Frank, R. A., 2003. Response context affects judgments of flavor components in foods and beverages. Food quality and preference, 14(2), 139-145. doi: 10.1016/S0950-3293(02)00073-3

Ferriol-González, C., Domingo-Calap, P., 2020. Phages for Biofilm Removal. Antibiotics, 9(5), 268. https://doi.org/10.3390/antibiotics9050268

Hall-Stoodley, L., Costerton, J. W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nature reviews microbiology, 2(2), 95-108. https://doi.org/10.1038/nrmicro821

Hanlon, G. W., Denyer, S. P., Olliff, C. J., Ibrahim, L. J., 2001. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. Applied and environmental microbiology, 67(6), 2746-2753. doi: 10.1128/AEM.67.6.2746-2753.2001

Jessen, B., Lammert, L., 2003. Biofilm and disinfection in meat processing plants. International biodeterioration & biodegradation, 51(4), 265-269. doi: 10.1016/S0964-8305(03)00046-5

Kaper, J. B., Nataro, J. P., Mobley, H. L., 2004. Pathogenic *Escherichia coli*. Nature reviews microbiology, 2(2), 123-140. doi: 10.1038/nrmicro818

Karmali, M. A., Petric, M., Lim, C., Fleming, P. C., Steele, B. T., 1983. *Escherichia coli* cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. The Lancet, 322(8362), 1299-1300. doi: 10.1016/s0140-6736(83)91167-4.

Kelly, D., McAuliffe, O., Ross, R. P., Coffey, A., 2012. Prevention of *Staphylococcus aureus* biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives. Letters in Applied Microbiology, 54(4), 286-291. doi: 10.1111/j.1472-765X .2012.03205.x.

Knezevic, P., Obreht, D., Curcin, S., Petrusic, M., Aleksic, V., Kostanjsek, R., Petrovic, O., 2011. Phages of *Pseudomonas aeruginosa*: response to environmental factors and in vitro ability to inhibit bacterial growth and biofilm formation. Journal of applied microbiology, 111(1), 245-254. doi: 10.1111/j.1365-2672.2011.05043.x.

Merritt, J. H., Kadouri, D. E., O'Toole, G. A., 2006. Growing and analyzing static biofilms. Current protocols in microbiology, (1), 1B-1. doi: 10.1002/9780471729259.mc01b01s00

Naves, P., del Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., Rodriguez-Cerrato, V., Ponte M.C., Soriano, F., 2008. Measurement of biofilm formation by clinical isolates of *Escherichia coli* is method-dependent. Journal of Applied Microbiology, 105(2), 585–590. https://doi.org/10.1111/j.1365-2672.2008.03791.x

Pires, D., Sillankorva, S., Faustino, A., Azeredo, J., 2011. Use of newly isolated phages for control of *Pseudomonas aeruginosa* PAO1 and ATCC 10145 biofilms. Research in microbiology, 162(8), 798-806. doi: 10.1016/j.resmic.2011.06.010.

Parsons, B. D., Zelyas, N., Berenger, B. M., Chui, L., 2016. Detection, characterization, and typing of Shiga toxin-producing *Escherichia coli*. Frontiers in microbiology, *7*, 478. doi: 10.3389/fmicb.2016.00478.

Picozzi, C., Antoniani, D., Vigentini, I., Foschino, R., 2017. Genotypic Characterization and Biofilm Formation of Shiga-toxin producing *Escherichia coli*. FEMS Microbiology Letters, fnw291. doi: 10.1093/femsle/fnw291

Pires, D. P. P., Oliveira, H. A. M., Melo, L. D. R., Kluskens, L., Sillankorva, S., Azeredo, J., 2016. Bacteriophage depolymerases: novel polysaccharide degrading enzymes. Appl. Microbiol. Biotechnol.; 100(5):2141-51. doi: 10.1007/s00253-015-7247-0

Simões, M., Simões, L. C., Machado, I., Pereira, M. O., Vieira, M. J., 2006. Control of flow-generated biofilms with surfactants: evidence of resistance and recovery. Food and Bioproducts Processing, 84(4), 338-345. https://doi.org/10.1205/fbp06022

Somers, E. B., & Lee Wong, A. C., 2004. Efficacy of two cleaning and sanitizing combinations on *Listeria monocytogenes* biofilms formed at low temperature on a variety of materials in the presence of ready-to-eat meat residue. Journal of food protection, 67(10), 2218-2229. doi: 10.4315/0362-028x -67.10.2218.

Sulakvelidze A., Barrow P., 2005. Phage therapy in animals and agribusiness. Bacteriophages: Biology and Applications, CRC Press, Boca Raton, Fla. pp. 335-380

Van Houdt, R., & Michiels, C. W., 2005. Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. Research in microbiology, 156(5-6), 626-633. doi: 10.1016/j.resmic.2005.02.005.

Viazis, S., Akhtar, M., Feirtag, J., Diez-Gonzalez, F., 2011. Reduction of *Escherichia coli* O157: H7 viability on hard surfaces by treatment with a bacteriophage mixture. International journal of food microbiology, 145(1), 37-42. https://doi.org/10.1016/j.ijfoodmicro.2010.11.021

Vogeleer, P., Tremblay, Y. D., Mafu, A. A., Jacques, M., Harel, J., 2014. Life on the outside: role of biofilms in environmental persistence of Shiga-toxin producing *Escherichia coli*. Frontiers in microbiology, 5, 317. https://doi.org/10.3389/fmicb.2014.00317

Wang, R., Bono, J. L., Kalchayanand, N., Shackelford, S., Harhay, D. M., 2012. Biofilm Formation by Shiga Toxin–Producing *Escherichia coli* O157:H7 and Non-O157 Strains and Their Tolerance to Sanitizers Commonly Used in the Food Processing Environment. Journal of Food Protection, 75(8), 1418–1428. https://doi.org/10.4315/0362-028X.JFP-11-427

Zhang, Y., Shigemura, K., Duc, H. M., Shen, C., Huang, H. H., Sato, J., Masuda, Y., Honjoh, K.-I., Miyamoto, T., 2020. Effects of bacteriophage on inhibition and removal of mixed biofilm of enterohemorrhagic *Escherichia coli* O157: H7 and O91: H. LWT, 134, 109945. https://doi.org/10.1016/j.lwt.2020.109945

4.3 Application of Bacteriophages to control pathogenic *Escherichia coli* related to Urinary Tract Infection and sequencing of bacteriophage and bacterial whole genomes.

This part of the work was done at the Department of Pathology, University of California San Diego under the supervision of Prof. David T. Pride, MD, PhD.

4.3.1 Abstract

Urinary Tract Infection (UTI) is one of the most common infection in the world and *E. coli* is the main microorganism involved. In this work, six bacteriophages were used against 270 pathogenic *E. coli* isolated from patients with UTI and analyzed by whole genome sequencing (WGS) and transmission electron microscope (TEM). The bacteria were first screened for the presence of temperate bacteriophage through Mitomycin C induction. Bacteria that showed to be resistant to bacteriophages were also sequenced. The results showed a presence of inducible prophage in 17,4% of bacteria. 262 out of 270 bacterial strains were sensitive to at least one phage. Sequencing and TEM imaging confirmed that the bacteriophages can be ascribed to *Myoviridae* and no pathogenicity or lysogenesis related genes were found. The bacterial sequences did not show CRISPR gene related to the bacteriophages used.

4.3.2 Short introduction

Escherichia coli is a Gram-negative bacterium. It can grow both in aerobic and anaerobic condition and can be characterized by motile elements: pili and flagella. *E. coli* is one of first bacteria studied and often used in laboratories due to its versatility. It is a common member of microbiota of humans and other mammals (Croxen *et al*., 2013); some *E. coli* have acquired virulence factors that increase the ability to adapt to new environment but cause also diseases in humans (Kaper *et al*., 2004). These virulence factors can be encoded by genetic mobile elements and can be locked in the bacterial genome, transmitted to daughter cells (Kaper *et al*., 2004). Pathogenic *E. coli* could be a cause of intestinal or extraintestinal infections. In particular, the intestinal *E. coli* group can be divided into several categories: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) (Köhler and Dobrindt, 2011). Extraintestinal *E. coli* (ExPEC) isolates can be divided in: uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC); the ExPEC are able to infect different anatomical sites (Dale and Woodforf*,* 2015). ExPEC bacteria were defined by Peirano *et al*., (2013) by the presence at least of two virulence factors within their genome: *papA* and/or *papC* (P fimbriae major subunit and assembly), *sfa/foc* (S and F1C fimbriae), *afa/draBC* (Dr-binding adhesins), *kpsM II* (group 2 capsule) and *iutA* (aerobactin receptor). UPEC strains are the leading cause of Urinary tract infections (UTIs), a very common disease that affects 40% of the women at least once during their lifetime (Micali *et al*., 2014). The presence of UTIs infection refers to the presence of a large number, generally higher than 10^5 UFC/ml, of bacteria in the urine (Terlizzi *et al.,* 2017); UPEC are 65-75% of the total microorganisms in community-acquired uncomplicated and complicated UTIs (Flores-Mireles *et al*., 2015). UTIs are called uncomplicated when they affect heathy people with no abnormal urinary tract, causing cystitis and pyelonephritis (Hooton, 2012).

Complicated UTIs are instead associated with abnormal tract or compromised host defense (Flores-Mireles *et al*., 2015). Routinely, antibiotics are used to treat UTIs according to their ability to resolve the infections quickly. This choice had become more complicated due to the worldwide increase in antimicrobial resistance among UPECs (Hooton, 2012). Meier *et al*. (2011) studied the antimicrobial resistance of community-acquired UTI highlighting an increasing of drug resistance. The antibiotics studied were extended-spectrum β-lactamases (amoxicillin–clavulanic acid, 69,6% resistance), quinolones (ciprofloxacin, 84,8% resistance; norfloxacin, 83,9% resistance), and trimethoprim–sulfa-methoxazole (75,9% resistance), nitrofurantoin (15% resistance) and fosfomycin (0% resistance). Overall, clinical antibiotic treatment was compromised by the increase in antimicrobial resistance in UPEC strains (Nicolle, 2011). In the last years with the spread of antimicrobial resistance, researchers are studying alternative strategies. Application of bacteriophage to humans, called phage therapy, and of their lytic proteins as an alternative or a supplement to antibiotics against multidrug resistant bacteria appears to be a promising strategy (Lin *et al.,* 2017). In presence of their host, bacteriophages are able to infect, multiply and kill using the host cell machinery and release the new virionic progeny, improving their number according to the number of infected bacteria (Sybesma *et al*., 2017). Hence, phage therapy is being re-evaluated for the treatment and prevention of bacterial infections in humans (Azam and Tanji, 2019). Generally, bacteriophages are considered safe by the Food and Drug Administration (FDA) according to the daily ingestion of bacteriophages, normally found in water and food (FDA, 2006).
The aim of this work was to test the efficacy of bacteriophages isolated from breeding farms against a large collection of pathogenic *E. coli* isolates related to UTIs. The whole genome of bacteriophages was sequenced to verify the safety of their application. In addition, bacterial genomes were sequenced to investigate the possible mechanisms of phage resistance.

4.3.3 Materials and methods

4.3.3.1 Culture condition

The pathogenic *E. coli* used in this work were clinical strains collected in San Diego area (Supplementary Table 1). The strains were streaked onto Eosin Methylene Blue Agar (EMBA), Levine (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. The plates were used in all the subsequent steps for inoculation of bacteria.

4.3.3.2 Prophage induction

In order to evaluate the presence of temperate bacteriophages in the collected bacteria, the strains were subjected to a DNA crosslinking agent: Mitomycin C. A single colony from EMBA plate of each strain was inoculated in 1 ml of LB Miller broth (Fisher bioreagents, Pittsburgh, USA) and incubated at 37 °C for 2 hours. Once in the exponential phase (OD_{600nm} ≃0,2), 3 µl of 0,5 ng/ml Mitomycin C (Sigma, Darmstadt, Germany) were added to the cultures and incubated for 4 hours at 37°C with shaking (120 rpm). After incubation, the cultures were centrifuged at 16000 *g* for 10 minutes. 5 µl of supernatant were spotted on a double layer of LB agar plate prepared as follow: 4 mL of melted LB soft agar (0,5%) supplemented with $CaCl_2$ stock solution to a final concentration of 0,01 M and 100 µL of exponential-phase of two *E. coli* strains (CNCTC 6896 and CNCTC 6246) sensitive to bacteriophages were added to a 10 ml culture tube. The entire content of the tube was spread on a LB plate (1,5% w/w agar), creating a double layer. After cooling, 5 µL of supernatant were spotted on the agar surface and the liquid was allowed to dry. Then, the plates were incubated upright overnight at 37 °C. The presence of prophages was revealed by a lysis in bacterial layer.

4.3.3.3 Host range

All the bacteria that did not show the presence of inducible prophage in the previous experiment, were used for the determination of the host range of the bacteriophages. Phages have been isolated from cattle herds as described in 4.1.3.3. Bacteriophages were stored at 4°C in SM buffer (0,05 M Tris-HCl buffer, pH 7,5, with 0,1M NaCl, 0,008 M $MgSO_4$) before use. A single bacterial colony from EMBA plates was inoculated in 1 ml of LB broth. 100 µL of the bacterial culture in exponential phase (OD_{600nm} ≃0,2) were added to 4 ml of melted LB soft agar and 40 µL of $CaCl_2$ 1M and then poured in a LB agar plate. Afterwards, a drop of 5 µL of each bacteriophage (~10^9 PFU/ml) was spotted on the bacterial layer and plates were incubated for 24 hours at 37°C. Bacterial sensitivity was evaluated based on the formation of clarification zones: the formation of clear plaques was interpreted as high sensitivity to the phage, while that of turbid plaques as low sensitivity and no sign of plaques as absence of lysis. The test was performed in duplicate.

4.3.3.4 Phage sequencing

After a three steps isolation process, 1 ml of phage in SM buffer was subjected to enzymatic treatment with 20 U of DNase I and RNase I_f (New England, Biolabs, Ipswich, Massachusetts, USA) in order to remove fragments of bacterial free nucleic acid in the filtrate, while saving the DNA present in the phage capsid. After 40 minutes at 37°C, the enzymes were inactivated with heat treatment at 75°C for 10 minutes. Phage DNA was extracted with a Silica column (QIAamp UltraSens Virus Kit, Hilden, Germany) according to manufacturer's instructions. After extraction, the DNA concentration was determined by the fluorometer Assay (Qubit™) and the DNA samples were diluted to 0,2 ng/µL for the library preparation. This was performed using the Nextera XT Library Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Fragment size was checked with 2100 Bioanalyzer (Agilent Technologies) using a high-sensitivity DNA kit

(Agilent Technologies) and quantified with the Qubit dsDNA HS Assay Kit (Invitrogen). The resulting paired-end sequence reads were generated using an Illumina iSeq 100 system. The whole bacteriophage genome was assembled with SPAdes (St. Petersburg genome assembler) version 3.14.0 (de novo assembly).

4.3.3.4 Bacterial sequencing

A colony of resistant bacteria from EMBA plates was inoculated in 1 mL of LB broth. After overnight growth at 37°C, 100 µL of bacterial culture was used for DNA extraction with silica column (DNeasy Blood & Tissue Kits, QIAamp, Hilden, Germany) according to manufacturer's instructions. DNA concentration was established through a fluorometric Assay (Qubit™) and 1 ng of DNA was used for library preparation (Nextera XT Library Preparation Kit, Illumina, San Diego, CA) as reported above. The samples were loaded in a MiniSeq system (Illumina).

4.3.3.5 Electron microscopy of bacteriophage morphology

Electron microscopy was performed on bacteriophages LF2 and FM10 dissolved in SM buffer at a concentration of 10^9 PFU/ml. 20 µL of each sample were placed on a Parafilm®M layer. A copper grid was placed on each droplet of sample and let for 10 minutes (fig. 10).

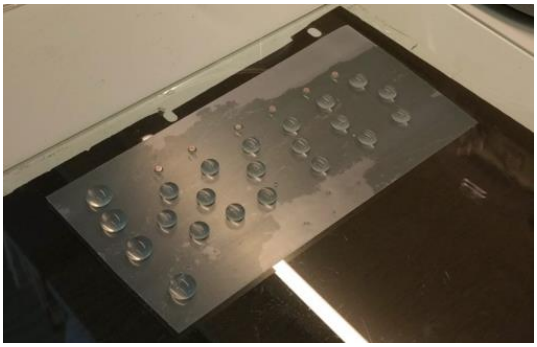


Figure 10. Copper grid containing phage samples.

Then, the grids were placed on 200 µL distilled water for 5 minutes; the operation was repeated three times. The grids were stained in with 2% uranyl acetate for 5 min and dried with a filter paper. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken with an Eagle 4 k (16 Megapixel) HS digital camera (FEI).

4.3.4 Results and discussion

4.3.4.1 Prophage induction

The collection of bacterial strains consisting of 327 bacteria (sup. table 1) isolated from patients with UTI infection was subjected to induction with Mytomicin C to test for the presence of prophages. The results indicated that 270 strains out of 327 (82,6%) did not have an inducible prophage since there was no sign of lysis in two different *E. coli* indicator strains. On the other side, 17,4% of bacteria showed a presence of prophage in both sensitive *E. coli* strains used.

4.3.4.2 Host range

Pathogenic *E. coli* strains, that did not show the presence of a temperate bacteriophage, were used to evaluate the ability of bacteriophages to lyse bacteria in *vitro* on LB plate. The induction step was done to avoid a masking result due to the release of prophage by bacteria and not by the addition of phage. UTI infections include cystitis and pyelonephritis and are estimated to be the second most common type of human infection; *E. coli* is the main bacterium associated with UTI (Tabasi *et al.,* 2015). The result showed that 262 out of 270 bacteria are sensitive to at least one bacteriophage (fig. 11). 8 *E. coli* strains showed to be resistant to 6 bacteriophages tested. Among the bacteria used in this work, 114 out of 265, that had information about antibiotic resistance, showed antidrug resistance to at least one antibiotic (sup. tab. 1). The antibiotic resistance information is provided for 265 out of 327 *E. coli* used (sup. tab. 1). Among the 8 phage resistant bacteria, 3 out of 7, showed antibiotic resistance, suggesting that it was not a relation between antimicrobial and phage resistance, different resistance mechanisms are involved. The high antimicrobial resistance in bacteria, also highlighted in our collection (43%), strengthens the hypothesis that bacteriophages can be a valid alternative or a support to antibiotics. Furthermore, as studied by Galtier

et al., (2018), the application of lytic bacteriophage to treat UPEC infection in murine model revealed much less impact on microbiota diversity than the use of antibiotics. Indeed, it has been largely reported that the composition of the microbiota influences several human disorders (Cenit *et al.,* 2017; Sampson *et al.,* 2016). Therefore, according to the specificity of bacteriophage application, their use could be helpful to reduce the use of antibiotics maintaining a preferable environment for human health. Each bacteriophage had different levels of efficacy and phage FM10 was the one with the widest host range being able to infect 242 out of 270 bacterial strains (fig. 11). In previous works on UPEC control through bacteriophage application, the best value for a single phage was 20 out of 53 (Freitag *et al.,* 2008) and with a phage cocktail to control UPEC was 10 out of 12 and 17 out of 21 (Manohar *et al.,* 2019). According to the large number of bacteria tested, the present result seems to be a valid application for controlling these pathogen bacteria.

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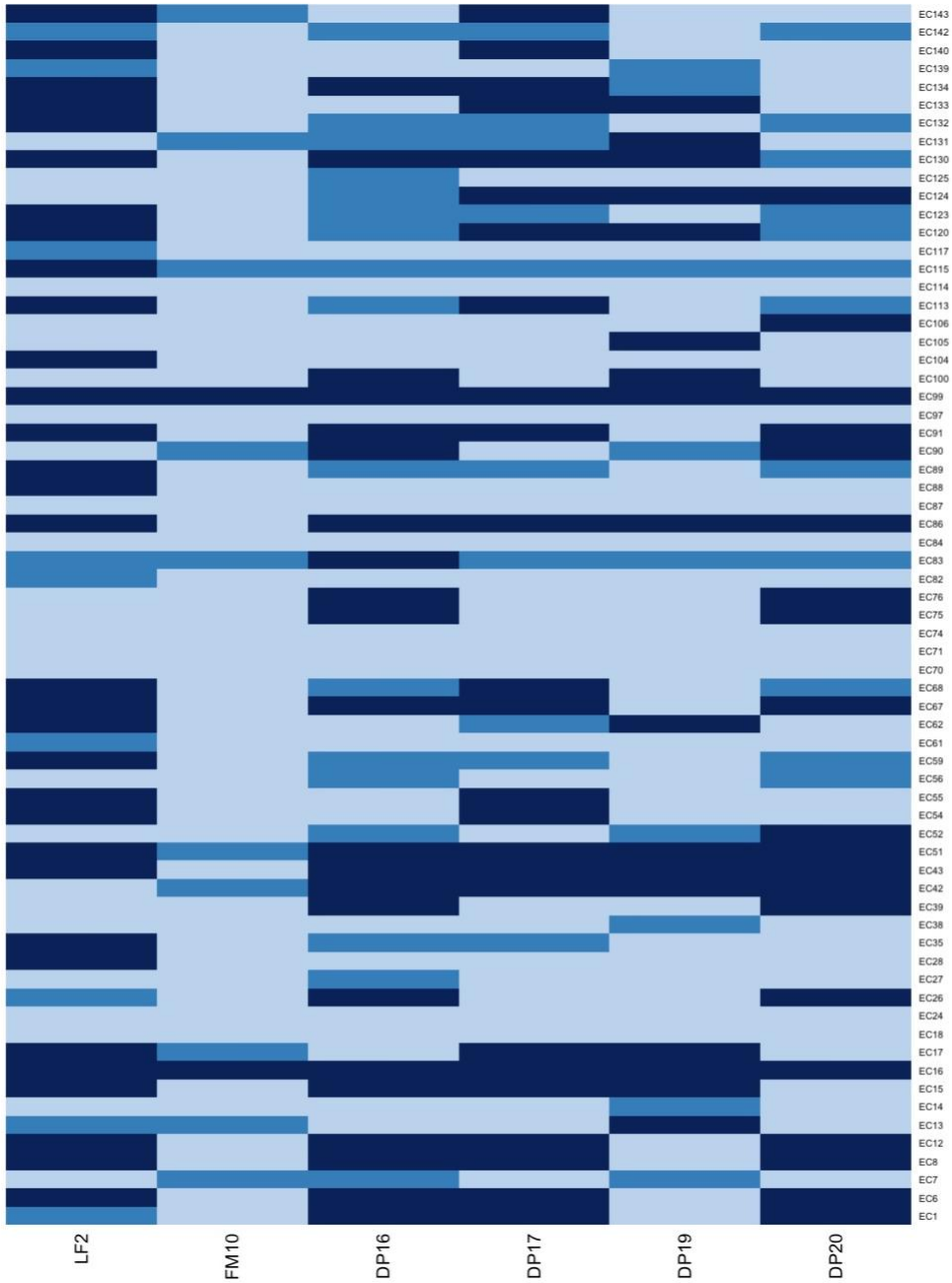
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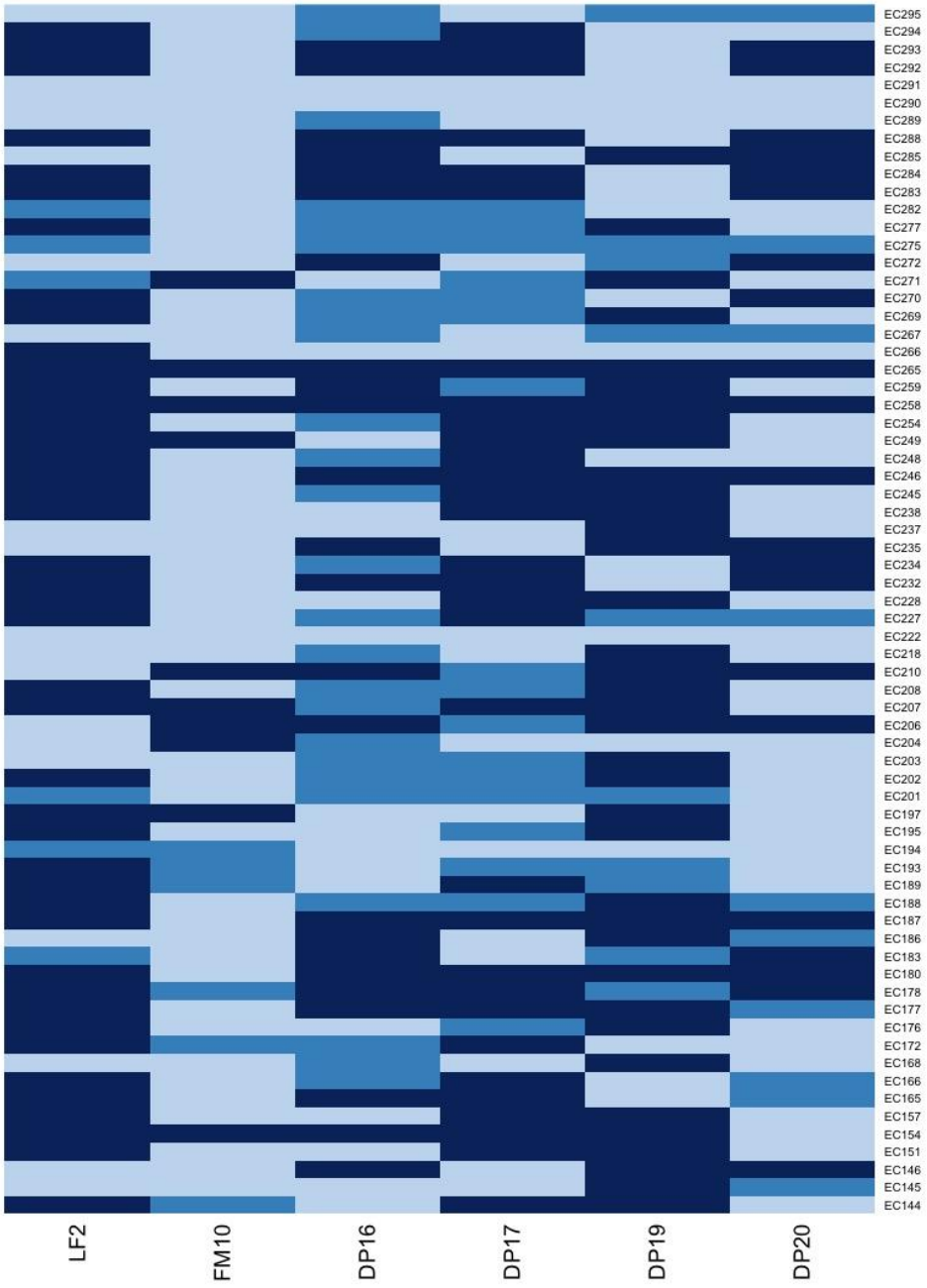
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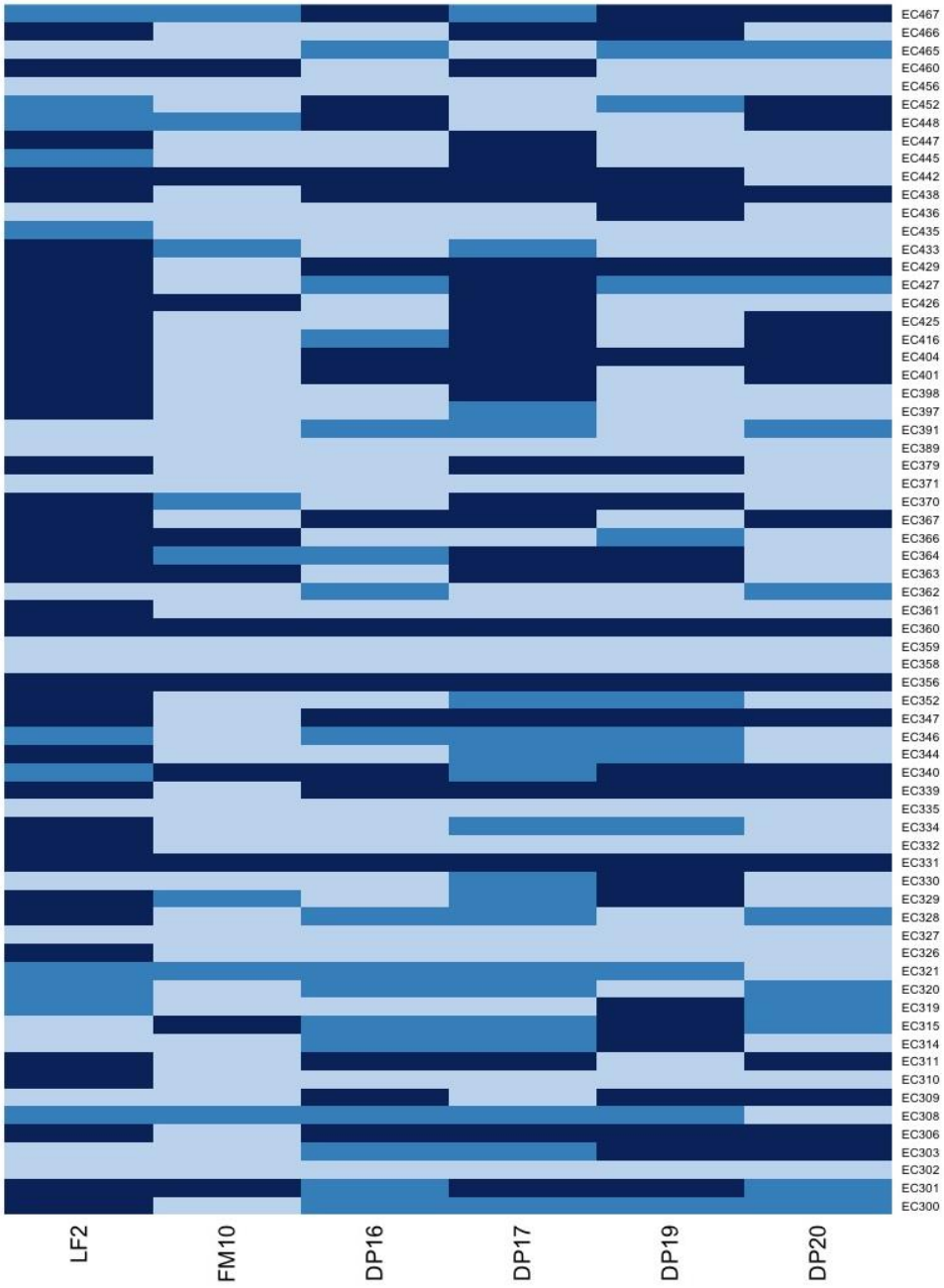
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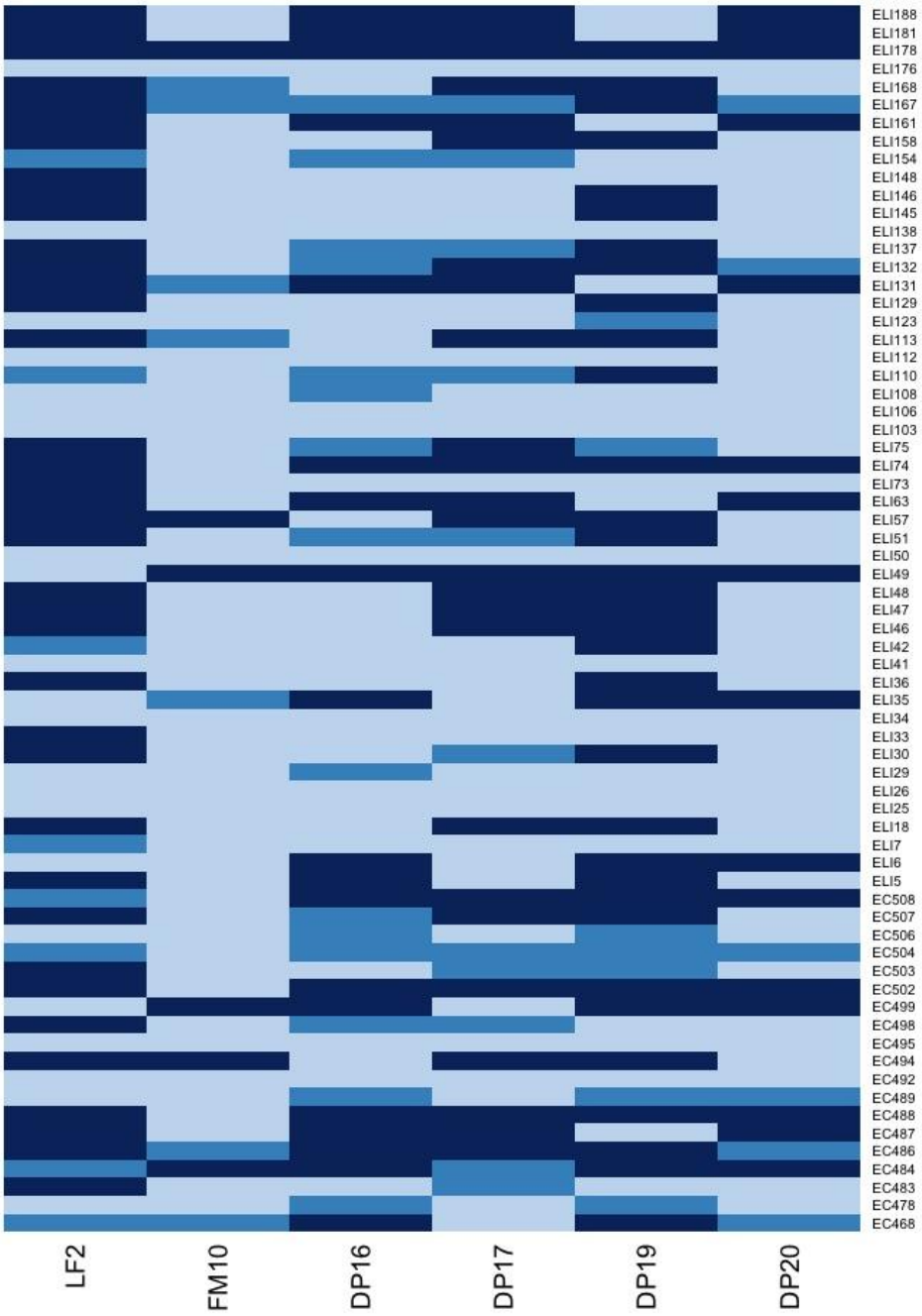
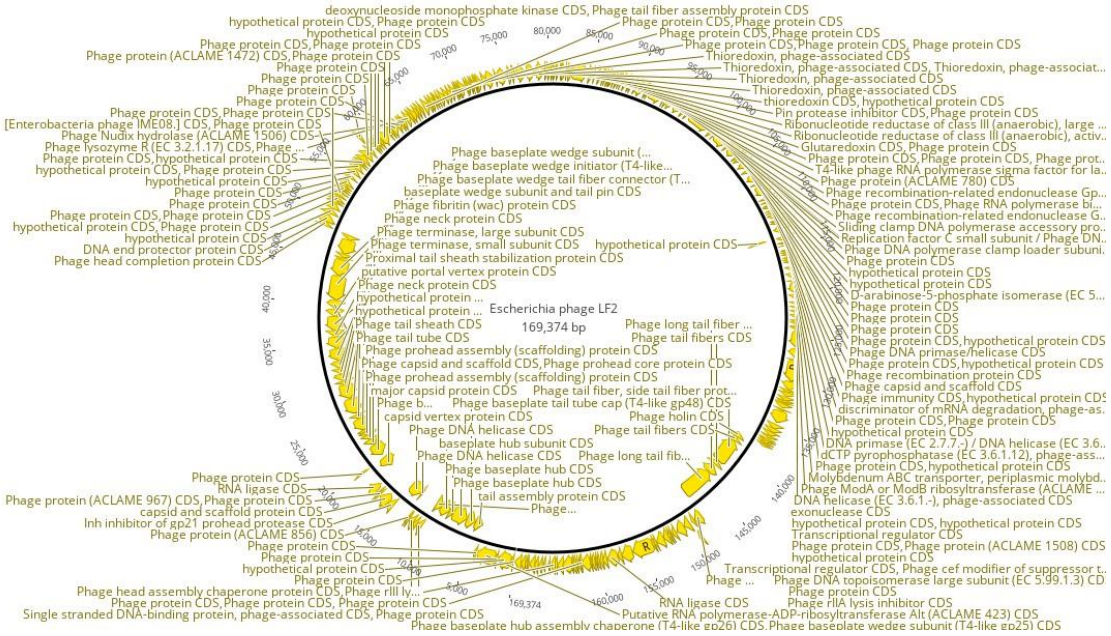


Figure 11. Heatmap of host range. On the x-axis bacteriophages used and on y-axis the bacteria used. White azure: lysis of bacteria; baby blue: turbid plaques; navy blue: no sign of lysis.

4.3.4.2 Phage genome sequencing

After the *de novo* assembling of the reads, the whole bacteriophage genomes were between 163’500 and 169’400 bp. Open Reading Frames (ORFs) were annotated and predicted with RAST webserver (https://rast.nmpdr.org/). The *E. coli* bacteriophage LF2 genome contains 273 coding sequences (CDS) (supplementary table 2), with a length of 169’374 bp (fig. 12a) and a guanine-cytosine content (GC-content) of 37.70%. The most related phage, according to PHAST search (http://phast.wishartlab.com), is *Escherichia* phage APCEc01 (Sequence ID: NC_029091.1) with 231 out of 273 CDS in common. The FM10 bacteriophage genome has 272 CDS (supplementary table 3), a GC-content of 40.64% and a length of 163’589 bp (fig. 12b). According to PHAST the most related phage (PHAGE_Entero_RB49_NC_005066) has 263 out of 272 CDS in common.

(a)



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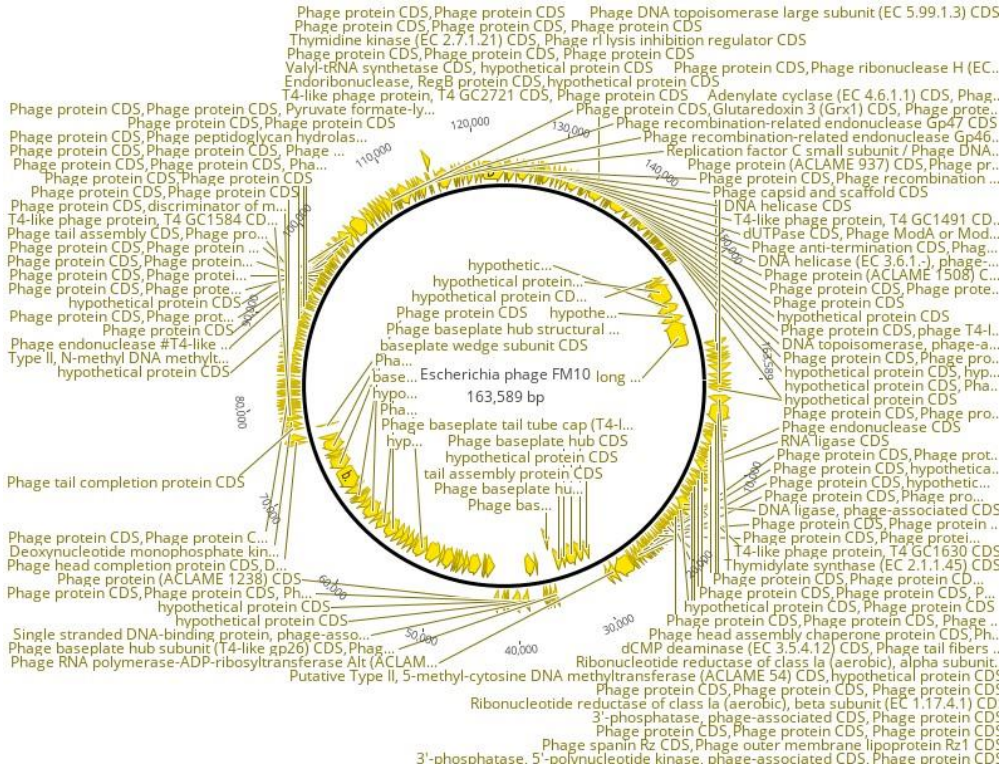

Figure 12. Whole genomes of LF2 (a) and FM10 (b) E. coli bacteriophages. Image elaboration by Geneious Prime 2020.

As concern the analysis of bacteriophage phylogeny there is no universal conserved gene for all phages, such as in bacterial taxonomic analyses where is it possible to use PCR-amplification of the 16S rDNA. Phylogenetic trees can be built on the basis of the conserved nucleotide sequences of the different genes that are present in the genome under study such as major capsid protein, terminase (Hylling *et al.,* 2020), neck protein (Lopes *et al.,* 2014). To establish the phylogenetic relationships among the isolated bacteriophages, three different trees were constructed according to the Jukes-Cantor genetic distance model using the Neighbor-Joining method on the common genes encoding for: major capsid, neck protein and large terminase subunit (fig. 13, 14 and 15) combining BLASTn and BLASTp phage

hits of phage. As described in the tree, bacteriophages FM10, DP16, DP17, DP19 and DP20 belonged to the same cluster in the major capsid phage tree (fig. 13) and large terminase subunit tree (fig. 15). In the neck protein tree (fig. 14) FM10 and DP17 belonged to the same cluster and DP16, DP19 and DP20 to another one, but they were closely related. FM2 was in different cluster in all the proposed trees. However, the bacteriophages used were closely related to *Escherichia coli* phages used in phage therapy (Dalmasso *et al.,* 2016).

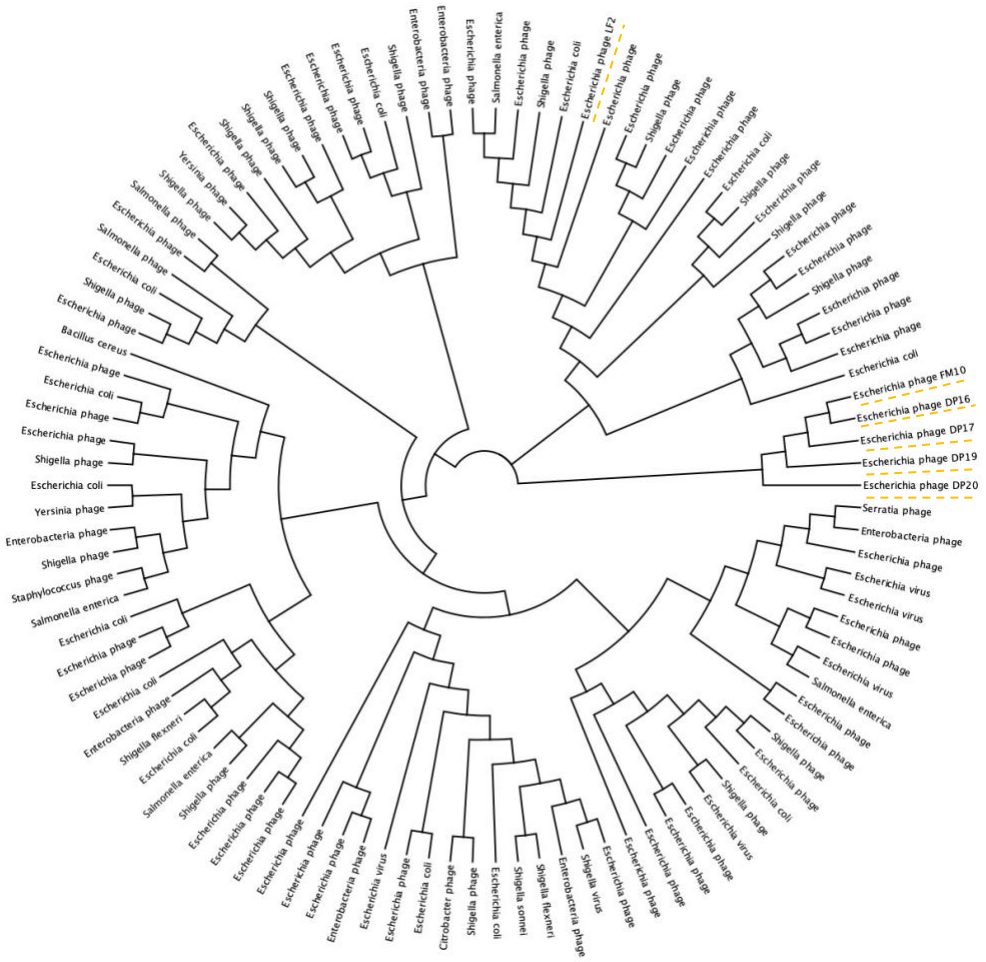

Figure 13. Phylogenetic tree of major capsid protein. Image elaboration by Geneious Prime 2020 (https://www.geneious.com).

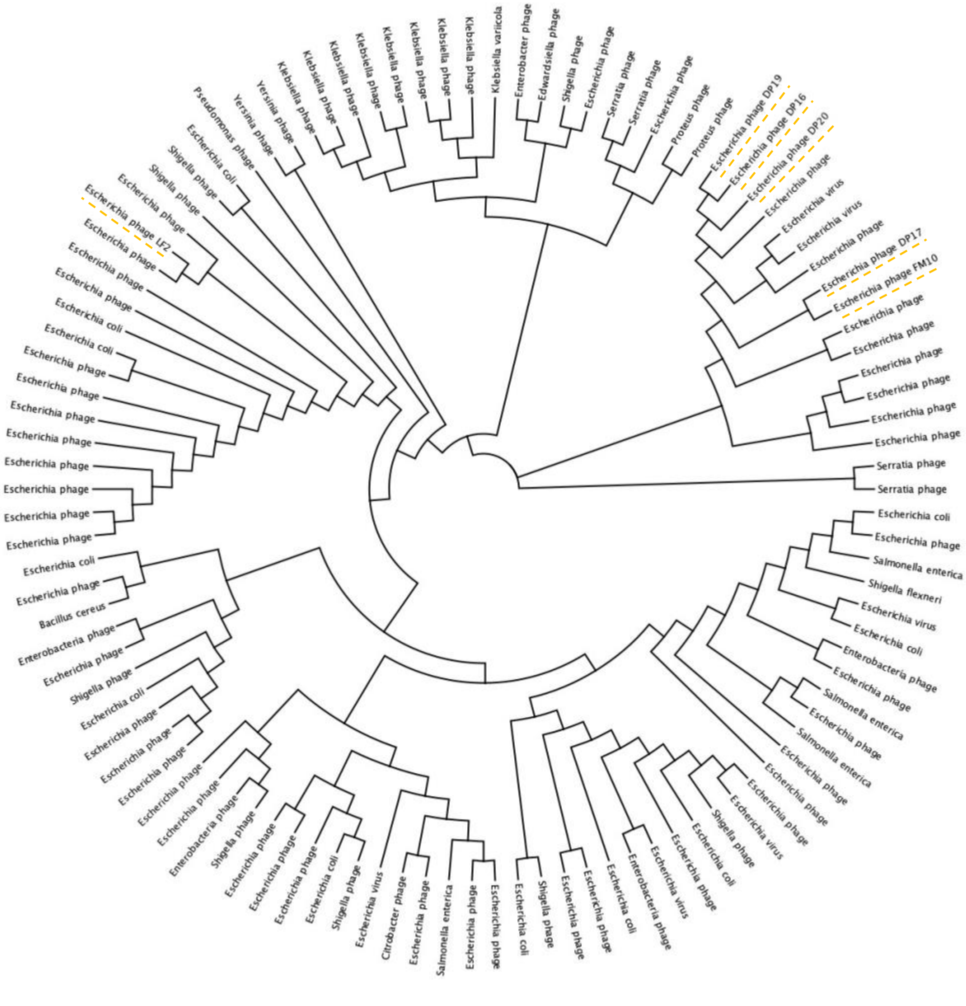

Figure 14. Phylogenetic tree of phage neck protein. Image elaboration by Geneious Prime 2020 (https://www.geneious.com).

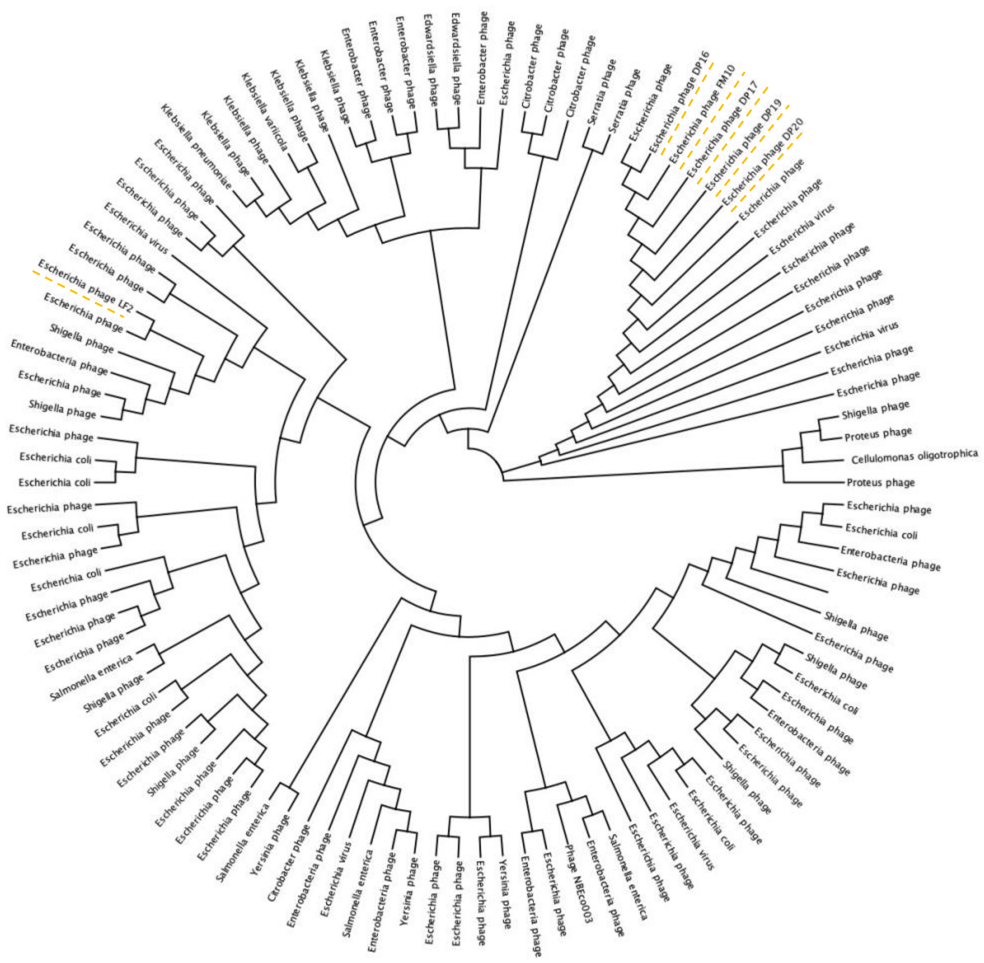

Figure 15. Tree of phage large terminase subunit. Image elaboration by Geneious Prime 2020 (https://www.geneious.com).

According to fig. 16 and 17, all the bacteriophages used in this work belonging to *Myoviridae* family, order of the *Caudovirales*. *Caudovirales* is the most abundant order, accounting for 96% of the reported bacterial virus. T4 bacteriophages also belong to the *Myoviridae* family (King *et al*., 2012). The proteomic trees were based on the first 100 genome-wide sequence similarities computed by tBLASTx using VIPtree (Nishimura *et al.,* 2017).

Trees of LF2 and FM10, that is close related to DP16, DP17, DP19 and DP20, are shown.

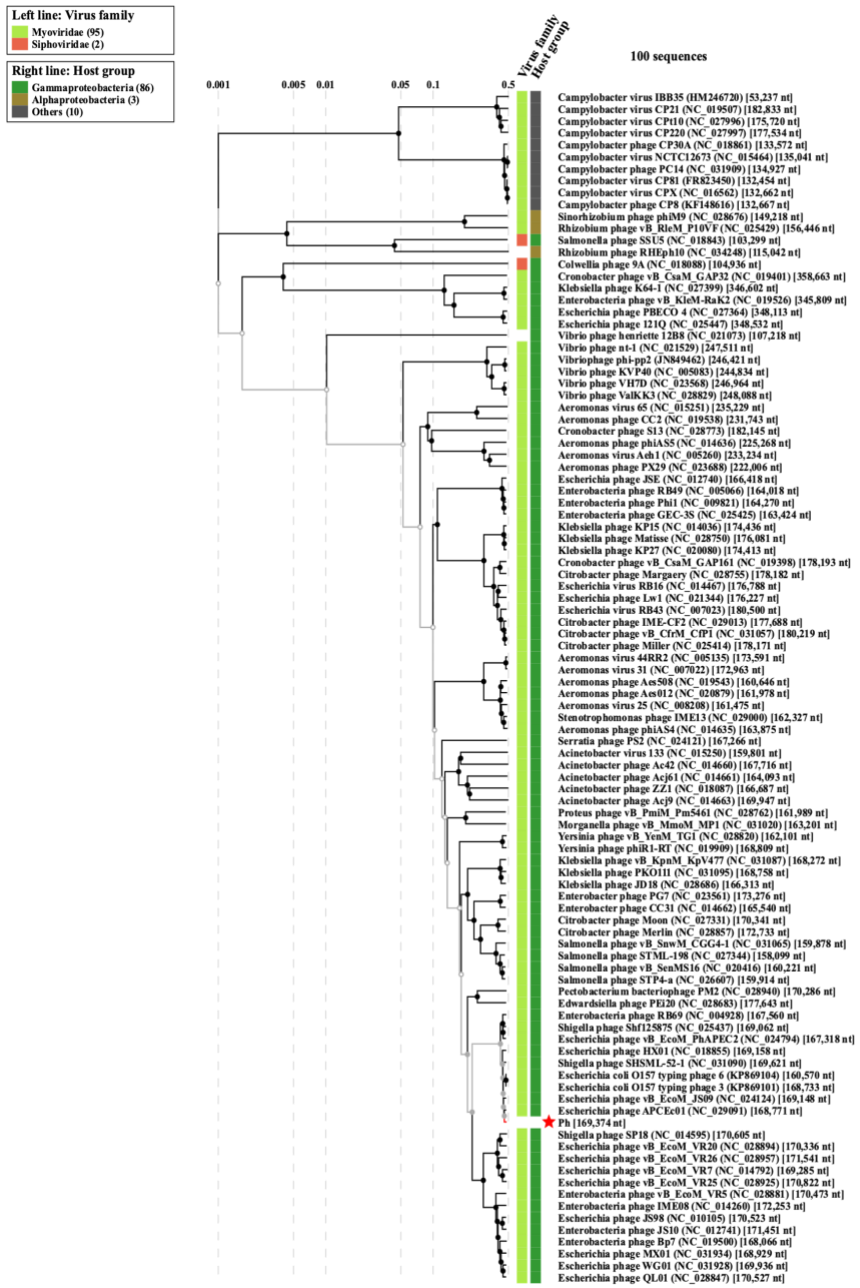

Figure 16. Proteomic tree of viral genome sequences based on the first 100 genome-wide sequence similarities computed by tBLASTx. The red star represents the bacteriophage LF2. Generate by ViPTree: the Viral Proteomic Tree server version 1.9.

Figure 17. Proteomic tree of viral genome sequences based on the first 100 genome-wide sequence similarities computed by tBLASTx. The red star represents the bacteriophage FM10. Generate by ViPTree: the Viral Proteomic Tree server version 1.9.

The absence of virulence and lysogenic genes is mandatory for the application of bacteriophage as biocontrol agents (Brussow, 2012). Observing the annotations reported in the supplementary tables 2 and 3, there were no CDS related to pathogenicity island, toxin production, antibiotic resistance genes, or lysogenic cycle. Therefore, it is possible to think of these phages as suitable candidates for phage therapy. In general, in studies in which bacteriophages are applied to humans (phage therapy), no cause of infection and no phage sequences have been identified in the treated people. Furthermore, no adverse health effects have been reported in the application of phages to humans (ANSES, 2014). The risk related to their application could be the transmission of genes in the host microbiota but, according to whole genome sequencing, no genes related to the transmission and/or lysogenesis were found in the bacteriophages used in this study. However, not all the CDS were assigned to a specific function or reported as hypothetical protein. Further studies need to be done before application to humans.

4.3.4.3 Bacterial sequencing

The reads generated by MiniSeq (Illumina) were assembled through Geneious software (https://www.geneious.com) using *E. coli* strain K-12 (substr. MG1655, complete genome) as reference. The bacterial genomes sequenced were chosen among the ones completely resistant to bacteriophages (fig. 11). The whole genomes of bacteria sequenced, composed by seven resistant ones plus one sensitive (EC71), had a length among 3’904’141 and 4’388’813 bp with a GC content of approximately 51% (tab. 7).

*Table 7. Bacteria sequenced; length of whole genome; Guanine-Cytosine (GC) content %; *sensitive bacteria to bacteriophages.*

Sequenced bacteria	Length	GC content %
EC16	4076380 bp	51.1
EC71*	4095911 bp	51.1
EC99	3904141 bp	49.7
EC258	4326314 bp	51
EC331	4419478 bp	50.9
EC356	4388813 bp	51
EC360	4092071 bp	51.1
EC265	4330577 bp	51.1

The aim of this part was to find out the factors related to phage resistance. Bacteria and bacteriophage have evolved together: bacteria have developed different mechanisms such as restriction-modification systems, abortive infection, clustered regularly interspaced short palindromic repeats (CRISPR) that can target and eliminate external DNA of plasmids and phages, mutation in specific surface receptor like proteins, polysaccharides, or lipopolysaccharides (LPS) present on the cell surface (Shabbir *et al.,* 2016). Several mechanisms involved in phage resistance were found in the bacteria studied. In particular, the entire genomes of the analyzed bacteria were submitted to CRISPRfinder tool and 34 different CRISPR region were found (https://crispr.i2bc.paris-saclay.fr/Server/). Some regions were present in more than one bacterium, but no trace of the corresponding spacers was reported in the bacteriophages used. It was not possible to find a relationship between resistant bacteria and bacteriophages used in this work using a metagenomic approach.

4.3.4.4 Bacteriophage morphology

Morphology was analyzed for bacteriophages LF2 and FM10 using TEM microscopy. TEM images showed that both bacteriophages had an icosahedral head and a contractile tail (fig. 18, 19 and 20). As can be seen, bacteriophage LF2 had a contracted tail in figure 18 with a length of about 143 nm and a relaxed tail in figure 19 with a length of approximately 225 nm. The bacteriophage FM10 had a length of about 244 nm. Then, according to phylogenetic trees (fig. 16 and 17) and the images, the two bacteriophages belong to *Myoviridae* family order of *Caudovirales*. Observing these images, it is not possible to determine the number of the short tail fibers.

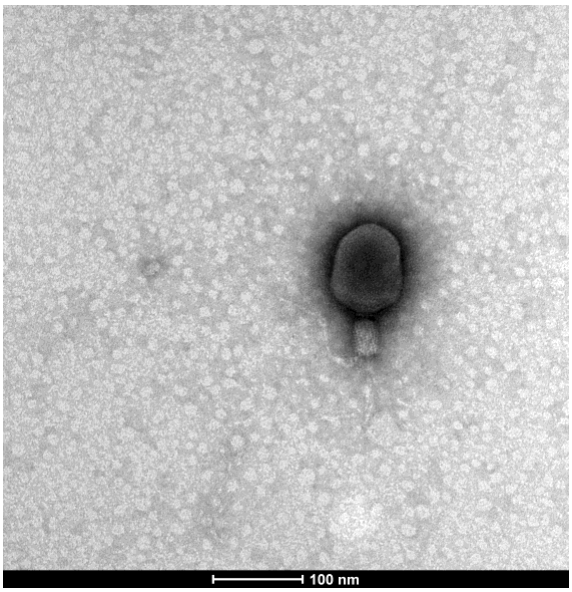

Figure 18. TEM image of bacteriophage LF2 with a contracted tail.

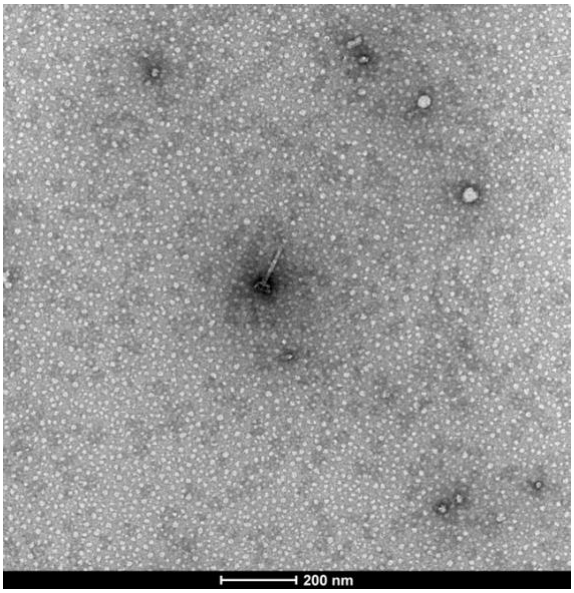

Figure 19. TEM image of bacteriophage LF2 with a relaxed tail.

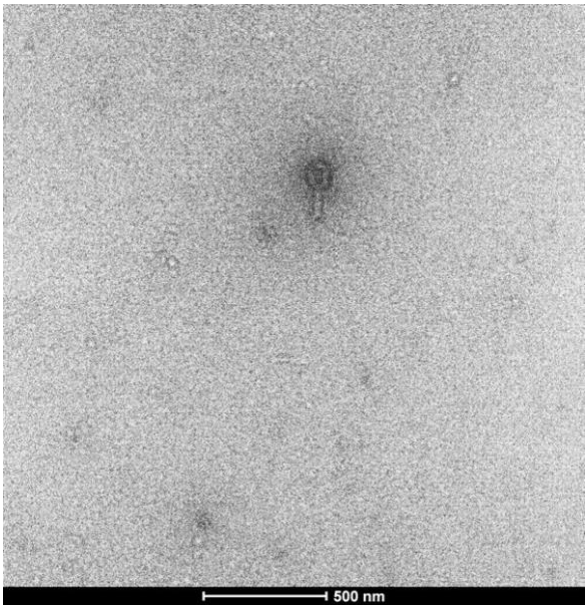

Figure 20. TEM image of bacteriophage FM10.

4.3.5 References

ANSES (French Agency for Food, Environmental and Occupational Health & Safety), 2014. Opinion of ANSES on the use of bacteriophages in foods of animal origin to control Listeria. 27 pp. Available online: https://www.anses.fr/en/system/files/BIORISK2013sa0111.pdf

Azam, A.H., Tanji, Y., 2019. Bacteriophage-host arm race: an update on the mechanism of phage resistance in bacteria and revenge of the phage with the perspective for phage therapy. Appl. Microbiol. Biotechnol. 103, 2121–2131. https://doi.org/10.1007/s00253-019-09629-x

Brussow, H., 2012. What is needed for phage therapy to become a reality in Western medicine? Virology; 434(2):138–42. pmid:23059181. doi: 10.1016/j.virol.2012.09.015.

Cenit, M. C., Sanz, Y., Codoñer-Franch, P., 2017. Influence of gut microbiota on neuropsychiatric disorders. World journal of gastroenterology, 23(30), 5486. https://dx.doi.org/10.3748%2Fwjg.v23.i30.5486

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., Finlay, B. B., 2013. Recent Advances in Understanding Enteric Pathogenic *Escherichia coli*. Clinical Microbiology Reviews, 26(4), 822–880. https://doi.org/10.1128/cmr.00022-13

Dale, A. P., Woodford, N., 2015. Extra-intestinal pathogenic *Escherichia coli* (ExPEC): disease, carriage and clones. *Journal of Infection*, *71*(6), 615-626. https://doi.org/10.1016/j.jinf.2015.09.009

Dalmasso, M., Strain, R., Neve, H., Franz, C. M., Cousin, F. J., Ross, R. P., Hill, C., 2016. Three new *Escherichia coli* phages from the human gut show promising potential for phage therapy. PloS one, 11(6), e0156773. doi:10.1371/journal.pone.0156773

Galtier, M., De Sordi, L., Maura, D., Arachchi, H., Volant, S., Dillies, M. A., Debarbieux, L., 2016. Bacteriophages to reduce gut carriage of antibiotic resistant uropathogens with low impact on microbiota composition. Environmental microbiology, 18(7), 2237-2245. https://doi.org/10.1111/1462-2920.13284

Hylling, O., Carstens, A. B., Kot, W., Hansen, M., Neve, H., Franz, C. M., Johansen, A., Hansen, L. H., 2020. Two novel bacteriophage genera from a groundwater reservoir highlight subsurface environments as underexplored biotopes in bacteriophage ecology. Scientific reports, 10(1), 1-9. https://doi.org/10.1038/s41598-020-68389-1

Hooton, T. M., 2012. Uncomplicated urinary tract infection. New England Journal of Medicine, 366(11), 1028-1037. doi: 10.1056/NEJMcp1104429

Flores-Mireles, A. L., Walker, J. N., Caparon, M., Hultgren, S. J., 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat. Rev. Microbiol. 13, 269–284. doi: 10.1038/nrmicro3432

Freitag, T., Squires, R. A., Schmid, J., 2008. Naturally occurring bacteriophages lyse a large proportion of canine and feline uropathogenic *Escherichia coli* isolates in vitro. Research in veterinary science, 85(1), 1-7. https://doi.org/10.1016/j.rvsc.2007.09.004

Lin, D. M., Koskella, B., Lin, H. C., 2017. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World journal of gastrointestinal

pharmacology and therapeutics, 8(3), 162. https://dx.doi.org/10.4292%2Fwjgpt.v8.i3.162

Lopes, A., Tavares, P., Petit, M. A., Guérois, R., Zinn-Justin, S., 2014. Automated classification of tailed bacteriophages according to their neck organization. BMC genomics, 15(1), 1027. doi: 10.1186/1471-2164-15-1027.

Kaper, J. B., Nataro, J. P., Mobley, H. L., 2004. Pathogenic *Escherichia coli*. Nature reviews microbiology, 2(2), 123-140. doi: 10.1038/nrmicro818

King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., 2012. Virus Taxonomy. San Diego, Elsevier.

Köhler, C. D., Dobrindt, U., 2011. What defines extraintestinal pathogenic *Escherichia coli*? International Journal of Medical Microbiology, 301(8), 642-647. doi: 10.1016/j.ijmm.2011.09.006.

Manohar, P., Tamhankar, A. J., Lundborg, C. S., Nachimuthu, R., 2019. Therapeutic characterization and efficacy of bacteriophage cocktails infecting *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* species. Frontiers in Microbiology, 10, 574. https://doi.org/10.3389/fmicb.2019.00574

Micali, S., Isgro, G., Bianchi, G., Miceli, N., Calapai, G., Navarra, M., 2014. Cranberry and recurrent cystitis: more than marketing? *Crit. Rev. Food Sci. Nutr.* 54, 1063–1075. doi: 10.1080/10408398.2011.625574

Meier, S., Weber, R., Zbinden, R., Ruef, C, Hasse, B., 2011. Extended-spectrum β-lactamase-producing Gram-negative pathogens in community-acquired urinary tract infections: an increasing challenge for antimicrobial therapy. Infection; 39:333-340. doi: 10.1007/s15010-011-0132-6.

Nicolle, L.E., 2011. Update in adult urinary tract infection. Curr. Infect. Dis. Rep.; 13:552-560. doi: 10.1007/s11908-011-0212-x.

Nishimura, Y., Yoshida, T., Kuronishi, M., Uehara, H., Ogata, H., Goto, S., 2017. ViPTree: the viral proteomic tree server. Bioinformatics. 1;33(15):2379-2380. doi: 10.1093/bioinformatics/btx157. PMID: 28379287.

Peirano, G., Mulvey, G. L., Armstrong, G. D., Pitout, J. D., 2013. Virulence potential and adherence properties of *Escherichia coli* that produce CTX-M and NDM β-lactamases. Journal of medical microbiology, 62(4), 525-530. https://doi.org/10.1099/jmm.0.048983-0

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., Challis, C., Schretter C. E., Rocha, S., Gradinaru, V., Chesselet, M. F., Keshavarzian, A., Shannon, K. M., Krajmalnik-Brown, R., Wittung-Stafshede, P., Knight, R., Mazmanian, S. K., 2016. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. Cell, 167(6), 1469-1480. https://doi.org/10.1016/j.cell.2016.11.018

Shabbir, M. A., Hao, H., Shabbir, M. Z., Wu, Q., Sattar, A., Yuan, Z., 2016. Bacteria vs. bacteriophages: parallel evolution of immune arsenals. Frontiers in microbiology, 7, 1292. https://doi.org/10.3389/fmicb.2016.01292

Sybesma, W., Zbinden R., Chanishvili, N., Kutateladze, M., Chkhotua A., Ujmajuridze A., Mehnert U., Kessler T.M., 2016. Bacteriophages as Potential Treatment for Urinary Tract Infections. Front. Microbiol. 7:465. doi: 10.3389/fmicb.2016.00465

Tabasi, M., Karam, M. R. A., Habibi, M., Yekaninejad, M. S., Bouzari, S., 2015. Phenotypic assays to determine virulence factors of uropathogenic *Escherichia coli* (UPEC) isolates and their correlation with antibiotic

resistance pattern. Osong public health and research perspectives, 6(4), 261-268. https://doi.org/10.1016/j.phrp.2015.08.002

Terlizzi, M. E., Gribaudo, G., Maffei, M. E., 2017. UroPathogenic *Escherichia coli* (UPEC) infections: virulence factors, bladder responses, antibiotic, and non-antibiotic antimicrobial strategies. Front. Microbiol., 8, 1566. https://doi.org/10.3389/fmicb.2017.01566

US Food and Drug Administration, 2006. “Food additives permitted for direct addition to food for human consumption; bacteriophage preparation”. Retrieved from https://www.govinfo.gov/content/pkg/FR-2006-08-18/pdf/E6-13621.pdf

Yap, M. L., Rossmann, M. G., 2014. Structure and function of bacteriophage T4. Future Microbiol. 9, 1319–1327. doi: 10.2217/fmb.14.91

Supplementary table 1. Characteristics of *E. coli* used in this work. In prophage column, x: absence of prophage; v: presence of prophage.

Strain	Pro-phage	Age	Sex	Antibiotic Resistance	Source
EC01	x	48	M	Ceftazidime, Ciprofloxacin, Ceftriaxone, Cefazolin, Cefepime, Cefoxitin, Ampicillin/Sulbactam, Piperacillin/Tazobactam	Body tissue, left middle finger tissue
EC06	x	90	F	ampicillin	urine
EC07	x	82	M	none	urine
EC08	x	44	F	ampicilline, Trimethoprim/Sulfamethoxazole	urine
EC11	v	80	F	none	urine
EC12	x	71	M	ciprofloxacin, cefoxitin, gentamicin, tobramycin	urine
EC13	x	67	F	none	urine
EC14	x	58	F	none	urine
EC15	x	81	F	none	urine
EC16	x	71	F	Trimethoprim/Sulfamethoxazole	urine
EC17	x	50	F	ampicillin, cefazolin	body tissue, right breast
EC18	x	76	M	ciprofloxacin, ampicillin, trimethoprim/sulfamethoxazole	sputum
EC24	x	33	M	Ampicillin, Ciprofloxacin, Trimethoprim/Sulfamethoxazole	urine
EC26	x	37	M	Ampicillin, Trimethoprim/Sulfamethoxazole	urine
EC27	x	50	F	Ampicillin, Trimethoprim/Sulfamethoxazole	urine
EC28	x	33	F	Ampicillin	urine
EC35	x	47	F	ampicillin	urine
EC38	x	58	F	none	urine
EC39	x	21	F	none	urine
EC41	v	54	F	none	urine
EC42	x	74	M	intermediate resistance to ampicillin	blood
EC43	x	55	F	ampicillin	blood
EC48	v	88	F	Ampicillin, Ciprofloxacin, Gentamicin, Tobramycin, Trimethoprim/sulfamethoxazole	urine
EC49	v	51	F	Ampicillin, Ciprofloxacin, Trimethoprim/sulfamethoxazole	urine
EC51	x	33	F	ampicillin, trimethoprim/sulfamethoxazole	urine
EC52	x	64	F	ampicillin	urine
EC54	x	20	F	ampicillin, sulbactam, trimethoprim/sulfamethoxazole	urine
EC55	x	24	M	none	urine
EC56	x	31	F	ampicillin, trimethoprim/sulfamethoxazole	urine
EC59	x	54	F	none	peritoneal fluid abdomen
EC61	x	66	M	ampicillin, trimethoprim/sulfamethoxazole	bone sacrum
EC62	x	70	F	none	sputum
EC63	v	29	F	ampicillin	urine

EC67	x	54	M	ciprofloxacin, gentamicin, tobramycin	urine
EC68	x				
EC70	x	87	F	none	urine
EC71	x	92	M	ampicillin, ciprofloxacin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	urine
EC72	x	35	F	none	urine
EC74	x	59	F	trimethoprim/sulfamethoxazole	urine
EC75	x	50	F	none	urine
EC76	x	69	F	none	urine
EC77	v	30	F	none	urine
EC82	x	63	F	ampicillin, ceftriaxone, cefazolin, cefoxitin, ampicillin/sulbactam	urine
EC83	x	64	M	none	urine
EC84	x	68	F	ampicillin, trimethoprim/sulfamethoxazole	urine
EC86	x	37	F	none	urine
EC87	x	82	F	none	urine
EC88	x	28	F	none	urine
EC89	x	37	F	ampicillin, trimethoprim/sulfamethoxazole	urine
EC90	x	88	F	nitrofurantoin	urine
EC91	x	33	M	ampicillin, cefazolin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	urine
EC96	v	62	F	ampicillin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	blood
EC97	x	62	F	ampicillin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	blood
EC99	x	32	F	none	urine
EC100	x	84	F	none	urine
EC103	v	86	F	none	urine
EC104	x	56	F	none	urine
EC105	x	52	M	ceftriaxone, certazidime, cefazolin, cefepime, cefoxitin, ampicillin/sulbactam, piperacillin/tazobactam, ampicillin	urine
EC106	x	77	F	none	urine
EC107	v	64	F	none	urine
EC110	v	32	F	ampicillin	urine
EC111	v	49	F	ampicillin, cefazolin, cefoxitin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole, piperacillin/tazobactam	blood
EC112	v	32	F	ampicillin	urine
EC113	x	69	M	ampicillin, ciprofloxacin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	blood
EC114	x	52	M	ceftazidime, ceftriaxone, cefazolin, cefepime, cefoxitin, ampicillin/sulbactam, piperacillin/tazobactam, ampicillin	urine
EC115	x	44	F	trimethoprim/sulfamethoxazole	urine
EC117	x	75	F	ampicillin, ceftazidime, ceftriaxone, cefazolin, cefepime, cefoxitin, ampicillin/sulbactam, piperacillin/tazobactam	blood
EC120	x	57	F	none	urine
EC123	x	75	F	none	urine

EC124	x	66	F	ampicillin, cipro, cefox, gentamicin, tobramycin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	urine
EC125	x	84	F	none	urine
EC126	v	44	M	none	urine
EC130	x	41	F	none	urine
EC131	x	81	F	ampicillin, ampicillin/sulbactam	urine
EC132	x	42	F	ampicillin, ampicillin/sulbactam	urine
EC133	x	34	F	none	urine
EC134	x	74	M	meropenem	urine
EC139	x	86	F	none	urine
EC140	x	80	F	none	urine
EC142	x	75	F	none	urine
EC143	x	30	F	ampicillin, ampicillin/sulbactam	urine
EC144	x	64	F	ceftazidime, ceftriaxone, cefazolin, cefepime, cefoxitin, ampicillin/sulbactam, piperacillin/tazobactam, ampicillin	urine
EC145	x	52	F	ertapenem	urine
EC146	x	86	F	ampicillin, gentamicin, tobramycin, ampicillin.sulbactam	urine
EC149	v	56	F	none	urine
EC151	x	84	M	none	urine
EC152	v	38	F	ampicillin, ampicillin/sulbactam	urine
EC154	x	50	F	ampicillin, cefazolin, cefoxitin, ampicillin/sulbactam	body site breast, right post anitbiotics
EC157	x	69	M	ampicillin, cefazolin, cefoxitin, ampicillin/sulbactam	body site esophagus
EC165	x	36	F	none	urine
EC166	x	80	F	none	urine
EC168	x	67	F	none	expectorated sputum
EC172	x	66	F	ampicillin	urine
EC173	v	84	F	ampicillin, cipro, ampicillin/sulbactam	urine
EC176	x	87	F	ampicillin, gentamicin, tobramycin, ampicillin/sulbactam	urine
EC177	x	90	F	ampicillin, cipro, cefazolin, gentamicin, tobramycin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	urine
EC178	x	23	F	ampicillin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	urine
EC180	x	67	F	none	expectorated sputum
EC183	x	43	F	none	urine
EC186	x	84	F	none	urine
EC187	x	61	F	none	urine
EC188	x	68	M	none	urine
EC189	x	53	M	none	urine
EC190	v	49	F	none	urine
EC193	x	55	M	gentamicin, tobramycin	blood

EC194	x	55	M	ampicillin, cipro, gentamicin, tobramycin, trimethoprim/sulfamethoxazole	blood
EC195	x	30	M	ampicillin, cipro, ceftazolin, cefoxitin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole, piperacillin/tazobactam	blood
EC196	v	73	M	ampicillin, ceftazidime, cipro, ceftriaxone, cefazolin, cefepime, cefoxitin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole, piperacillin/tazobactam	sputum
EC197	x	50	F	ampicillin, cefazolin, cefoxitin, ampicillin/sulbactam	body tissue breast, right
EC201	x	79	F	none	urine
EC202	x	71	F	none	urine
EC203	x	64	F	ceftazidime, cipro, ceftriaxone, cefazolin, cefepime, cefoxitin, gentamicin, tobramycin, ampicllin/sulbactam, trimethoprim/sulfamethoxazole, piperacillin/tazobactam	urine
EC204	x	75	M	ampicllin, gentamicin	urine
EC205	v	64	F	none	urine
EC206	x	66	F	none	urine
EC207	x	67	F	none	urine
EC208	x	88	F	none	urine
EC210	x	45	F	ampicillin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole	urine
EC218	x	53	F	none	urine
EC222	x	73	M	cipro, cefox,	urine
EC227	x	57	M	none	urine
EC228	x	54	F	none	urine
EC229	v	54	F	none	urine
EC232	x	72	M	none	urine
EC234	x	51	F	none	urine
EC235	x	64	F	none	urine
EC237	x	58	F	none	urine
EC238	x	54	F	none	urine
EC242	v	70	M	none	urine
EC245	x	62	M	none	body site knee, left
EC246	x	70	F	amp, apm/sulbact, trimeth/sulfa	urine
EC247	v	31	F	amp, amp/sulbact, cefazolin, cefox, trimeth/sulfa, cefepime, ceftazidime, cipro	urine
EC248	x	95	M	amp, cefazolin, amp/sulbact	urine
EC249	x	95	M	amp, cefazolin, cefox, amp/sulbact	urine
EC250	v	48	F	amp, cefox, amp/sulbact	urine
EC254	x	46	M	none	urine
EC257	v	43	F	none	urine
EC258	x	26	F	amp, amp/sulbact	urine
EC259	x	64	F	none	urine
EC260	v	68	F	none	urine

EC265	x	60	M	none	expectorated sputum
EC266	x	66	F	amp, ceftazidime, cipro, ceftriaxone, cefaz, cefepime, cefox, genta, tobra, amp/sulbat, trimeth/sulfa, piper/tazo	body tissue right, deep wound tissue femur
EC267	x	73	F	none	aspirate axilla, left
EC269	x	21	M	ceftazidime, cipro, ceftriax, cefaz, cefepime, cefox, genta, tobra, amp/sulbact, trimeth/sulfa, piper/tazo	expectorated sputum - cystic fibrosis
EC270	x	26	F	amp, amp/sulbact, trimeth/sulfa	body site cervix
EC271	x	32	F	amp, genta, tobray, trimeth/sulfa	urine
EC272	x	35	F	none	urine
EC275	x	39	F	N/A	urine
EC277	x	46	M	amp, ceftazidime, cipro, ceftriax, cefaz, cefepime, cefox, genta, tobra, amp/sulbac, trimeth/sulfa, piper/tazo	sputum ling, right
EC282	x	52	F	none	urine
EC283	x	83	F	none	urine
EC284	x	58	F	nitrofurantoin	urine
EC285	x	79	F	amp, cipro, genta, tobra, amp/sulbact	urine
EC288	x	87	F	none	urine
EC289	x	70	F	none	urine
EC290	x	75	F	amp, amp./sulbact	urine
EC291	x	34	F	amp, genta, amp/sulbact	urine
EC292	x	64	M	amp, amp/sulbact	urine
EC293	x	29	F	amp, amp/sulbact	urine
EC294	x	72	F	amp, amp/sulbact	urine
EC295	x	38	F	none	urine
EC300	x	32	M	ampicillin, cipro, genta, tobra, amp/sulbact, trimeth/sulfa	lesion leg
EC301	x	68	M	none	abscess anal skin e-swab
EC302	x	72	F	amp, cipro, amp/sulbact	urine
EC303	x	53	F	none	urine
EC306	x	39	M		urine
EC308	x	31	F	amp, cgenta, tobra, amp/sulbact, trimeth/sulfa	urine
EC309	x	36	F	none	urine
EC310	x	76	F	none	urine
EC311	x	71	M	cipro, genta, tobra	urine
EC314	x	59	M	none	blood
EC315	x	70	M	amp, cipro, ceftriax, cefaz, nitro, ceftaz	urine
EC319	x	31	F	none	urine
EC320	x	59	F	amp, amp/sulbact, trimeth/sulfa	urine
EC321	x	64	F	amp, cipro, cefaz, genta, tobra, amp.sulbact, trimeth/sulfa	urine

EC326	x	73	F	cipro, trimeth/sulfa	urine
EC327	x	70	F	none	urine
EC328	x	60	F	amp, amp/sulbact	urine
EC329	x	73	F	amp, trimeth/sulfa	urine
EC330	x	41	F	none	urine
EC331	x	12	F	none	urine
EC332	x	57	F	none	urine
EC334	x	19	F	none	urine
EC335	x	48	M	amp, amp/sulbact, trimeth/sulfa	urine
EC339	x	88	F	cipro, cefox	urine
EC340	x	74	F	amp, amp/sulbact	urine
EC341	v	55	F	cipro,	urine
EC344	x	49	F	none	urine
EC346	x	59	F	amp, amp/sulbact, trimeth/sulfa	urine
EC347	x	74	F	amp, amp/sulbact	urine
EC349	v	57	M	none	urine
EC352	x	71	F	none	urine
EC353	v	57	M	amp, ceftaz, cipro, ceftriax, cefaz, cefepime, cefox, amp/sulbact, piper/tazo	urine
EC356	x	20	F	none	urine
EC357	v	72	F	none	urine
EC358	x	56	F	amp, amp/sulbact	urine
EC359	x	69	F	none	urine
EC360	x	82	F	amp, amp/sulbact	urine
EC361	x	51	F	none	urine
EC362	x	73	F	none	aspirate breast
EC363	x	68	M	amp, amp/sulbact	sputum bronchus
EC364	x	30	M	amp, cipro, cefaz, cefox, amp/sulbact, trimeth/sulfa, piper/tazo	tracheal aspirate
EC366	x	63	F	amp, ceftaz, ceftriax, cefaz, cefox, amp/sulbact	urine
EC367	x	35	F	none	urine
EC370	x	77	F	amp, cipro, genta, tobra, trimeth/sulfa	urine
EC371	x	62	F	cipro, trimeth/sulfa	urine
EC374	v	35	F	none	urine
EC375	v	71	F	none	urine
EC379	x	82	M	amp, tobra, amp/sulbact, trimeth/sulfa, piper/tazo	urine
EC389	x	82	F	none	urine
EC391	x	60	M	none	urine

EC397	x	90	F	amp, amp/sulbact	urine
EC398	x	63	M	none	urine
EC401	x	79	F	none	urine
EC404	x	48	F	none	urine
EC405	v	25	F	none	urine
EC406	v	68	F	amp, amp/sulbact, trimeth/sulfa	urine
EC410	v	44	F	none	urine
EC415	v	26	F	amp, ceftaz, ceftriax, cefaz, cefox, amp/sulbact, trimeth/sulfa, piper/tazo	urine
EC416	x	42	F	none	urine
EC418	v	46	F	amp, amp/sulbact	urine
EC425	x	44	M	amp, cefaz, cefox, amp/sulbact	urine
EC426	x	91	F	amp, cipro, amp/sulbact, trimeth/sulfa	urine
EC427	x	61	F	cipro. trimeth/sulfa	urine
EC429	x	46	F	amp, amp./sulbact, trimeth/sulfa	urine
EC433	x	62	F	none	urine
EC435	x	34	F	amp, amp/sulbact, trimeth/sulfa	urine
EC436	x	85	F	amp, genta, tobra, amp/sulbact, trimeth/sulfa	urine
EC438	x	67	F	none	urine
EC442	x	54	F	ceftaz, cipro, ceftriax, cefaz, cefepime, cefox, genta, amp/sulbact, piper/tazo, amp	urine
EC443	v	25	F	none	urine
EC445	x	24	F	cipr, amp/sulbact, trimeth/sulfa, amp	sputum - cystic fibrosis
EC447	x	44	M	amp, amp/sulbact	blood
EC448	x	57	M	none	blood
EC452	x	20	F	none	placenta
EC456	x	22	F	amp, amp/sulbact	urine
EC460	x	20	F		expectorated sputum
EC465	x	49	F	amp, cipro, cefaz, genta, tobra, amp.sulbact, trimeth/sulfa	urine
EC466	x	18	F	none	urine
EC467	x	68	F	none	urine
EC468	x	48	F	none	urine
EC478	x	68	F	none	urine
EC482	v	77	F	trimeth/sulfa	urine
EC483	x	17	F	none	urine
EC484	x	45	F	none	urine
EC485	v	37	F	amp, amp/sulbact	urine
EC486	x	22	F	amp, trimeth/sulfa	urine

EC487	x	69	F	amp, amp/sulbact, piper/tazo	urine
EC488	x	69	F	none	urine
EC489	x	101	F	none	urine
EC492	x	67	F	amp, amp/sulbact	urine
EC493	v	33	F	amp, amp/sulbact	urine
EC494	x	101	F	none	urine
EC495	x	65	F	none	urine
EC498	x	23	F	none	urine
EC499	x	74	F	amp, amp/sulbact	urine
EC500	v	57	F	amp, ceftaz, ceftrix, cefaz, cefox, amp./sulbact	urine
EC501	v	68	F	amp, genta, amp/sulbact, trimeth/sulfa	urine
EC502	x	74	F	amp, amp/sulbact, trimeth/sulfa	urine
EC503	x	26	F	none	urine
EC504	x	73	F	none	urine
EC505	v	97	F	none	urine
EC506	x	36	F	amp, amp/sulbact	urine
EC507	x	64	F	amp, amp/sulbact	urine
EC508	x	48	F	none	urine
EC509	v	58	F	none	urine
ELI3	v	NP	NP	NP	urine
ELI5	x	NP	NP	NP	urine
ELI6	x	NP	NP	NP	urine
ELI7	x	NP	NP	NP	urine
ELI18	x	NP	NP	NP	urine
ELI19	v	NP	NP	NP	urine
ELI25	x	NP	NP	NP	urine
ELI26	x	NP	NP	NP	urine
ELI29	x	NP	NP	NP	urine
ELI30	x	NP	NP	NP	urine
ELI33	x	NP	NP	NP	urine
ELI34	x	NP	NP	NP	urine
ELI35	x	NP	NP	NP	urine
ELI36	x	NP	NP	NP	urine
ELI37	v	NP	NP	NP	urine
ELI41	x	NP	NP	NP	urine
ELI42	x	NP	NP	NP	urine

ELI43	v	NP	NP	NP	urine
ELI44	v	NP	NP	NP	urine
ELI45	v	NP	NP	NP	urine
ELI46	x	NP	NP	NP	urine
ELI47	x	NP	NP	NP	urine
ELI48	x	NP	NP	NP	urine
ELI49	x	NP	NP	NP	urine
ELI50	x	NP	NP	NP	urine
ELI51	x	NP	NP	NP	urine
ELI57	x	NP	NP	NP	urine
ELI58	v	NP	NP	NP	urine
ELI63	x	NP	NP	NP	urine
ELI73	x	NP	NP	NP	urine
ELI74	x	NP	NP	NP	urine
ELI75	x	NP	NP	NP	urine
ELI103	x	NP	NP	NP	urine
ELI106	x	NP	NP	NP	urine
ELI108	x	NP	NP	NP	urine
ELI110	x	NP	NP	NP	urine
ELI111	v	NP	NP	NP	urine
ELI112	x	NP	NP	NP	urine
ELI113	x	NP	NP	NP	urine
ELI123	x	NP	NP	NP	urine
ELI129	x	NP	NP	NP	urine
ELI130	v	NP	NP	NP	urine
ELI131	x	NP	NP	NP	urine
ELI132	x	NP	NP	NP	urine
ELI137	x	NP	NP	NP	urine
ELI138	x	NP	NP	NP	urine
ELI145	x	NP	NP	NP	urine
ELI146	x	NP	NP	NP	urine
ELI147	v	NP	NP	NP	urine
ELI148	x	NP	NP	NP	urine
ELI151	v	NP	NP	NP	urine
ELI154	x	NP	NP	NP	urine
ELI158	x	NP	NP	NP	urine

ELI159	v	NP	NP	NP	urine
ELI161	x	NP	NP	NP	urine
ELI167	x	NP	NP	NP	urine
ELI168	x	NP	NP	NP	urine
ELI176	x	NP	NP	NP	urine
ELI177	v	NP	NP	NP	urine
ELI178	x	NP	NP	NP	urine
ELI181	x	NP	NP	NP	urine
ELI188	x	NP	NP	NP	urine

Supplementary table 2. Coding sequence (CDS) of bacteriophage FM2.

Name	Type	Minimum	Maximum	Length	Direction
hypothetical protein CDS	CDS	168854	169429	576	reverse
Phage protein CDS	CDS	168202	168804	603	reverse
Phage protein CDS	CDS	167838	168212	375	reverse
Phage protein CDS	CDS	167500	167859	360	reverse
Phage protein (ACLAME 1472) CDS	CDS	167198	167503	306	reverse
Phage protein CDS	CDS	166916	167188	273	reverse
Phage protein CDS	CDS	166709	166906	198	reverse
Phage protein CDS	CDS	166407	166646	240	reverse
hypothetical protein CDS	CDS	165422	166378	957	reverse
hypothetical protein CDS	CDS	165141	165341	201	reverse
Phage protein CDS	CDS	164777	165082	306	reverse
Phage protein CDS	CDS	164089	164775	687	reverse
Phage protein CDS	CDS	163598	164089	492	reverse
Phage protein CDS	CDS	163365	163601	237	reverse
Phage Nudix hydrolase (ACLAME 1506) CDS	CDS	162917	163375	459	reverse
Phage lysozyme R (EC 3.2.1.17) CDS	CDS	162394	162882	489	reverse
Phage protein CDS	CDS	162116	162397	282	reverse
Phage endonuclease CDS	CDS	161644	162057	414	reverse
Phage protein CDS	CDS	161376	161630	255	reverse
Phage protein CDS	CDS	160997	161311	315	reverse
Phage protein CDS	CDS	160642	160971	330	reverse
Phage protein CDS	CDS	159930	160469	540	reverse
Phage protein CDS	CDS	159625	159933	309	reverse
Autonomous glycyl radical cofactor CDS	CDS	159256	159618	363	reverse
Phage protein CDS	CDS	159032	159256	225	reverse
Phage protein CDS	CDS	158776	159042	267	reverse
Phage protein CDS	CDS	158561	158776	216	reverse

Phage endonuclease CDS	CDS	158041	158499	459	reverse
hypothetical protein CDS	CDS	157490	158032	543	reverse
Valyl-tRNA synthetase CDS	CDS	157146	157493	348	reverse
Phage protein CDS	CDS	156686	157153	468	reverse
Phage protein CDS	CDS	156477	156689	213	reverse
Phage protein CDS	CDS	156274	156480	207	reverse
Phage protein CDS	CDS	156095	156277	183	reverse
Thymidine kinase CDS	CDS	155504	156085	582	reverse
Phage protein CDS	CDS	155264	155476	213	reverse
Phage rI lysis inhibition regulator (ACLAME 1105) CDS	CDS	154949	155251	303	reverse
Phage protein CDS	CDS	154668	154847	180	reverse
Phage protein CDS	CDS	154454	154660	207	reverse
Phage protein CDS	CDS	154286	154396	111	reverse
Phage protein CDS	CDS	154110	154289	180	reverse
Phage protein CDS	CDS	153578	154108	531	reverse
Phage protein CDS	CDS	153095	153568	474	reverse
Thioredoxin, phage-associated CDS	CDS	152109	153095	987	reverse
hypothetical protein CDS	CDS	151815	152081	267	reverse
Thioredoxin, phage-associated CDS	CDS	150716	151696	981	reverse
Thioredoxin, phage-associated CDS	CDS	150291	150578	288	reverse
Thioredoxin, phage-associated CDS	CDS	149705	150232	528	reverse
Thioredoxin, phage-associated CDS	CDS	148647	149642	996	reverse
Thioredoxin, phage-associated CDS	CDS	147632	148591	960	reverse
Thioredoxin, phage-associated CDS	CDS	146619	147569	951	reverse
Thioredoxin, phage-associated CDS	CDS	146314	146619	306	reverse
hypothetical protein CDS	CDS	145901	146314	414	reverse
thioredoxin CDS	CDS	145645	145908	264	reverse
hypothetical protein CDS	CDS	145352	145648	297	reverse
hypothetical protein CDS	CDS	144977	145333	357	reverse
Phage protein CDS	CDS	144678	144848	171	reverse
Pin protease inhibitor CDS	CDS	144263	144676	414	reverse
Phage protein CDS	CDS	144074	144253	180	reverse
recombination endonuclease VII CDS	CDS	143562	144035	474	reverse
Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2) CDS	CDS	141748	143565	1818	reverse
Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4) CDS	CDS	141281	141751	471	reverse
Phage protein CDS	CDS	141187	141303	117	reverse
Phage protein CDS	CDS	140955	141170	216	reverse
Glutaredoxin CDS	CDS	140345	140668	324	reverse
Phage protein CDS	CDS	139996	140178	183	reverse

Phage protein CDS	CDS	139751	139999	249	reverse
Phage protein CDS	CDS	139450	139743	294	reverse
Phage protein CDS	CDS	139308	139442	135	reverse
Phage protein CDS	CDS	139111	139311	201	reverse
Phage protein CDS	CDS	138808	139047	240	reverse
Phage protein CDS	CDS	138409	138741	333	reverse
Phage protein CDS	CDS	138185	138412	228	reverse
Phage protein CDS	CDS	137919	138188	270	reverse
T4-like phage RNA polymerase sigma factor for late transcription CDS	CDS	137289	137846	558	reverse
Phage protein (ACLAME 855) CDS	CDS	137090	137299	210	reverse
Phage protein (ACLAME 780) CDS	CDS	136765	137088	324	reverse
Phage protein CDS	CDS	136584	136790	207	reverse
Phage protein CDS	CDS	136371	136544	174	reverse
Phage recombination-related endonuclease Gp47 CDS	CDS	135282	136301	1020	reverse
Phage protein CDS	CDS	135028	135285	258	reverse
Phage protein CDS	CDS	134802	135041	240	reverse
Phage recombination-related endonuclease Gp46 CDS	CDS	133117	134805	1689	reverse
Phage protein CDS	CDS	132874	133062	189	reverse
Phage RNA polymerase binding protein (EC 2.7.7.6) (ACLAME 854) CDS	CDS	132445	132861	417	reverse
Sliding clamp DNA polymerase accessory protein, phage associated CDS	CDS	131716	132402	687	reverse
Replication factor C small subunit / Phage DNA polymerase clamp loader subunit CDS	CDS	130678	131640	963	reverse
Phage DNA polymerase clamp loader subunit Gp62 CDS	CDS	130113	130676	564	reverse
Phage endoribonulcease translational repressor of early genes, regA CDS	CDS	129742	130110	369	reverse
hypothetical protein CDS	CDS	126949	129660	2712	reverse
D-arabinose-5-phosphate isomerase (EC 5.3.1.13) CDS	CDS	126273	126908	636	reverse
Phage protein CDS	CDS	126133	126276	144	reverse
Phage protein CDS	CDS	124406	126091	1686	reverse
hypothetical protein CDS	CDS	124020	124406	387	reverse
Phage protein CDS	CDS	122803	123963	1161	reverse
Phage protein CDS	CDS	122567	122806	240	reverse
hypothetical protein CDS	CDS	121809	122525	717	reverse
Phage protein CDS	CDS	120910	121809	900	reverse
Phage protein CDS	CDS	120360	120908	549	reverse
Phage recombination protein CDS	CDS	119089	120261	1173	reverse
Phage capsid and scaffold CDS	CDS	118755	119096	342	reverse
Phage DNA primase/helicase CDS	CDS	117303	118745	1443	reverse
Phage protein CDS	CDS	116840	117214	375	reverse
hypothetical protein CDS	CDS	116467	116784	318	reverse
discriminator of mRNA degradation, phage-associated CDS	CDS	116282	116470	189	reverse

Phage immunity CDS	CDS	115842	116210	369	reverse
Phage immunity CDS	CDS	115532	115780	249	reverse
hypothetical protein CDS	CDS	115180	115473	294	reverse
Phage protein CDS	CDS	114528	115178	651	reverse
Phage protein CDS	CDS	114329	114526	198	reverse
hypothetical protein CDS	CDS	113842	114309	468	reverse
DNA primase (EC 2.7.7.-) / DNA helicase (EC 3.6.1.-), phage-associated CDS	CDS	112780	113802	1023	reverse
hypothetical protein CDS	CDS	112586	112783	198	forward
dCTP pyrophosphatase (EC 3.6.1.12), phage-associated (ACLAME 965) CDS	CDS	111976	112497	522	reverse
Phage protein CDS	CDS	111803	111976	174	reverse
hypothetical protein CDS	CDS	111589	111813	225	reverse
hypothetical protein CDS	CDS	111350	111586	237	reverse
Phage protein CDS	CDS	110807	111055	249	reverse
Phage protein CDS	CDS	110631	110810	180	reverse
hypothetical protein CDS	CDS	110167	110631	465	reverse
Molybdenum ABC transporter, periplasmic molybdenum-binding protein ModA (TC 3.A.1.8.1) CDS	CDS	109986	110165	180	reverse
Phage protein CDS	CDS	109825	109989	165	reverse
hypothetical protein CDS	CDS	109188	109769	582	reverse
Phage ModA or ModB ribosyltransferase (ACLAME 972) CDS	CDS	108522	109130	609	reverse
putative anti-sigma factor CDS	CDS	107623	108369	747	reverse
Phage protein CDS	CDS	107309	107620	312	reverse
DNA helicase (EC 3.6.1.-), phage-associated CDS	CDS	105999	107312	1314	reverse
exonuclease CDS	CDS	105312	105989	678	reverse
hypothetical protein CDS	CDS	104752	105246	495	reverse
hypothetical protein CDS	CDS	104234	104752	519	reverse
Transcriptional regulator CDS	CDS	103805	104224	420	reverse
Transcriptional regulator CDS	CDS	103221	103745	525	reverse
Phage cef modifier of suppressor tRNAs (ACLAME 1240) CDS	CDS	102936	103163	228	reverse
Phage protein CDS	CDS	102526	102936	411	reverse
Phage protein (ACLAME 1508) CDS	CDS	102344	102523	180	reverse
hypothetical protein CDS	CDS	101919	102341	423	reverse
Phage DNA topoisomerase large subunit (EC 5.99.1.3) CDS	CDS	100038	101855	1818	reverse
Phage protein CDS	CDS	98901	100001	1101	reverse
Phage rIIA lysis inhibitor CDS	CDS	98608	98808	201	reverse
Phage rIIA lysis inhibitor CDS	CDS	96382	98595	2214	reverse
hypothetical protein CDS	CDS	95425	96372	948	reverse
hypothetical protein CDS	CDS	95096	95383	288	reverse
Phage endonuclease CDS	CDS	94603	95079	477	reverse
Phage protein CDS	CDS	94271	94534	264	reverse

hypothetical protein CDS	CDS	94081	94191	111	reverse
Phage protein CDS	CDS	93820	94020	201	reverse
hypothetical protein CDS	CDS	93297	93743	447	reverse
Acridine resistance CDS	CDS	93098	93244	147	reverse
Phage protein CDS	CDS	92958	93098	141	reverse
DNA topoisomerase, phage-associated CDS	CDS	91628	92953	1326	reverse
Putative transcriptional regulator MotA (ACLAME 1235) CDS	CDS	90810	91442	633	reverse
Phage anti-restriction nuclease CDS	CDS	90470	90799	330	reverse
hypothetical protein CDS	CDS	90012	90473	462	reverse
Phage anti-restriction nuclease CDS	CDS	89731	90012	282	reverse
hypothetical protein CDS	CDS	89435	89554	120	reverse
Phage protein CDS	CDS	89146	89445	300	reverse
Phage protein CDS	CDS	88995	89156	162	reverse
hypothetical protein CDS	CDS	88676	88948	273	reverse
Phage holin CDS	CDS	88016	88675	660	forward
Phage tail fibers CDS	CDS	87455	88006	552	forward
Phage tail fiber, side tail fiber protein Stf CDS	CDS	84287	87424	3138	forward
Phage tail fibers CDS	CDS	83613	84278	666	forward
Phage long tail fiber CDS	CDS	82423	83550	1128	forward
Phage long tail fiber CDS	CDS	78539	82414	3876	forward
Phage ribonuclease H (EC 3.1.26.4) CDS	CDS	77518	78435	918	reverse
Phage double-stranded DNA binding protein #T4-like dsbA, late transcriptional regulation #T4 GC1668 CDS	CDS	77241	77510	270	reverse
Transcriptional regulator CDS	CDS	76925	77263	339	reverse
Phage DNA helicase loader CDS	CDS	76275	76928	654	reverse
Single stranded DNA-binding protein, phage-associated CDS	CDS	75254	76156	903	reverse
Phage protein CDS	CDS	74748	75140	393	reverse
Phage protein CDS	CDS	74437	74685	249	reverse
hypothetical protein CDS	CDS	74180	74434	255	reverse
Dihydrofolate reductase, phage-associated CDS	CDS	73605	74192	588	reverse
Thymidylate synthase (EC 2.1.1.45) CDS	CDS	72748	73608	861	reverse
Phage protein CDS	CDS	72492	72746	255	reverse
Phage protein (ACLAME 596) CDS	CDS	72187	72492	306	reverse
Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1) CDS	CDS	69941	72196	2256	reverse
Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1) CDS	CDS	68709	69887	1179	reverse
Phage endonuclease CDS	CDS	68272	68682	411	reverse
RNA ligase CDS	CDS	67092	68216	1125	reverse
Putative phage alc transcription terminator (ACLAME 1242) CDS	CDS	66530	67030	501	reverse
Phage spanin Rz CDS	CDS	66186	66542	357	reverse
Phage outer membrane lipoprotein Rz1 CDS	CDS	65899	66189	291	reverse

Phage protein CDS	CDS	65684	65902	219	reverse
Phage protein CDS	CDS	65327	65626	300	reverse
Phage protein CDS	CDS	65136	65327	192	reverse
3'-phosphatase, 5'-polynucleotide kinase, phage-associated CDS	CDS	64240	65139	900	reverse
Phage protein CDS	CDS	64052	64240	189	reverse
Phage protein CDS	CDS	63844	64059	216	reverse
Phage protein CDS	CDS	63561	63836	276	reverse
Phage protein CDS	CDS	63263	63499	237	reverse
hypothetical protein CDS	CDS	63151	63276	126	reverse
2-keto-3-deoxy-D-arabino-heptulosonate-7- phosphate synthase I alpha (EC 2.5.1.54) CDS	CDS	62150	63142	993	reverse
dCMP deaminase (EC 3.5.4.12) CDS	CDS	61569	62150	582	reverse
Phage tail fibers CDS	CDS	61271	61567	297	reverse
Phage head assembly chaperone protein CDS	CDS	60881	61213	333	reverse
Phage rIII lysis inhibitor accessory CDS	CDS	60508	60756	249	reverse
1,4-alpha-glucan (glycogen) branching enzyme, GH-13-type (EC 2.4.1.18) CDS	CDS	60033	60212	180	reverse
hypothetical protein CDS	CDS	59534	59902	369	reverse
Phage protein CDS	CDS	59094	59459	366	reverse
hypothetical protein CDS	CDS	58444	59058	615	reverse
Phage protein CDS	CDS	58192	58389	198	reverse
Phage protein CDS	CDS	57984	58202	219	reverse
hypothetical protein CDS	CDS	57533	57991	459	reverse
hypothetical protein CDS	CDS	56721	57536	816	reverse
Phage protein CDS	CDS	56442	56711	270	reverse
hypothetical protein CDS	CDS	54952	56445	1494	reverse
Phage protein CDS	CDS	54764	54952	189	reverse
Putative RNA polymerase-ADP-ribosyltransferase Alt (ACLAME 423) CDS	CDS	52621	54708	2088	reverse
Phage protein CDS	CDS	52269	52562	294	reverse
tail assembly protein CDS	CDS	51274	52236	963	forward
Phage baseplate tail tube cap (T4-like gp48) CDS	CDS	50165	51274	1110	forward
Phage baseplate hub CDS	CDS	48384	50156	1773	forward
Phage baseplate hub CDS	CDS	47917	48387	471	forward
baseplate hub subunit CDS	CDS	46734	47906	1173	forward
Phage baseplate CDS	CDS	45985	46737	753	forward
Phage baseplate hub assembly chaperone (T4-like gp26) CDS	CDS	45311	45937	627	reverse
Phage baseplate wedge subunit (T4-like gp25) CDS	CDS	44913	45311	399	reverse
Single stranded DNA-binding protein, phage-associated CDS	CDS	44419	44913	495	reverse
Phage protein CDS	CDS	44195	44419	225	reverse
Phage protein (ACLAME 856) CDS	CDS	43995	44162	168	reverse
Phage DNA helicase CDS	CDS	43706	43936	231	forward

Phage DNA helicase CDS	CDS	42166	43680	1515	forward
Inh inhibitor of gp21 prohead protease CDS	CDS	41447	42115	669	reverse
capsid and scaffold protein CDS	CDS	40307	41437	1131	reverse
Phage protein (ACLAME 967) CDS	CDS	40011	40205	195	reverse
Phage protein CDS	CDS	39763	40014	252	reverse
RNA ligase CDS	CDS	38645	39643	999	reverse
capsid vertex protein CDS	CDS	37329	38612	1284	forward
Phage protein CDS	CDS	36958	37227	270	reverse
major capsid protein CDS	CDS	35337	36905	1569	forward
Phage prohead assembly (scaffolding) protein CDS	CDS	34507	35319	813	forward
Phage prohead assembly (scaffolding) protein CDS	CDS	33832	34473	642	forward
Phage capsid and scaffold CDS	CDS	33407	33832	426	forward
Phage prohead core protein CDS	CDS	33171	33407	237	forward
putative portal vertex protein CDS	CDS	31600	33171	1572	forward
Phage tail tube CDS	CDS	31024	31515	492	forward
Phage tail sheath CDS	CDS	28929	30911	1983	forward
Phage terminase, large subunit CDS	CDS	27063	28898	1836	forward
Phage terminase, small subunit CDS	CDS	26585	27079	495	forward
Proximal tail sheath stabilization protein CDS	CDS	25754	26575	822	forward
Phage neck protein CDS	CDS	24937	25701	765	forward
Phage neck protein CDS	CDS	24009	24935	927	forward
Phage fibritin (wac) protein CDS	CDS	22528	23976	1449	forward
hypothetical protein CDS	CDS	20945	22528	1584	forward
baseplate wedge subunit and tail pin CDS	CDS	20289	20948	660	forward
hypothetical protein CDS	CDS	18484	20289	1806	forward
Phage baseplate wedge tail fiber connector (T4-like gp9) CDS	CDS	17612	18484	873	forward
Phage baseplate wedge subunit (T4-like gp8) CDS	CDS	16535	17539	1005	forward
Phage baseplate wedge initiator (T4-like gp7) CDS	CDS	13444	16542	3099	forward
Phage baseplate wedge subunit (T4-like gp6) CDS	CDS	11474	13447	1974	forward
Putative phospholipase (ACLAME 172) CDS	CDS	11172	11465	294	forward
Phage protein (ACLAME 782) CDS	CDS	10696	11169	474	forward
Phage baseplate hub structural protein / Phage lysozyme R (EC 3.2.1.17) CDS	CDS	8917	10650	1734	forward
baseplate wedge subunit CDS	CDS	8342	8917	576	forward
Phage head completion protein CDS	CDS	7831	8280	450	reverse
DNA end protector protein CDS	CDS	7007	7828	822	reverse
hypothetical protein CDS	CDS	6320	6904	585	reverse
deoxynucleoside monophosphate kinase CDS	CDS	5532	6266	735	reverse
Phage tail fiber assembly protein CDS	CDS	5297	5527	231	reverse
hypothetical protein CDS	CDS	4842	5297	456	reverse
Phage protein CDS	CDS	4477	4764	288	reverse

[Enterobacteria phage IME08.] CDS	CDS	4143	4406	264	reverse
Phage protein CDS	CDS	3888	4073	186	reverse
Phage protein CDS	CDS	3524	3886	363	reverse
Phage protein CDS	CDS	3237	3527	291	reverse
Phage protein CDS	CDS	2717	3232	516	reverse
tRNA-Met-CAT	tRNA	2564	2637	74	reverse
tRNA-Arg-TCT	tRNA	2484	2557	74	reverse
Phage protein CDS	CDS	2118	2462	345	reverse
hypothetical protein CDS	CDS	1269	1733	465	reverse
hypothetical protein CDS	CDS	642	1145	504	reverse
Phage protein CDS	CDS	418	582	165	reverse
Phage protein CDS	CDS	144	371	228	reverse
hypothetical protein CDS	CDS	2	73	72	reverse

Supplementary table 3. Coding Sequence (CDS) of bacteriophage FM10.

Name	Minimum	Maximum	Length	Direction
hypothetical protein CDS	162782	163588	807	forward
putative single-stranded DNA binding protein CDS	161694	162662	969	forward
Phage DNA helicase loader CDS	160953	161633	681	forward
Phage late transcriptional regulator #T4-like phage gp33, activator #T4 GC1667 CDS	160689	160943	255	forward
Phage double-stranded DNA binding protein #T4-like dsbA, late transcriptional regulation #T4 GC1668 CDS	160421	160696	276	forward
Adenylate cyclase (EC 4.6.1.1) CDS	159817	160416	600	forward
Phage ribonuclease H (EC 3.1.26.4) CDS	158859	159806	948	forward
Phage protein CDS	158671	158856	186	forward
long tail fiber proximal subunit CDS	154856	158596	3741	reverse
hypothetical protein CDS	153683	154822	1140	reverse
hypothetical protein CDS	151990	153480	1491	reverse
hypothetical protein CDS	148989	151919	2931	reverse
Phage protein CDS	148709	148948	240	reverse
hypothetical protein CDS	148019	148675	657	reverse
Phage protein CDS	147735	147983	249	forward
Phage protein CDS	147427	147738	312	forward
Phage protein CDS	147070	147402	333	forward
Phage protein CDS	146693	147061	369	forward
Phage protein CDS	146491	146682	192	forward
Phage protein CDS	146189	146467	279	forward
hypothetical protein CDS	145419	146177	759	forward

Phage protein CDS	145193	145348	156	forward
Phage protein CDS	144911	145123	213	forward
hypothetical protein CDS	144599	144841	243	forward
hypothetical protein CDS	144335	144535	201	forward
Phage protein CDS	144168	144338	171	forward
Phage protein CDS	143891	144163	273	forward
hypothetical protein CDS	143618	143824	207	forward
Phage protein CDS	143110	143505	396	forward
Phage protein CDS	142909	143106	198	forward
DNA topoisomerase, phage-associated CDS	141542	142906	1365	forward
Phage protein CDS	141172	141534	363	forward
Phage protein CDS	140876	141169	294	forward
Phage protein CDS	140513	140830	318	forward
phage T4-like protein, rIIA-rIIB membrane associated lysis inhibitor # T4 GC 1698 CDS	139506	140498	993	forward
Phage protein CDS	139313	139444	132	forward
hypothetical protein CDS	137193	139301	2109	forward
Phage protein CDS	136921	137178	258	forward
Phage protein CDS	136715	136909	195	forward
Phage protein CDS	136528	136722	195	forward
Phage protein CDS	136126	136461	336	forward
Phage DNA topoisomerase large subunit (EC 5.99.1.3) CDS	134273	136096	1824	forward
Phage protein (ACLAME 1508) CDS	134000	134173	174	forward
hypothetical protein CDS	133330	133998	669	forward
Phage protein CDS	133086	133328	243	forward
Phage protein CDS	132917	133084	168	forward
DNA helicase (EC 3.6.1.-), phage-associated CDS	131515	132906	1392	forward
Phage protein CDS	131201	131515	315	forward
Phage anti-termination CDS	130446	131201	756	forward
Phage ModA or ModB ADP-ribosyltransferase (EC 2.4.2.-) (ACLAME 972) CDS	129881	130384	504	forward
dUTPase CDS	129293	129811	519	forward
DNA primase (EC 2.7.7.-) / DNA helicase (EC 3.6.1.-), phage-associated CDS	128250	129278	1029	forward
Phage protein CDS	128037	128240	204	forward
Phage protein CDS	127882	128040	159	forward
T4-like phage protein, T4 GC1491 CDS	127713	127889	177	forward
Phage protein CDS	127342	127716	375	forward
Phage protein CDS	126676	127311	636	forward
DNA helicase CDS	125213	126625	1413	forward
Phage capsid and scaffold CDS	124899	125216	318	forward
Phage recombination protein CDS	123773	124843	1071	forward
Phage protein CDS	123573	123761	189	forward

DNA polymerase (EC 2.7.7.7), phage-associated CDS	120833	123511	2679	forward
Phage endoribonulcease translational repressor of early genes, regA CDS	120388	120750	363	forward
Phage DNA polymerase clamp loader subunit Gp62 CDS	119808	120386	579	forward
Replication factor C small subunit / Phage DNA polymerase clamp loader subunit CDS	118834	119808	975	forward
Sliding clamp DNA polymerase accessory protein, phage associated CDS	118082	118768	687	forward
Phage protein CDS	117674	118051	378	forward
Phage RNA polymerase binding protein (EC 2.7.7.6) (ACLAME 854) CDS	117292	117669	378	forward
Phage protein CDS	117118	117261	144	forward
Phage recombination-related endonuclease Gp46 CDS	115362	117044	1683	forward
hypothetical protein CDS	115118	115381	264	forward
Phage protein CDS	114549	115121	573	forward
Phage protein CDS	114257	114574	318	forward
Phage protein CDS	114009	114260	252	forward
Phage recombination-related endonuclease Gp47 CDS	112929	113954	1026	forward
Phage protein CDS	112551	112838	288	forward
Phage protein CDS	112316	112549	234	forward
T4-like phage RNA polymerase sigma factor for late transcription CDS	111799	112332	534	forward
Phage protein (ACLAME 1470) CDS	110289	111758	1470	forward
Phage protein CDS	110002	110289	288	forward
Phage protein CDS	109822	109980	159	forward
Glutaredoxin 3 (Grx1) CDS	109563	109832	270	forward
Phage protein CDS	109090	109566	477	forward
Phage protein CDS	108264	109088	825	forward
Phage protein CDS	107787	108251	465	forward
hypothetical protein CDS	107255	107782	528	forward
Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4) CDS	106741	107244	504	forward
hypothetical protein CDS	106599	106751	153	forward
Phage protein CDS	105996	106523	528	forward
Phage protein CDS	105436	105978	543	forward
Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2) CDS	103473	105335	1863	forward
Phage protein CDS	102934	103428	495	forward
hypothetical protein CDS	101701	102879	1179	forward
Phage protein CDS	101078	101644	567	forward
Phage protein CDS	100791	101051	261	forward
Phage endonuclease #T4-like phage gp49, endonuclease VII #T4 GC1525 CDS	100253	100726	474	forward
Glutaredoxin, phage-associated #T4-like nrdC, glutaredoxin, PF00462 #T4 GC1531 CDS	99947	100228	282	forward
Type II, N-methyl DNA methyltransferase (group alpha) CDS	99126	99938	813	forward
Phage protein CDS	98695	99126	432	forward
Phage protein CDS	98487	98684	198	forward

Phage protein CDS	98176	98397	222	forward
Phage protein CDS	97890	98183	294	forward
Phage protein CDS	97531	97875	345	forward
Phage protein CDS	97187	97495	309	forward
Phage protein CDS	96898	97176	279	forward
Phage protein CDS	96610	96819	210	forward
Phage protein CDS	96252	96590	339	forward
Phage protein CDS	95992	96252	261	forward
Phage protein CDS	95776	95973	198	forward
Phage protein CDS	95532	95786	255	forward
Phage protein CDS	95101	95535	435	forward
Phage protein CDS	94893	95090	198	forward
Phage protein CDS	94592	94882	291	forward
Phage tail assembly CDS	93711	94490	780	forward
Phage protein CDS	93435	93656	222	forward
Phage protein CDS	93064	93348	285	forward
Phage protein CDS	92717	93067	351	forward
Phage protein CDS	92382	92720	339	forward
Phage protein CDS	92183	92392	210	forward
hypothetical protein CDS	91456	91854	399	forward
Phage rI lysis inhibition regulator CDS	91167	91469	303	forward
Thymidine kinase (EC 2.7.1.21) CDS	90502	91098	597	forward
Phage protein CDS	90209	90502	294	forward
Phage protein CDS	89998	90216	219	forward
Phage protein CDS	89807	90001	195	forward
hypothetical protein CDS	89362	89817	456	forward
Valyl-tRNA synthetase CDS	88987	89346	360	forward
hypothetical protein CDS	88389	88979	591	forward
Endoribonuclease, RegB protein CDS	87881	88303	423	forward
Phage protein CDS	87541	87876	336	forward
Phage protein CDS	87238	87483	246	forward
Phage protein CDS	87047	87241	195	forward
T4-like phage protein, T4 GC2721 CDS	86327	87025	699	forward
Pyruvate formate-lyase (EC 2.3.1.54) CDS	85824	86240	417	forward
Phage protein CDS	85688	85813	126	forward
Phage protein CDS	85203	85688	486	forward
Phage peptidoglycan hydrolase CDS	84751	85140	390	forward
Phage protein CDS	84531	84722	192	forward
Phage tail length tape-measure protein CDS	84314	84529	216	forward
Phage protein CDS	84132	84311	180	forward

Phage protein CDS	83727	83885	159	forward
Phage protein CDS	83133	83453	321	forward
Phage protein CDS	82914	83111	198	forward
Phage protein CDS	82717	82914	198	forward
Phage protein CDS	82533	82715	183	forward
Phage protein CDS	82198	82524	327	forward
Phage protein CDS	81930	82118	189	forward
Phage protein CDS	81732	81920	189	forward
Phage protein CDS	81492	81719	228	forward
Phage protein CDS	81240	81425	186	forward
Phage protein CDS	80745	80963	219	forward
Phage protein CDS	80566	80748	183	forward
Phage protein CDS	80345	80569	225	forward
Phage protein CDS	80238	80348	111	forward
Phage protein CDS	80044	80241	198	forward
discriminator of mRNA degradation, phage-associated CDS	79851	80054	204	forward
Phage protein CDS	79683	79841	159	forward
Phage protein CDS	79242	79616	375	forward
T4-like phage protein, T4 GC1584 CDS	78667	79131	465	forward
Phage tail fiber assembly protein CDS	78404	78664	261	forward
Deoxynucleotide monophosphate kinase (EC 2.7.4.13) #T4-like phage gp1 #T4 GC1586 CDS	77715	78371	657	forward
Phage tail completion protein CDS	77109	77699	591	forward
Phage protein CDS	76665	76919	255	reverse
DNA end protector protein CDS	75813	76634	822	forward
Phage head completion protein CDS	75328	75801	474	forward
Phage baseplate wedge subunit CDS	74718	75272	555	reverse
Phage baseplate hub structural protein / Phage lysozyme R (EC 3.2.1.17) CDS	72907	74706	1800	reverse
Phage protein (ACLAME 782) CDS	72332	72898	567	reverse
Phage-encoded phospholipase (ACLAME 172) CDS	72039	72332	294	reverse
baseplate wedge subunit CDS	70138	72042	1905	reverse
baseplate wedge subunit CDS	67055	70141	3087	reverse
baseplate wedge subunit CDS	66067	67062	996	reverse
Phage protein CDS	65699	65956	258	reverse
Phage protein CDS	65379	65711	333	reverse
Phage protein CDS	64540	65379	840	reverse
baseplate wedge tail fiber connector CDS	63610	64464	855	reverse
baseplate wedge subunit and tail pin CDS	61811	63613	1803	reverse
hypothetical protein CDS	61165	61809	645	reverse
hypothetical protein CDS	59753	61153	1401	reverse
hypothetical protein CDS	57984	59753	1770	reverse

Phage neck protein CDS	57005	57937	933	reverse
Phage neck protein CDS	56228	56968	741	reverse
tail sheath stabilizer and completion protein CDS	55368	56201	834	reverse
Phage terminase, small subunit CDS	54857	55354	498	reverse
Phage terminase, large subunit CDS	53037	54860	1824	reverse
Phage tail sheath CDS	50999	53002	2004	reverse
tail tube protein CDS	50431	50925	495	reverse
Phage portal vertex of the head CDS	48807	50372	1566	reverse
Phage prohead core protein CDS	48567	48806	240	reverse
Phage capsid and scaffold CDS	48147	48554	408	reverse
Phage prohead assembly (scaffolding) protein CDS	47440	48135	696	reverse
Phage prohead assembly (scaffolding) protein CDS	46614	47408	795	reverse
major capsid protein CDS	44956	46554	1599	reverse
Phage protein CDS	44489	44626	138	reverse
Phage protein CDS	44303	44455	153	reverse
hypothetical protein CDS	42977	44218	1242	reverse
Phage protein (ACLAME 1238) CDS	42389	42976	588	forward
Phage protein CDS	41538	41975	438	forward
Phage protein CDS	41216	41491	276	forward
Phage protein CDS	41019	41216	198	forward
hypothetical protein CDS	39603	40817	1215	forward
hypothetical protein CDS	38612	39337	726	forward
hypothetical protein CDS	37039	38541	1503	reverse
DNA helicase, phage-associated CDS	36759	36995	237	reverse
Phage protein (ACLAME 856) CDS	36519	36695	177	forward
Single stranded DNA-binding protein, phage-associated CDS	36090	36497	408	forward
Phage baseplate wedge subunit (T4-like gp25) CDS	35656	36042	387	forward
Phage baseplate hub subunit (T4-like gp26) CDS	35018	35647	630	forward
Phage baseplate CDS	34177	34947	771	reverse
Phage baseplate hub subunit CDS	33090	34199	1110	reverse
Phage baseplate hub CDS	32572	33090	519	reverse
hypothetical protein CDS	30842	32575	1734	reverse
Phage baseplate tail tube cap (T4-like gp48) CDS	29784	30842	1059	reverse
tail assembly protein CDS	28852	29784	933	reverse
hypothetical protein CDS	28334	28819	486	forward
Phage RNA polymerase-ADP-ribosyltransferase Alt (ACLAME 423) CDS	27513	28337	825	forward
hypothetical protein CDS	25383	27434	2052	forward
Phage protein CDS	25102	25314	213	forward
Phage protein CDS	24851	25030	180	forward
Phage protein CDS	24264	24782	519	forward

Phage protein CDS	23933	24262	330	forward
hypothetical protein CDS	23217	23933	717	forward
Phage protein CDS	23047	23220	174	forward
Phage protein CDS	22727	22978	252	forward
Phage protein CDS	22475	22642	168	forward
Phage protein CDS	22117	22398	282	forward
hypothetical protein CDS	21777	22046	270	forward
Phage protein CDS	21400	21654	255	forward
Phage protein CDS	20840	21148	309	forward
Phage protein CDS	20502	20789	288	forward
Phage protein CDS	20142	20420	279	forward
Phage protein CDS	19817	20137	321	forward
Phage protein CDS	19615	19830	216	forward
Phage protein CDS	19403	19612	210	forward
Phage protein CDS	19138	19416	279	forward
Phage protein CDS	18946	19125	180	forward
Phage protein CDS	18714	18881	168	forward
Phage protein CDS	18352	18645	294	forward
Phage protein CDS	18143	18334	192	forward
Phage protein CDS	17765	18085	321	forward
Phage protein CDS	17568	17747	180	forward
Phage protein CDS	17184	17495	312	forward
hypothetical protein CDS	16620	17096	477	forward
DNA ligase, phage-associated CDS	15131	16627	1497	forward
T4-like phage protein, T4 GC1630 CDS	14665	15144	480	forward
Phage protein CDS	14511	14675	165	forward
Phage head assembly chaperone protein CDS	14105	14428	324	forward
Phage tail fibers CDS	13749	14063	315	forward
dCMP deaminase (EC 3.5.4.12) CDS	13250	13756	507	forward
Phage protein CDS	13025	13228	204	forward
3'-phosphatase, 5'-polynucleotide kinase, phage-associated CDS	12147	13025	879	forward
Phage outer membrane lipoprotein Rz1 CDS	11695	12147	453	forward
Phage spanin Rz CDS	11432	11698	267	forward
RNA ligase CDS	10212	11330	1119	forward
Phage endonuclease CDS	9764	10225	462	forward
Phage protein CDS	9315	9713	399	forward
Phage protein CDS	9095	9244	150	forward
Phage protein CDS	8808	9026	219	forward
Phage protein CDS	8522	8740	219	forward
3'-phosphatase, phage-associated CDS	7945	8421	477	forward

Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1) CDS	6788	7948	1161	forward
Phage protein CDS	6605	6772	168	forward
Phage protein CDS	6432	6605	174	forward
Phage protein CDS	6265	6432	168	forward
hypothetical protein CDS	5739	6125	387	forward
Putative Type II, 5-methyl-cytosine DNA methyltransferase (ACLAME 54) CDS	5356	5664	309	forward
Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1) CDS	3057	5300	2244	forward
Thymidylate synthase (EC 2.1.1.45) CDS	1756	2988	1233	forward
hypothetical protein CDS	858	1427	570	forward
Phage protein CDS	513	842	330	forward
Phage protein (ACLAME 937) CDS	1	465	465	forward

4.4 Stressors influencing temperate phage release by Shiga toxin-producing *Escherichia coli*

4.4.1 Abstract

STEC is a foodborne pathogen and the main sources of contamination are cheese, milk, mixed red meat and vegetables.
Three STEC strains were selected for their ability to release stx-phages and the prophages were characterized through a RAPD-PCR fingerprint. The ability to release prophages was evaluated by Real Time qPCR, after a stress related to the cheese making process: addition of NaCl at three different concentrations (1, 1,5 and 2% w/v), presence of lactic acid (0,5, 1,5 and 3% v/v), anaerobic condition, pasteurization treatment (72ºC for 15 seconds), UV irradiation and in presence of antibiotics (ciprofloxacin, nalidixic acid and norfloxacin).
Two out of three isolated prophages showed the same RAPD fingerprint with both primers used. Induction of the prophages proved that the addition of NaCl at 1,5 and 2% significantly increased the release compared to the control; on the other side, the addition of lactic acid had a significant repressive effect.

4.4.2 Short introduction

Escherichia coli is a commensal bacterium of the gastrointestinal tract of humans and warm-blooded animals, but it is also present in water and soil (Brenner *et al.,* 2005; Liu, 2019). In most cases it is harmless to his host, but some strains are characterized by the presence of virulence factors, that can cause disease in humans (Croxen *et al.,* 2013). Among pathogenic *E. coli*, Shiga toxin-producing (STEC) strains represent the main important group for foodborne pathogenesis (Castro *et al.,* 2017) since it can cause gastrointestinal diseases, bloody diarrhea and can potentially develop into hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Yang *et al.,* 2017).

In 2018, in Europe, STEC strains caused 2,28 cases of infections per 100'000 population with an increase of 39% (1,64) compared to 2017, representing the third zoonoses reported (EFSA, 2019). The main *reservoir* of STEC is cattle, followed by sheep and goats (EFSA, 2019), and less frequently by other animals such as poultry, pigs, birds, dogs, horses, deers and flies. Direct contact with contaminated water or animals is also relevant as a route of transmission to humans (fig. 21) (Twardoń *et al.,* 2005).

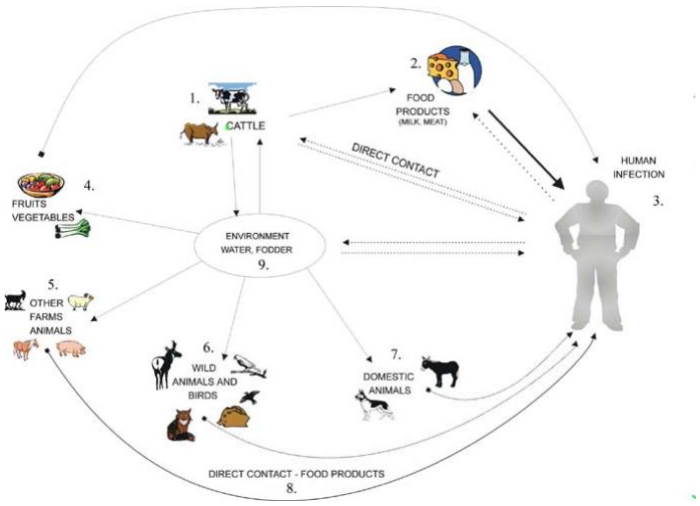

Figure 21. Epidemiology of STEC (Twardon et al., 2005).

The most common serotype is O157:H7, but in recent years there is a growing public health interest in other STEC serotypes, the so called non-O157. Indeed, non-O157 STEC are more commonly associated with acute diarrhea than O157 and have the potential to cause large disease (Valillis *et al.,* 2018). The main sources of food contamination are cheese, milk, mixed red meat and vegetables (EFSA, 2019). In a recent case of STEC infection reported in Europe, EFSA assumed that a cheese produced in Romania was the possible cause of 25 cases in Italy and Romania, with the death of three patients (EFSA, 2016).

In general, STEC strains are a great concern to the dairy industry due to their high virulence. In fact, they can cause outbreaks in humans even with a small amount of ingested cells (5-50) (Farrokh *et al.,* 2013).

The expression of Shiga toxins, the main cause of virulence, is related to the presence of prophages located in the bacterial chromosome, named stx-phages. Shiga toxin is an exotoxin produced only by STEC and *S. dysenteriae* serotype 1 and is characterized by an AB_5 structure containing an enzymatic A subunit that is not covalently associated with the five B subunits (Yang *et al.,* 2017). The A subunits play a role in inhibition of protein synthesis and in cell damage by apoptosis (Yang *et al.,* 2015). The five B subunits bind the globotriaosylceramide receptor (Gb3) on the surface of eukaryotic cells, then the toxin is internalized by endocytosis and through the trans-Golgi network and endoplasmic reticulum affects the ribosome (Pacheco and Sperandio, 2012).

After a stress or spontaneously, temperate bacteriophages can move from lysogenic to lytic cycle with the possibility to increase the spread of *stx*-genes among *E. coli* (Lenzi *et al.,* 2016). Antibiotics have been reported as prophage inducer. Indeed, in the clinical treatment of STEC infections the use of antibiotics is controversial due to the release of prophages and the overproduction of toxins (McGannon *et al*., 2010). Furthermore, it is possible to notice several stressors in the food industry involved in the same phenomena. For example, during cheese making process, the addition of

NaCl and changes in temperature and in pH can be considered as potential stressors and induce bacteriophage release and toxin transmission.

The objectives of this work were to characterize temperate bacteriophages from STEC strains and to evaluate the influence of stress factors, some related to cheese making process, on transduction phenomena through a quantitative Real-time PCR (qPCR).

4.4.3 Materials and methods

4.4.3.1 Bacterial growth condition

The STEC strains used (supplementary table 4) were streaked on Tryptone Bile X-Glucuronide (TBX) (Merck, Darmstadt, Germany) agar plates and incubated at 37°C for 24 hours. A single colony was transferred in a 10 mL LB broth (Alfa Aesar, Karlsruhe, Germany) tube and incubated overnight at 37°C. The overnight culture was centrifuged at 1200 *g* for 15 minutes (Centrifuge 5415 D, Eppendorf, Hamburg, Germany), the supernatant was discarded, and the pellet was resuspended in LB broth added with 20% (v/v) glycerol and stored at -20°C until further use.

4.4.3.2 Bacteriophage DNA extraction and RAPD-PCR

The STEC strains were subjected to phage induction by adding 1 µg/mL of Norfloxacin (Sigma-Aldrich, St. Louis, USA) to cells at exponential phase (OD_{600nm}=0,2-0,3). After 6 hours of incubation at 37°C, the solutions were centrifuged at 4800 *g* for 10 min (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) and filtered through a 0,45 µm membrane filters (Minisart Syringe filter). Then, 50 mL of crude bacteriophage filtrate were precipitated by addition of 10% (w/v) of polyethylene glycol (PEG) 6000 (Merck, Darmstadt, Germany) and 0,5 M NaCl. After 6 hours at 4°C, the solution was centrifuged at 4800 *g* for 10 min (Centrifuge 5415 D) and the supernatant discarded. The pellet was resuspended in 400 µL of SM (100mM NaCl, 8mM $MgSO_4$, 50mM Tris-HCl, pH 7,5, 0,01% gelatin) buffer and incubated overnight at 4°C.
The suspension was subjected to an enzymatic treatment with 5 µL of DNase (20 mg/mL) (Roche, Mannheim, Germany) and 10 µL of RNase (5 mg/mL) (Merck, Darmstadt, Germany) at 37ºC for 60 min to remove any non-phage nucleic acids. Subsequently, the enzymes were inactivated by heat treatment at 75ºC for 10 min.

A PCR amplification of the 16S rRNA gene was performed to verify the presence of bacterial DNA using BSF-8 (5' AGAGTTTGATCCTGGCTCAG 3') and BSR-1541 (5'AAGGAGGTGATCCAGCCGCA 3') primers. The PCR products were processed by electrophoresis and if no bacterial DNA was detected the extraction process went on.

Then, 50 µL of EDTA (0,5 M; pH 8), 50 µL of SDS 10% (w/v) and 2,5 µL of Proteinase K (20 mg/mL) were added to the phage suspensions followed by incubation at 37ºC for 1 hour. Subsequently, 400 µL of saturated phenol were added, gently mixed and centrifuged at 13400 *g* for 10 min (Centrifuge 5415 D). The aqueous phase was transferred to a new tube and 200 µL of saturated phenol and 200 µL of chloroform: isoamyl alcohol (24:1) were added and centrifuged as previously described. The liquid phase was transferred in a new tube and added with 200 µL of sodium acetate (3 M; pH 7) and 600 µL of isopropanol, for DNA precipitation. After centrifugation under the same condition, the pellet was resuspended in 200 µL of Ethanol 70% (v/v), followed by centrifugation at 13400 *g* for 4 min. Ethanol was then discarded and pellet dried. After drying at 37ºC for 60 min, the pellet was resuspended in 50 µL of TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8) and the DNA was stored at -20ºC until further usage.

Random amplification of polymorphic DNA (RAPD) reactions were performed on bacteriophage DNA using two primers: M13 (5'-GAGGGTGGCGGTTCT-3') (Rossetti and Giraffa, 2005) and OPL5 (5'-ACGCAGGCAC-3') (Gutiérrez *et al*., 2011). The reaction mixes are reported in Table 8. The thermal cycles used are the ones reported in literature (Rossetti and Giraffa, 2005; Gutierrez *et al*., 2011). The RAPD-PCR products were run in agarose gel (1%) added with 0,4 µg/mL of ethidium bromide in TAE buffer (Tris-acetate 40 mM, ETDA 1 mM, pH 8) at 90 V for 60 min (Mini-Sub® Cell GT Cell, Bio-Rad, Hercules, USA). Two microliters of 10-kbp DNA marker ("All size DNA Mass Ladder", LeGene Biosciences, San Diego, CA, USA) were also loaded in the gel. Gels were visualized in the Gel Doc™ XR imaging system (Bio-Rad, Hercules,

USA) and analysed with Quantity One 1-D Analysis Software. Phage DNA was also tested for the presence of *stx* and *eae* genes as described in 4.1.3.7.

Table 8. RAPD-PCR reaction mixes.

Reagent	Initial concentration	Reaction mix	
		M13-RAPD	OPL5-RAPD
Reaction buffer (containing $MgCl_2$ 1,5 mM)	10X	1 X	1 X
$MgCl_2$	25 mM	–	1 mM
dNTPs	10 mM	200 µM	200 µM
Primer	10 µM	0,05 µM	0,8 µM
Taq polymerase*	5 U/µL	1 U	1 U
DNA template		80ng	80ng
Final volume		25 µL	25 µL

4.4.3.3 Assessment of bacteriophage inducers by qPCR

STEC strains in exponential phase (OD_{600nm}=0,2-0,3) were subjected to 0,5, 1,5 and 3% (v/v) lactic acid, 1, 1,5% and 2% (w/v) NaCl, anaerobic growth in LB tubes, pasteurization at 72ºC for 15 sec (calculated with a control tube equipped with a thermometer), UV irradiation (20 cm distance for 60 sec), as stress factors related with cheese production. Furthermore, strains were exposed to ciprofloxacin (0,5 µg/mL), nalidixic acid (3 µg/mL) and norfloxacin (1 µg/mL) (Sigma-Aldrich, St. Louis, USA).

After incubation at 37ºC for 16 h, the samples were centrifuged at 4800 *g* for 10 min (Centrifuge 5415 D) and filtered through 0,45 µm filters (Minisart® Sartorius). To remove bacterial DNA, 100 µL aliquots were treated with

DNase and RNase (10 mg/mL each) at 37°C for 30 min followed by heat treatment at 100°C for 10 min to inactivate the enzymes.

The assay was designed for 15-µl reactions (QPCR Green Master Mix LRox 1X, Biotechrabbit, Hennigsdorf, Germany) containing 400 nM of primers *stx1F* (5'ATAAATCGCCATTCGTTGACTAC 3') and *stx1R* (5' AGAACGCCCACTGAGATCATC 3') (EU-RL VTEC_Method_01_Rev 0, 2013). Real-time qPCR assays were carried out in a MasterCycler® ep Realplex (Eppendorf AG) with an initial denaturation at 95ºC for 3 min and 40 cycles as follows: 95ºC for 15 sec, 60ºC for 30 sec, 65ºC for 30 sec. A standard curve was obtained by 5-point interpolation of 10-fold serial dilutions of a bacterial gDNA extracted from STEC strain 225R-A carring the *stx1* and *eae* virulence genes. The DNA concentration was measured through a spectrophotometric lecture at 260_{nm} and the DNA copy number was calculated using a ThermoFisher tool: (https://www.thermofisher.com/it/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html).

Each experiment was replicated 4 times for each strain. The analysis of variance with post-hoc Tukey HSD (Honestly Significant Difference) was performed using the open-source software: R Core Team (R Core Team, 2017), "agricolae" and "ggplot2" packages for graphic processing.

4.4.4 Results and discussion

4.4.4.1 Bacteriophage RAPD-PCR

In order to select the bacterial strains able to release *stx*-phage after a stress, 35 STEC strains (sup. tab. 4) were subjected to phage induction using Norfloxacin. Three strains: 225R-A, 229RACH and F1-1 showed a sign of lysis in LB double layer agar plates and the presence of an amplification signal after PCR amplification of filtrates on *stx* genes. These strains were used for PCR-RAPD analysis and the quantification of phage release by a Real Time qPCR.

Random amplification of polymorphic DNA (RAPD) is a molecular technique used to generate genomic fingerprints based on the amplification of short sequence giving a genotypic differentiator among bacterial or phage samples (Gutiérrez *et al*., 2011). In order to determine diversity among the selected temperate bacteriophage, the reactions were performed using primers M13 (Rossetti and Giraffa, 2005) and OPL5 (Gutiérrez *et al*., 2011). The amplification products were processed by electrophoresis, and the resulting profiles are shown in Figure 22 and 23. The temperate bacteriophages were called: vB_Eco225R-A and vB_Eco229RACH, which presented identical profiles with both primers used, but distinctive from that of the third one vB_EcoF1-1.

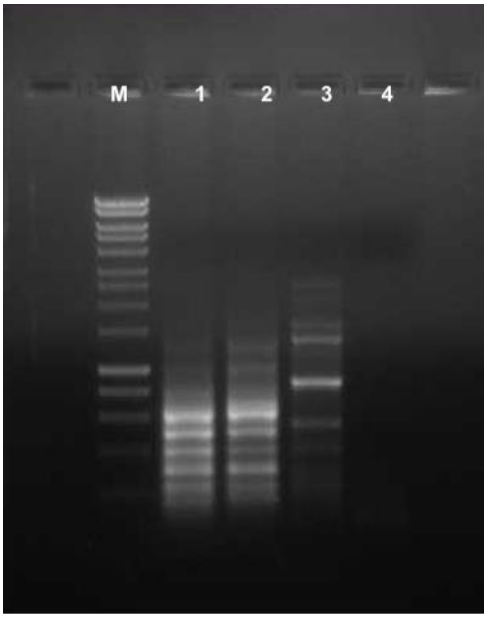

Figure 22. Bacteriophage fingerprinting: RAPD-PCR using M13 primer. M: all size DNA Ladder; 1: vB_Eco225R-A; 2: vB_Eco229RACH; 3: vB_EcoF1-1; 4: No template.

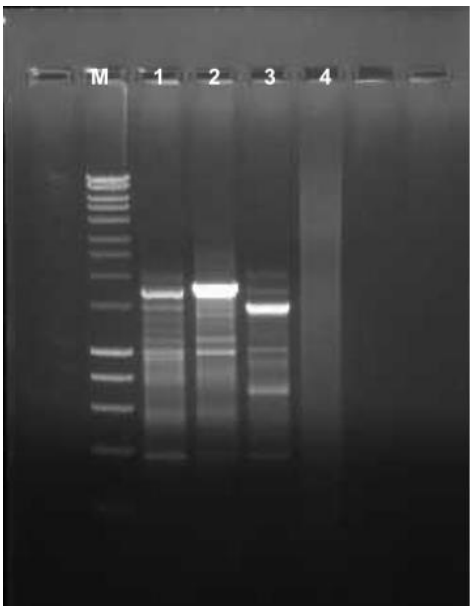

Figure 23. Bacteriophage fingerprinting: RAPD-PCR using OPL5 primer. M: all size DNA Ladder; 1: vB_Eco225R-A; 2: vB_Eco229RACH; 3: vB_EcoF1-1; 4: No template.

Furthermore, the two *E. coli* strains that hosted the temperate phages with the similar RAPD fingerprinting do not have the same serotype. In fact, 229RACH is an O111 while 225R-A is an O26. The two bacterial strains origin from two different patients. The other strain (F1-1) is another O26. Since Stx-bacteriophages can have a broad host ranges within *Enterobacteriaceae* (Allison, 2007), it is reasonable that the two bacteria used are infected with the same phage encoding *stx1*-gene.

4.4.4.2 Influence of stressors on bacteriophage induction

Pathogenic bacteria often have multiple temperate bacteriophages within their genome, which can be directed to a lytic cycle in response to a stress. One reason could be that any integration would provoke a battle with other prophages and bacterial defences; during this process some phages lose and some remain active producing progeny virions that are released in the environment (Argov *et al.,* 2019). The bacterium activated the SOS response, a complex response to DNA damage that includes induction of gene capable of blocking cell division and promoting mutation, recombination and DNA repair (McKenzie *et al.,* 2000).

In this work, three STEC strains were exposed to several stressors related to the cheese-making process: sodium chloride at three different concentrations (1%, 1,5% and 2% w/v), lactic acid (0,5%, 1,5% and 3% v/v), pasteurization, UV irradiation, deprivation of oxygen and antibiotics relevant for laboratory or clinical settings: ciprofloxacin, nalidixic acid and norfloxacin.

Evaluation was performed by Real Time qPCR amplification of the *stx1* gene. This gene was chosen because it was common among the three bacteria used and detectable also in their prophage.

SYBR Green qPCR can establish the amount of any double stranded DNA (Peng *et al.,* 2018). To quantify the amount of DNA contained in samples, a

5-point standard curve was used. The linear relationship of Ct versus Log (copies/mL) was: Y= -3,496x + 43,068, R^2=0,9983 (fig. 24).

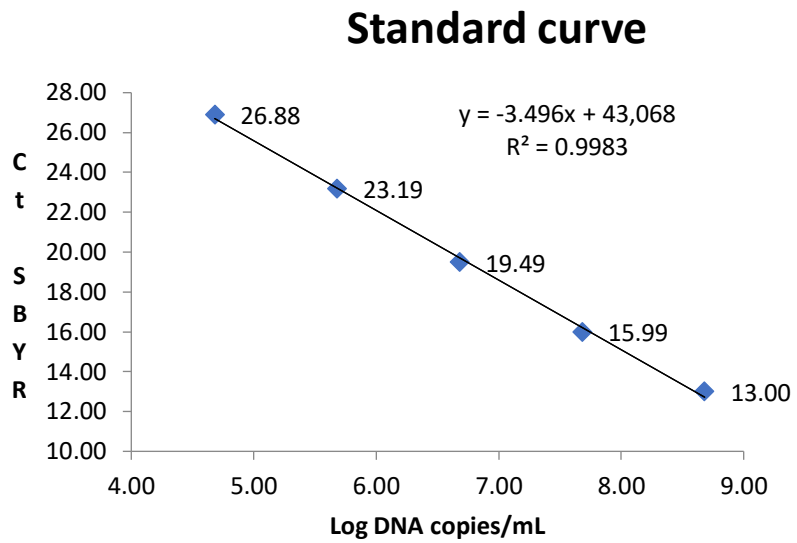

Figure 24. Standard curve of 225R-A DNA from SYBR Green qPCR Log DNA copies/ml calculated from serial dilution from 1:10 to 1:100000.

The addition of salt was one of the first antimicrobials used for food preservation and is still a popular method in food industry. Sodium chloride in cheese varies between 0,7% and 6,0%, depending on the type of cheese and the method of salting (Bansal and Mishra, 2020). The addition of salt improves the attributes of flavour, texture and appearance and has also an antimicrobial action against undesirable microorganisms.

During this work, temperate bacteriophages were studied under hypotonic conditions. The results are reported in supplementary table 5 and summarized in table 9. As can be seen from average values in Table 9, phage induction increased in direct proportion to NaCl concentration. In fact, the average values were: 8,19, 7,97 and 7,15 for the addition of 2%, 1,5% and 1% NaCl, respectively, compared to spontaneous induction of 6,49. In particular, there were significant differences for the addition of 1,5% and 2% in improving phage release compared to the control and no significant

difference for the addition of 1% NaCl (P<0,05). In a study conducted by Harris *et al.* (2012) to simulate meat process, two STEC strains were submitted to different levels of NaCl (0%, 1%, 2%, 3% w/v). Regarding the same concentration used in this study (2%), the result indicated the equivalent behaviour in improve phage release. Conversely, a salt supplementation of 3% reduced phage release.

Table 9. Summary of SYBR qPCR results organized by stressors. Values with different letters are significantly different groups (P<0,05) assigned by One-way ANOVA (Analysis Of Variance) with post-hoc Tukey HSD (Honestly Significant Difference). na: nalidixic acid; Ox: oxygen deprivation; Sp: spontaneous release; T°: heat treatment; No: norfloxacin; Ci: ciprofloxacin; LacAc: Lactic acid.

Stressors	Average Log copies/mL	std	n	groups
2%NaCl	8,19	0,91	12	a
1,5%NaCl	7,97	0,74	12	ab
UV	7,43	0,88	12	abc
1%NaCl	7,15	0,94	12	bcd
Ox	6,69	0,72	12	cde
Sp	6,49	1,00	12	cde
T°	6,29	0,95	12	de
na	6,24	0,66	12	de
No	6,04	0,46	12	ef
Ci	5,85	0,64	12	ef
LacAc	5,30	0,32	36	f

Commonly, physical methods are used to inactivate pathogenic microorganisms, as well as microorganisms and their enzymes that cause spoilage, ensuring food safety and extending shelf life (Yousef and Balasubramaniam, 2012). The effects of heat treatment (72 ºC for 15 sec), ionizing irradiation by exposure to UV light and oxygen deprivation were investigated by quantifying phage release after 16 hours incubation at 37ºC. UV induction of bacteriophage is a well know technique and is also used to

reduce microbial load in food industry. In this study, UV irradiation increased phage induction with a mean value of 7,43 compared to spontaneous induction of 6,49 Log DNA copies/mL. Additionally, oxygen scarcity improved phage release with a mean value of 6,69 Log DNA copies/mL. In both cases, no significant differences were found (P<0,05) (tab. 9). Heat treatment slightly reduced the spontaneous release with no significant differences (tab. 9). After this treatment, the inoculated bacteria in exponential phase were eliminated. The phenomenon was confirmed by the fact that non colony grew after plating in TBX agar plates after 48 hours incubation at 37ºC. It is possible to hypothesize that the presence of phage *stx-1* gene reported in the samples was due to the release that took place before treatment, during exponential growth.

To mimic the stress caused by the decrease in pH due to lactic acid bacteria during coagulation step in cheese making process, three STEC strains were added with lactic acid at three different concentrations (0,5, 1,5 and 3% v/v). The pH influences the growth of STEC strains since they were able to resist to a minimum of pH 4 for almost 8 hours until the disappearance of viability (Molina *et al.,* 2003). In a study conducted using Cheddar cheeses as a model system, it was demonstrated that among NaCl, pH and protonated lactic acid addition, the low pH was primarily responsible for controlling pathogen, including STEC (Oh *et al.,* 2014).

The data reported in sup. tab. 5 showed a substantially homogeneous result in the three lactic acid additions (0,5, 1,5 and 3%v/v) performed. Therefore, the data in table 9 (containing the average of each addition and the statistical analysis) about the lactic acid addition were reported as lactic acid addition considering them 12 repetitions for each bacterial strain used, regardless the amount of acid added. As we can notice, the addition of lactic acid reduced significatively the prophage release with an average of 5,30 compared to spontaneous release 6,49 (P<0,05) log DNA copies/mL. This finding was in agreement with a previous research that showed how *stx* induction was inhibited at a pH lower than 5,5 (Imamovic and Muniesa, 2012). Furthermore,

in another study in which the phage release was evaluated by adding 1,5 and 3% of lactic acid, temperate phages could not be detected (Bonanno *et al.,* 2017).

Table 10 shows the phage release by the three strains with the value of 7,22, 6,17 and 6,05 Log DNA copies/mL for the strains 225R-A, F1-1 and 229RACH, respectively. The strain 225R-A was significantly different from the other two (P <0,05).

Table 10. Summary of SYBR qPCR organized by different strains. Values with different letters are significantly different groups (P< 0,05) assigned by One-way ANOVA (Analysis Of Variance) with post-hoc Tukey HSD (Honestly Significant Difference). Copies: Log phage DNA copies/ml; Std: standard deviation; n: number of replicates; groups: assigned by Tukey HSD test.

Bacteria	Average Log copies/mL	std	n	groups
225R-A	7,22	1,26	52	a
F1-1	6,17	0,90	52	b
229RACH	6,05	0,96	52	b

So, some stressors related to cheese making process can improve or decrease the phage release, influencing the safety of the process. Furthermore, the presence of free stx-phage could be a potential cause of false positive in food samples analyzed by PCR (Bonanno *et al*., 2017).

Results are summarized in Figure 25 where it can be noticed that the median of spontaneous release is lower than the median of 2%, 1,5%, 1% NaCl, UV irradiation, heat treatment and oxygen deprivation. So, these stressors improve the phage release. Unexpectedly, the addition of all three antibiotics decreases, even if not significantly, the amount of phage release compared to the control.

Significant differences from the control were found for the addition of 2 and 1,5% NaCl with the improvement of phage release and a significant decrease due to the addition of lactic acid (tab. 9) (P<0,05). No significant differences were found for the other applied stressors.

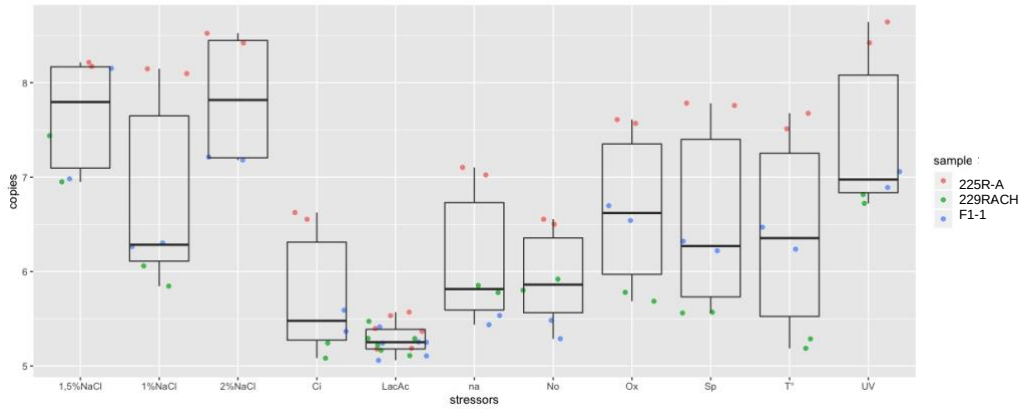

Figure 25. Box plots representing the distribution of the phage DNA copies/ml for each stressor. Ci: ciprofloxacin; LacAc: Lactic acid; na: nalidixic acid; No: norfloxacin; Ox: oxygen deprivation; Sp: spontaneous release; T°: heat treatment.

4.4.5 References

Allison, H. E., 2007. Stx-phages: Drivers and mediators of the evolution of STEC and STEC-like pathogens. Future Microbiology, 2(2), 165-174. https://doi.org/10.2217/17460913.2.2.165

Argov, T., Sapir, S.R., Pasechnek, A., Azulay, G., Stadnyuk, O., Rabinovich, L., Sigal, N., Borovok, I., Herskovits, A. A., 2019. Coordination of cohabiting phage elements supports bacteria–phage cooperation. Nat. Commun. 10, 5288. https://doi.org/10.1038/s41467-019-13296-x

Bansal, V., Mishra, S. K., 2020. Reduced-sodium cheeses: Implications of reducing sodium chloride on cheese quality and safety. Comprehensive Reviews in Food Science and Food Safety, 19(2), 733-758. https://doi.org/10.1111/1541-4337.12524

Bonanno, L., Delubac, B., Michel, V., Auvray, F., 2017. Influence of stress factors related to cheese-making process and to STEC detection procedure on the induction of Stx phages from STEC O26: H11. Frontiers in microbiology, 8, 296. https://doi.org/10.3389/fmicb.2017.00296

Brenner, D. J., Krieg, N. R., Staley, J. T., Garrity, G. M., Brenner, D. J., Vos, P. De, Noel, R., 2005. Bergey's Manual of Systematic Bacteriology (2nd ed.). Springer, Boston, MA.

Castro, V. S., Carvalho, R. C. T., Conte-Junior, C. A., Figuiredo, E. E. S., 2017. Shiga-toxin producing *Escherichia coli*: pathogenicity, supershedding, diagnostic methods, occurrence, and foodborne outbreaks. Comprehensive Reviews in Food Science and Food Safety, 16(6), 1269-1280. https://doi.org/10.1111/1541-4337.12302

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., Finlay, B. B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin. Microbiol. Rev. 26, 822–880. Doi: 10.1128/CMR.00022-13

European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA), 2016. Multi-country outbreak of Shiga toxin-producing *Escherichia coli* infection associated with haemolytic uraemic syndrome. Available from: http://ecdc.europa. Eu/en/publications/Publications/RRA-Escherichia-coli-O26- Romania-Italy-April2016.pdf

EFSA, ECDC, 2019. The European Union One Health 2018 Zoonoses Report. EFSA Journal, 17(12), 5926. https://doi.org/10.2903/j.efsa.2019.5926

European Union Reference Laboratory (EURL), 2013. Identification and Characterization of Verocytotoxin-Producing *Escherichia coli* (VTEC) by Real Time PCR Amplification of the Main Virulence Genes and the Genes Associated with the Serogroups Mainly Associated With Severe Human Infections. EU-RL VTEC_Methods. Retrieved from www.iss.it/vtec

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., Thevenot, D., Condron, R., De Reu K, Govaris, A., Heggum, K., Heyndrickx, M., Hummerjohann, J., Lindsay, D., Miszczycha, S., Moussiegt, S., Verstraete, K., 2013. Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. Int. J. Food Microbiol. 162, 190–212. Doi: 10.1016/j.ijfoodmicro.2012.08.008

Gutiérrez, D., Martín-Platero, A. M., Rodríguez, A., Martínez-Bueno, M., García, P., Martínez, B., 2011. Typing of bacteriophages by randomly amplified polymorphic DNA (RAPD)-PCR to assess genetic diversity. FEMS Microbiology Letters, 322(1), 90–97. https://doi.org/10.1111/j.1574-6968.2011.02342.x

Harris, S. M., Yue, W., Olsen, S. A., Hu, J., Means, W. J., McCormick, R. J., Zhu, M., 2012. Salt at concentrations relevant to meat processing enhances Shiga toxin 2 production in *Escherichia coli* O157:H7. Int. J. Food Microbiol., 159(3), 186–192. https://doi.org/10.1016/j.ijfoodmicro.2012.09.007

Imamovic, L., Muniesa, M., 2012. Characterizing RecA-Independent Induction of Shiga toxin2-Encoding Phages by EDTA Treatment. PloS ONE 7(2): e32393. https://doi.org/10.1371/journal.pone.0032393

Lenzi, L. J., Lucchesi, P. M. A., Medico, L., Burgán, J., Krüger, A., 2016. Effect of the Food Additives Sodium Citrate and Disodium Phosphate on Shiga Toxin-Producing *Escherichia coli* and Production of stx-Phages and Shiga toxin. Frontiers in Microbiology, 7(June), 1–7. https://doi.org/10.3389/fmicb.2016.00992

Liu, D., 2019. *Escherichia coli*. In Encyclopedia of Microbiology (4th Edition), pp. 171-182. https://doi.org/10.1016/B978-0-12-801238-3.02291-1

McGannon, C. M., Fuller, C. A., Weiss, A. A., 2010. Different classes of antibiotics differentially influence Shiga toxin production. Antimicrobial Agents and Chemotherapy, DOI: 10.1128/AAC.01783-09

McKenzie, G. J., Harris, R. S., Lee, P. L., Rosenberg, S. M., 2000. The SOS response regulates adaptive mutation. Proceedings of the National Academy of Sciences, *97*(12), 6646-6651. DOI: 10.1073/pnas.120161797.

Molina, P.M., Parma, A.E., Sanz, M.E., 2003. Survival in acidic and alcoholic medium of Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157:H7 isolated in Argentina. BMC Microbiol 3, 17. https://doi.org/10.1186/1471-2180-3-17

Oh, J. H., Vinay-Lara, E., McMinn Jr, R., Glass, K. A., Johnson, M. E., Steele, J. L., 2014. Evaluation of NaCl, pH, and lactic acid on the growth of Shiga toxin-producing *Escherichia coli* in a liquid Cheddar cheese extract. Journal of dairy science, 97(11), 6671-6679. https://doi.org/10.3168/jds.2014-7946

R Core Team (2017). R: A Language and Environment for Statistical Computing. Vienna: R Found. Stat. Comput. URL http://www.R-project.org/

Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. Journal of Microbiological Methods, 63(2), 135–144. https://doi.org/10.1016/j.mimet.2005.03.001

Pacheco, A.R., Sperandio, V., 2012. Shiga toxin in enterohemorrhagic *E. coli*: regulation and novel anti-virulence strategies. Front. Cell. Infect. Microbiol. 2:81. Doi: 10.3389/fcimb.2012.00081

Peng, X., Nguyen, A., Ghosh, D., 2018. Quantification of M13 and T7 bacteriophages by TaqMan and SYBR green qPCR. Journal of virological methods, *252*, 100-107. Doi: 10.1016/j.jviromet.2017.11.012.

Twardoń, J., Sobieszczańska, B., Gonet, A., Błaszkowska, M., 2005. Epidemiology of Shiga-like toxin–producing *Escherichia coli* strains (STEC). Veterinary Medicine, 8(4), 03. http://www.ejpau.media.pl/volume8/issue4/art-03.html

Valilis, E., Ramsey, A., Sidiq, S., DuPont, H.L., 2018. Non-O157 Shiga toxin-producing *Escherichia coli*-a poorly appreciated enteric pathogen: Systematic review. Int. J. Infect. Dis. 76, 82–87. Doi: 10.1016/j.ijid.2018.09.002.

Yang, S.C., Hung, C.F., Aljuffali, I.A., Fang, J.Y., 2015. The roles of the virulence factor *IpaB* in *Shigella* spp. In the escape from immune cells and invasion of epithelial cells. Microbiol Res 181:43–51. Doi: 10.1016/j.micres. 2015.08.006.

Yang, S. C., Lin, C. H., Aljuffali, I. A., Fang, J. Y., 2017. Current pathogenic *Escherichia coli* foodborne outbreak cases and therapy development. Archives of Microbiology, 199(6), 811-825. https://doi.org/10.1007/s00203-017-1393-y

Yousef, A. E., Balasubramaniam, V. M., 2012. Physical Methods of Food Preservation in Doyle, M. P., Buchanan R. L. (Ed.), Food Microbiology: Fundamentals and Frontiers, 4th edition. American Society of Microbiology Press, Washington, DC.

Supplementary table 4. List of STEC strains used.

Strain	Sample source	Serogroup
214CH	Human stool	O157
214R-ACH	Human stool	O26
214R-MCH-B	Human stool	O157
224SMA-GS	Human stool	ND
225R-A	Human stool	O26
226BB	Human stool	O157
227MCH	Human stool	O157
227Rosa	Human stool	ND
228GS	Human stool	O145
229B-ACH	Human stool	ND
229M-AS	Human stool	ND
229PRAL-ACH	Human stool	O26
229PRAL-AS	Human stool	ND
229RACH	Human stool	O111
229Rosa-A	Human stool	ND
231PCH-A	Human stool	ND
232AS-B-LUC	Human stool	ND
233P-CH-A	Human stool	ND
239R-A	Human stool	ND
242CH	Human stool	O157
242Rossa	Human stool	O157
243RACH	Human stool	O26
L12-2	Raw Goat Milk	O26
L36-2	Raw Goat Milk	ND
F1-1	Goat's Milking Filter	O26
F10-4	Goat's Milking Filter	O26
F80-1	Goat's Milking Filter	ND
F80-2	Goat's Milking Filter	ND
F80-3	Goat's Milking Filter	ND
F80-4	Goat's Milking Filter	ND
F90-1	Goat's Milking Filter	ND
F90-3	Goat's Milking Filter	ND
F93-3	Goat's Milking Filter	O26
F95-2	Goat's Milking Filter	O26
F95-3	Goat's Milking Filter	O26

Supplementary table 5. Results of SYBR qPCR. Std: standard deviation; LacAc: Lactic acid; Ox: oxygen deprivation; T°: heat treatment; Sp: spontaneous release; na: nalidixic acid; No: norfloxacin; Ci: ciprofloxacin.

		Log DNA copies/ml, replicates:					
bacteria	**stressors**	**1**	**2**	**3**	**4**	**average**	**std**
225R-A	1%NaCl	8,090	8,035	8,548	8,586	8,315	0,292
225R-A	1,5%NaCl	8,118	8,164	9,331	9,170	8,696	0,644
225R-A	2%NaCl	8,402	8,525	9,416	9,394	8,934	0,546
229RACH	1%NaCl	6,165	5,993	6,854	7,202	6,553	0,570
229RACH	1,5%NaCl	6,920	7,369	7,822	8,120	7,558	0,526
229RACH	2%NaCl	6,828	7,089	8,592	8,287	7,699	0,871
F1-1	1%NaCl	6,334	6,365	7,080	6,492	6,568	0,348
F1-1	1,5%NaCl	8,096	6,949	7,929	7,661	7,659	0,506
F1-1	2%NaCl	7,157	7,129	8,796	8,702	7,946	0,928
225R-A	UV	8,402	8,673	8,570	8,787	8,608	0,164
229RACH	UV	6,803	6,720	6,605	6,706	6,708	0,081
F1-1	UV	7,017	6,868	7,030	7,011	6,981	0,076
225R-A	0,5%LacAc	5,747	5,776	5,543	5,495	5,640	0,142
225R-A	1,5%LacAc	5,618	5,481	4,904	4,860	5,216	0,390
225R-A	3%LacAc	5,641	5,475	5,093	5,222	5,358	0,247
225R-A	Ox	7,495	7,535	7,599	7,523	7,538	0,044
229RACH	Ox	5,942	5,867	5,473	6,662	5,986	0,495
F1-1	Ox	6,700	6,565	6,391	6,476	6,533	0,132
229RACH	0,5%LacAc	5,507	5,701	5,030	4,980	5,305	0,355
22RACH	1,5%LacAc	5,467	5,424	5,024	4,845	5,190	0,305
229RACH	3%LacAc	5,561	5,561	4,892	5,483	5,374	0,324
225R-A	T°	7,601	7,441	7,287	7,347	7,419	0,137
229RACH	T°	5,481	5,558	5,002	4,986	5,257	0,305
F1-1	T°	6,505	6,311	6,024	5,986	6,206	0,246
F1-1	0,5%LacAc	5,524	5,656	4,911	4,873	5,241	0,407
F1-1	1,5%LacAc	5,421	5,530	4,820	4,804	5,144	0,386
F1-1	3%LacAc	5,533	5,387	4,908	4,999	5,206	0,301
225R-A	Sp	7,684	7,707	7,564	8,146	7,775	0,255
22RACH	Sp	5,776	5,770	5,313	5,414	5,568	0,240
F1-1	Sp	6,379	6,296	5,923	5,945	6,136	0,236
225R-A	na	6,986	7,057	6,973	7,224	7,060	0,116
229RACH	na	5,939	5,999	6,335	6,042	6,079	0,176
F1-1	na	5,747	5,673	5,587	5,316	5,581	0,188

225R-A	No	6,577	6,531	6,781	6,501	6,598	0,126
229RACH	No	6,053	5,959	6,042	5,848	5,975	0,095
F1-1	No	5,558	5,710	5,313	5,665	5,562	0,177
225R-A	Ci	6,574	6,637	6,866	6,668	6,686	0,126
229RACH	Ci	5,404	5,524	5,307	5,219	5,363	0,131

5 GENERAL CONCLUSION

In recent years, with the spread of the antibiotic resistant genes, there is a new interest on bacteriophage application not only for the study of the biological implication they have, but also for the control of pathogenic bacteria in different fields. During this work novel bacteriophages were isolated in cattle herds and applied with positive results to control a collection of 31 STEC strains, including O157 and non-O157 serogroups and a collection of 270 strains related to Urinary Tract Infections. Only 8 bacteria showed to be resistant to all the tested bacteriophages.

In addition, a cocktail made with different phages proved to be able to control STEC strains in a challenge test on fresh cucumbers, reducing microbial load of at least two log cycles, at both the temperatures tested. Phages were also efficient in preventing 43,64% of biofilm formation.

To ensure the safety of their application, the bacteriophages were sequenced confirming that no genes related to pathogenesis or antibiotic resistance were present in the whole genome. Indeed, the Shiga toxin transmission is under phage control. Furthermore, in this work was demonstrated that, by simulating a cheese making process, the addition of salt can increase the release of temperate bacteriophages, but the spreading of genes is limited by the low pH.

According to the results obtained in this thesis, resistance to antibiotic and to phages is provided by different mechanisms, suggesting that the formulation of a phage cocktail active on different pathogenic *E. coli* can be for example, directly applied on crops or during the washing step of some Ready to eat food, preventing infections of multi-drug resistant bacteria by inactivating them before infection occurs.

Thus, the isolated bacteriophages that have been here characterized and studied, provide a promising tool for improving food safety through the control of pathogenic *E. coli*.

Furthermore, several bacteriophage preparations have already been granted as Generally Recognized as Safe (GRAS) by the FDA and already available on the market (e.g., SalmoFresh™, ListShield™, PhagheGuard™ and EcoShield) (Moye et al., 2018). However, European law has not yet approved the use of bacteriophage for the food industry.

Nevertheless, the EFSA opinions in 2009 and 2016 on a general application of phages to control pathogens in food and on a specific phage product received a positive evaluation. It is therefore not strange to think that in the near future, bacteriophages will also be authorized and used in Europe.

6 IMPLICATIONS AND FUTURE DIRECTIONS

Based on the best knowledge acquired, this study appears to be the first one on bacteriophages isolated from cattle herds in Milan area and applied to a large collection of different types of pathogenic *E. coli*, including STEC (O157 and non O157) and *E. coli* related to Urinary tract infections. Hence, as the number of multi-drug resistant bacteria (including *E. coli*) continue to increase, it is crucial to invest in alternatives: the application of bacteriophages, also considering the results obtained during this study, seems to be one of the most suitable.

However, after gene annotation of bacteriophage genomes, a vast gap in knowledge can be noted due to the presence of several unknown genes. Little is known about bacteriophages and research in this field will improve the knowledge and at the same time the safety of their use. The tests performed on the human model (the so-called phage therapy) would also be of relevant interest, but this is not the subject of my thesis and of my PhD course

Advances in bacteriophage research and growing interest are likely to be a key point for the future of food safety because the main advantage of this strategy is the ability of bacteriophage to evolve with bacteria, to run faster than bacteria, in a race that also involved research to find new bacteriophages to make our planet a safer place...

A concept similar to the one told by Lewis Carroll in Alice's Adventures in Wonderland: “Now, here, you see, it takes all the running you can do, to keep in the same place.”

7. APPENDICES

Lists of publication:

Peer reviewed:

- **Mangieri N.,** Picozzi C., Cocuzzi R. and Foschino R. (2020). Evaluation of a Potential Bacteriophage Cocktail for the Control of Shiga-Toxin Producing *Escherichia coli* in Food. Frontiers in Microbiology. 11:1801. doi: 10.3389/fmicb.2020.01801;

- Beccalli M., Picozzi C., **Mangieri N.**, Vigentini I. and Foschino R. (2019). Assessment of Microbial Populations in the Manufacture of Vacuum-Packaged Ready-to-Eat Roast Beef and in a Related Production Plant. Journal of Food Protection, Vol. 82, No. 1, 2018, Pages 58–64 doi: 10.4315/0362-028X.JFP-18-147;

- Cordero-Bueso G., **Mangieri N**., Maghradze D., Foschino R., Valdetara F., Cantoral J. M. and Vigentini I. (2017). Wild Grape-Associated Yeasts as Promising Biocontrol Agents against *Vitis vinifera* Fungal Pathogens. Frontiers in Microbiology; 8: 2025. doi: 10.3389/fmicb.2017.02025;

PRESENTATIONS AND POSTERS:

- **Mangieri N.** (2020). Bacteriophage application for controlling pathogenic *Escherichia coli.* Proceedings of the Workshop on PhD Research in Food Systems, Milan, September 14-18. ISBN 978-88-945582-0-3. (oral presentation).

- **Mangieri N.**, Viera R., Foschino R. and Picozzi C. (2019). Evidence of the same stx-phage in two different *E. coli* serotypes. 5th International conference on microbial diversity. Catania, Italy, September 25-27. (poster presentation).

- **Mangieri N**. (2018). Bacteriophage application to control non-O157 Shiga-toxin producing *Escherichia coli.* XXIII Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology; Oristano, September 19-21; ISBN 978-88-907678-6-9 (Poster presentation).

- **Mangieri N.,** Foschino R. and Picozzi C. (2018). Can the induction of stx phages be influenced by stressors in cheese-making process? 10th International symposium on Shiga toxin (Verocytotoxin) Producing *Escherichia coli* Infections; Florence, May 6-8; (Poster presentation).

- Cordero-Bueso G., **Mangieri N**., Foschino R., Maghradze D., Ruiz-Munoz M., Cantoral J. M. and Vigentini I. (2018). Levaduras aisladas de la vid silvestre y diferentes sistemas de cultivo del viñedo como estrategia para el biocontrol de hongos fitopatógenos. Microbiologia industrial y biotecnologia microbiana: actas del VII CMIBM 2018. ISBN: 978-84-949056-3-6 (Oral presentation).

- Picozzi C., **Mangieri N**., Antoniani D., Vigentini I. and Foschino R. (2017). Different occurence of biofilm producing STEC in dairy and human isolates. Microbial diversity 2017 (Poster presentation).

- Cordero-Bueso G., Vigentini I., Foschino R., Maghradze D., **Mangieri N**. and Cantoral J. M. (2017). Wild Grape-Associated Yeasts as a Promising Strategy of Biocontrol against *Vitis vinifera* Fungal Pathogens. FEMS 2017 (Poster presentation).

- Cordero-Bueso G., **Mangieri N.**, Foschino R., Maghradze D., Cantoral J. M. and Vigentini I. (2017). Wild grape-associated yeasts as a promising strategy of biocontrol against *Vitis vinifera* fungal pathogens. The YeSVitE Conference. ISBN 979-12-200-2601-7 (Oral presentation).