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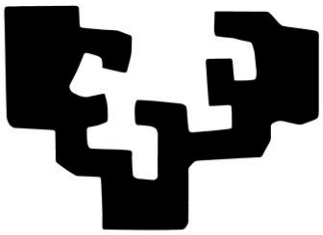

Universidad del País Vasco

Euskal Herriko Unibertsitatea

Isolation, characterization and selection of new *Campylobacter* specific bacteriophages to use as biocontrol agents

Ibai Nafarrate Mendez

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Eskerrik asko ama y aita, por toda la paciencia que habéis tenido y todo lo que me habéis ayudado con todo, con pequeñas y grandes cosas, los ánimos que me habéis dado y todo lo que habéis preocupado por que estuviera bien y todo fuese como tenía que ir. Y a mi hermano, que aunque ha estado mucho tiempo fuera también me ha animado.

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Espero no olvidarme de nadie…

Eskerrik asko!!!!

Abstract

Campylobacter is a high priority issue for food safety and public health since it causes campylobacteriosis, the most common food borne zoonotic illness in humans, and the incidence of antibiotic resistant *Campylobacter* strains is increasing. The application of *Campylobacter* specific bacteriophages is a promising strategy to complement currently existing intervention measures. The objective of this work was to develop an innovative *Campylobacter* biocontrol tool based on the application of specific bacteriophages. Three hundred and four *Campylobacter* specific bacteriophages were isolated from broiler meat (280) and broiler and swine faecal droppings (six and 18). According to their genome size, they were classified within the campylophages groups II (18) and III (286) and after a first host range analysis against 19 *Campylobacter* strains, 59 bacteriophages were selected. Their genetic diversity was determined by RFLP and RAPD-PCR analyses, and their lytic spectrum assessed against 41*Campylobacter* strains of diverse species, sources and antimicrobial resistance. Ten bacteriophages were then selected and their lytic spectrum was evaluated against 110 *Campylobacter* strains of different geographical origins (Spain, Italy and Poland) and antimicrobial resistance profiles. These bacteriophages belonging to both groups, II and III, showed specific but complementary host ranges, being able to infect antibiotic resistant *Campylobacter* isolates from the three countries. All of them remained stable and active at pH values from 2 to 9 and temperatures from 4 to 42 °C. In addition, those bacteriophages that displayed short latent periods and large burst sizes were considered to select five bacteriophages as the most promising candidates for the development of a *Campylobacter* biocontrol tool for applications from primary production to food consumption.

Laburpena

Campylobacter lehentasunezko gaia da elikagaien segurtasunerako eta osasun publikorako, campilobacteriosis izenez ezagutzen den zoonosiaren erantzulea delako eta antibiotikoekiko erresistenteak diren anduien intzidentzia handia delako. *Campylobacter* bakteriofago espezifikoak erabiltzea etorkizun handiko estrategia da gaur egun dauden biokontrol neurriak osatzeko. Ikerketa honen helburua bakteriofagoen aplikazioan oinarritutako *Campylobacter*ren biokontrol tresna berritzailea garatzea izan zen. Hirurehun eta lau bakteriofago isolatu ziren oilasko haragiaren (280) eta oilasko eta txerri gorotzen (sei eta 18) laginetatik. Bakteriofago horiek II. (18) eta III. (286) taldeetan sailkatu ziren haien genoma tamainaren arabera. *Campylobacter* espezieko 19 anduiren aurkako gaitasun litikoa aztertu ondoren, 59 bakteriofago hautatu ziren. Bakteriofago horien dibertsitate genetikoa RFLP eta RAPD-PCR bidez aztertu zen, eta haien espektro litikoa, espezie, jatorri eta antibiotikoekiko sentikortasunaren profil desberdineko 41 *Campylobacter* anduiren aurrean ebaluatu zen. Hamar bakteriofago aukeratu ziren eta haien especktro litikoa jatorri geografiko (Espainia, Italia eta Polonia) eta antibiotikoekiko sentikortasun-profil desberdinetako 110 *Campylobacter* anduiren aurka ebaluatu zen. Bi taldeetako bakteriofago horiek gaitasun litiko espezifikoa eta haien artean komplementarioa erakutsi zuten, eta gai izan ziren hiru herrialdeetatik zetozen antibiotikoekiko erresistenteak ziren anduiak infektatzeko. Guztiak egonkor eta aktibo mantendu ziren 2 eta 9 arteko pH-an eta 4 eta 42 ° C arteko tenperaturan, gainera, latentzia-aldi laburrak eta leherketa-tamaina handiak erakutsi zituzten bakteriofagoak hartu ziren kontuan, bost bakteriofago hautatzeko elikagaien ekoizpen-kate osoan aplikatu ahal izango den *Campylobacter* biokontrolerako tresna bat garatzeko.

Resumen

Campylobacter supone un gran problema para la seguridad alimentaria y la salud pública por ser el patógeno alimentario responsable de la zoonosis conocida como campilobacteriosis y por su alta incidencia de cepas resistentes a antibióticos. El empleo de bacteriófagos específicos de *Campylobacter* es una prometedora estrategia para complementar las medidas existentes de intervención y control de este patógeno. El objetivo de este trabajo fue desarrollar una herramienta innovadora, basada en la aplicación de bacteriófagos, para el biocontrol de *Campylobacter*. Se aislaron 304 bacteriófagos a partir de muestras de carne de pollo (280) y excrementos de pollo y cerdo (seis y 18), que, según su tamaño de genoma, se clasificaron dentro de los grupos II (18) y III (286). Tras un primer análisis de su capacidad lítica contra 19 cepas de *Campylobacter* se seleccionaron 59 bacteriófagos. Se estudió la diversidad genética de éstos mediante las técnicas de RFLP y RAPD-PCR y se evaluó su espectro lítico frente a 41 cepas de *Campylobacter* de diferentes especies, fuentes y perfiles de sensibilidad a antibióticos. Se seleccionaron diez bacteriófagos que se enfrentaron a 110 cepas de *Campylobacter* de diferentes orígenes geográficos (España, Italia y Polonia) y con diferentes perfiles de sensibilidad a antibióticos. Estos bacteriófagos pertenecientes a ambos grupos II y III mostraron una capacidad lítica específica al tiempo que complementaria y fueron capaces de infectar cepas resistentes a antibióticos provenientes de los tres países. Todos se mantuvieron estables y activos a pH de entre 2 y 9 y temperaturas de entre 4 a 42 ° C. Se consideraron, además, aquellos bacteriófagos que tenían períodos de latencia cortos y grandes tamaños de explosión para seleccionar cinco bacteriófagos como los más prometedores para el biocontrol de *Campylobacter* en toda la cadena de producción de alimentos.

INDEX

JUSTIFICATION

AND

OBJETIVES

The present work, aimed to obtain a Ph.D. degree, has been performed from 2016 to 2020 in the Bacteriophages Research Group of the Food Quality, Safety and Identity Area of the Food Research Division of AZTI. During this period, I held a training grant to young researchers in the scientific-technological and business environment of the Basque agricultural and food sector, from the Department of Agriculture, Fisheries and Food of the Basque Government.

During the first three years of my thesis, my work was carried out within the project "BERRIPHAGE: new bacteriophage-based tools for the biological control of foodborne pathogens", financial supported by the Department of Agriculture, Fisheries and Food of the Basque Government. Within the framework of the BERRIPHAGE project, my thesis was focused in the study of *Campylobacter*-specific bacteriophages. This work resulted also in the fruitful and close collaboration with the Group UFI 11/25 "Microbios y Salud", at the Department of Immunology, Microbiology and Parasitology of the University of the Basque Country (UPV / EHU).

During the last year of my thesis, I worked in the European Project entitled "C-SNIPER: *Campylobacter*-Specific Nullification via Innovative Phage-mediated Enteropathogen Reduction", coordinated by AZTI and financial supported by EIT-Food, No. 19241, aimed to develop a new bacteriophage-based solution to control the prevalence of *Campylobacter* in poultry.

Several strategies have been proposed to control *Campylobacter* at different stages of the production chain, but none of them has completely eliminate this food borne pathogen and it still is the most reported zoonotic problem. Moreover, the development and spread of antimicrobial resistant *Campylobacter* species has become a threat to

public health. Therefore, there is an urgent need to develop an alternative non-antibiotic based strategy to control this pathogen within the farm-to-fork process. Among the different existing alternatives, one of the most promising strategies is the use of specific bacteriophages as biocontrol agents.

The working hypothesis of this doctoral thesis was: ***Campylobacter*-specific bacteriophages have the potential to be used as innovative strategies to control this food borne pathogen from primary production to food consumption.**

Our general objective, to demonstrate this hypothesis, was to develop an innovative *Campylobacter* biocontrol tool based on the application of *Campylobacter* specific bacteriophages.

These four partial objectives were developed to achieve our general aim:

1.- To isolate new *Campylobacter* specific bacteriophages effective against different species of *Campylobacter*.

2.- To characterize different *Campylobacter* isolates of diverse species in order to stablish a collection of target bacteria from different sources to analyse the lytic spectrum of new *Campylobacter* specific bacteriophages.

3.- To evaluate the host specificity and genetic diversity of new *Campylobacter* specific bacteriophages.

4.- To select the most appropriate *Campylobacter* specific bacteriophage candidates to be used as biocontrol agents according to their technological properties.

EXPERIMENTAL DESIGN

In order to achieve the objectives proposed, the following experimental design was performed:

Objective 1: To isolate new *Campylobacter* specific bacteriophages effective against different species of *Campylobacter*.

Study 1: Efficient isolation of *Campylobacter* bacteriophages from chicken skin, analysis of several isolation protocols. Manuscript accepted with minor revisions by *Food Microbiology*.

Seven new and already published *Campylobacter* specific bacteriophage isolation methods were assessed on the basis of an efficient bacteriophage recovery. These protocols were assayed in broiler skin matrices with known concentrations of bacteriophages. The detection limit and recovery efficiency of each method were measured. The most successful method was applied for the isolation of new *Campylobacter* specific bacteriophages from different broiler samples, including necks, thighs and wings. *Campylobacter* isolates from broiler skin or faeces samples were used as host bacteria. The new recovered bacteriophages were purified and stored for further analysis.

Objective 2: To characterize different *Campylobacter* isolates of diverse species in order to stablish a collection of target bacteria from different sources to analyse the lytic spectrum of new *Campylobacter* specific bacteriophages.

Study 2: Molecular typing and antimicrobial susceptibility of *Campylobacter* spp. isolates in northern Spain. Manuscript submitted to *International Journal of Food Microbiology*.

A collection of 89 *Campylobacter* isolates were isolated from human clinical samples, retail broiler and faecal droppings of broiler and swine in the north area of Spain. The identification of *Campylobacter* species was performed by multiplex PCR and the genetic diversity was analysed using *fla*A-RFLP (*fla*A-Restriction Fragment Length Polymorphism) and PFGE (Pulsed-Field Gel Electrophoresis) genotyping methods. The antimicrobial susceptibility/resistance of the *Campylobacter* isolates to ciprofloxacin (a fluoroquinolones agent), erythromycin (a macrolide agent) and tetracycline (a tetracycline agent) was determined by disk diffusion as described by the guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST). Furthermore, the correlation between specific genotype and antibiotic susceptibility/resistance profile was assessed. These new *Campylobacter* isolates were stored to later use in the analysis of the lytic ability of 304 new bacteriophages.

Objective 3: To evaluate the host specificity and genetic diversity of new *Campylobacter* specific bacteriophages.

Study 3: Host specificity and genetic diversity of new *Campylobacter* specific bacteriophages. Manuscript in preparation.

The collection of 304 new *Campylobacter* specific bacteriophages mainly isolated in the first study was characterized. Genome size of the bacteriophages was determined by PFGE (Pulsed-Field Gel Electrophoresis) and classified within campylophages of groups II (180 kb) or III (140 kb). Genetic diversity of the bacteriophages was analysed using the restriction endonucleases usually applied for *Campylobacter* specific bacteriophages, and the technique of RAPD-PCR (Random Amplification of Polymorphic DNA - Polimerase Chain Reaction), that has never been used for

campylophages characterization although its application was successfully employed for other bacteriophages. Furthermore, the lytic spectrum of the new *Campylobacter* specific bacteriophages was assessed against the collection of novel *Campylobacter* isolates recovered from broiler, swine and human clinical samples and accurately characterized in the second study.

Objective 4: To select the most appropriate *Campylobacter* specific bacteriophage candidates to be used as biocontrol agents according to their technological properties.

Study 4: Promising *Campylobacter* specific bacteriophage candidates for biocontrol applications Manuscript in preparation.

The most effective ten *Campylobacter* specific bacteriophages, selected from the third study according to their classification within group II or group III campylophages, lytic spectrum and lysis degrees, were further characterized by assessing their most relevant technological properties. Their lytic spectrum was determined against a new collection of *Campylobacter* strains of different antibiotic-resistance profiles and geographical origins (Spain, Italy and Poland), that were previously characterized by rep-PCR (repetitive element palindromic - PCR). Their latent period and burst size were also determined in a single round of replication. Their stability and lytic activity at broad ranges of temperatures (from 4 to 42 °C) and pH values (from 2 to 9) were evaluated. This work allowed the selection of the most promising candidates to be used as biocontrol agents in *Campylobacter*-specific cocktails for applications from primary production to food consumption.

INTRODUCTION

1. *Campylobacter* genus

Originally, this genus was described as members of the genus *Vibrio* and the first description was made by Theodore Escherich in 1886. In 1913, McFadyean and Stockman firstly described the isolation of *Vibrio fetus* from aborted bovine foetuses. Later in 1927, Smith and Orcutt isolated a group of bacteria from the faeces of cattle with diarrhoea which they called *Vibrio jejuni*. In 1944, Doyle classified a different microorganism isolated from faeces of swine with diarrhoea as *Vibrio coli* (Vandamme et al., 2010; Vandamme et al., 2000). The genus *Campylobacter* was firstly introduced in 1963 by Sebald and Veron to differentiate these strict microaerophilic, non-fermentative and with a DNA with low G+C content bacteria from the traditional members of the genus *Vibrio*. After that, in 1973, Veron and Chatelain published the first accepted taxonomy of the genus *Campylobacter* (Wassenaar and Newell, 2006).

The taxonomy of the genus has been substantially changed several times. Currently, following *Bergey's Manual of Systematic Bacteriology*, (Garrity et al., 2005). *Campylobacter* genus together with *Arcobacter*, *Dehalospirillum* and *Sulfurospirillum* belongs to the *Campylobacteriaceae* family. This family with *Helicobacteraceae* and *Hydrogenimonaceae* are included in the *Campylobacterales* order of the class *Epsilonproteobacteria*. *Campylobacter* genus contains 32 officially described species and 11 subspecies (Table 1), which cluster in five discrete phylogenetic groups. In all of them pathogenic microorganisms are present highlighting the clinical relevance of the genus (Figure 1) (Costa and Iraola, 2019). The species of *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter lari* and *Campylobacter upsaliensis* are the most important in the public health field (EFSA, 2019a).

Table 1. Species and subspecies of *Campylobacter* genus.

Species	Subspecies
Campylobacter avium	
Campylobacter blaseri	
Campylobacter canadensis	
Campylobacter coli	
Campylobacter concisus	
Campylobacter corcagiensis	
Campylobacter cuniculorum	
Campylobacter curvus	
Campylobacter fetus	*Campylobacter fetus* subsp. *fetus* *Campylobacter fetus* subsp. *testudinum* *Campylobacter fetus* subsp. *venerealis*
Campylobacter geochelonis	
Campylobacter gracilis	
Campylobacter helveticus	
Campylobacter hepaticus	
Campylobacter hominis	
Campylobacter hyointestinalis	*Campylobacter hyointestinalis* subsp. *hyointestinalis* *Campylobacter hyointestinalis* subsp. *lawsonii*
Campylobacter iguaniorum	
Campylobacter insulaenigrae	
Campylobacter jejuni	*Campylobacter jejuni* subsp. *doylei* *Campylobacter jejuni* subsp. *jejuni*
Campylobacter lanienae	
Campylobacter lari	*Campylobacter lari* subsp. *concheus* *Campylobacter lari* subsp. *lari*
Campylobacter mucosalis	
Campylobacter ornithocola	
Campylobacter peloridis	
Campylobacter pinnipediorum	*Campylobacter pinnipediorum* subsp. *caledonicus* *Campylobacter pinnipediorum* subsp. *pinnipediorum*
Campylobacter rectus	
Campylobacter showae	
Campylobacter sputorum	
Campylobacter subantarcticus	
Campylobacter troglodytis	
Campylobacter upsaliensis	
Campylobacter ureolyticus	
Campylobacter volucris	

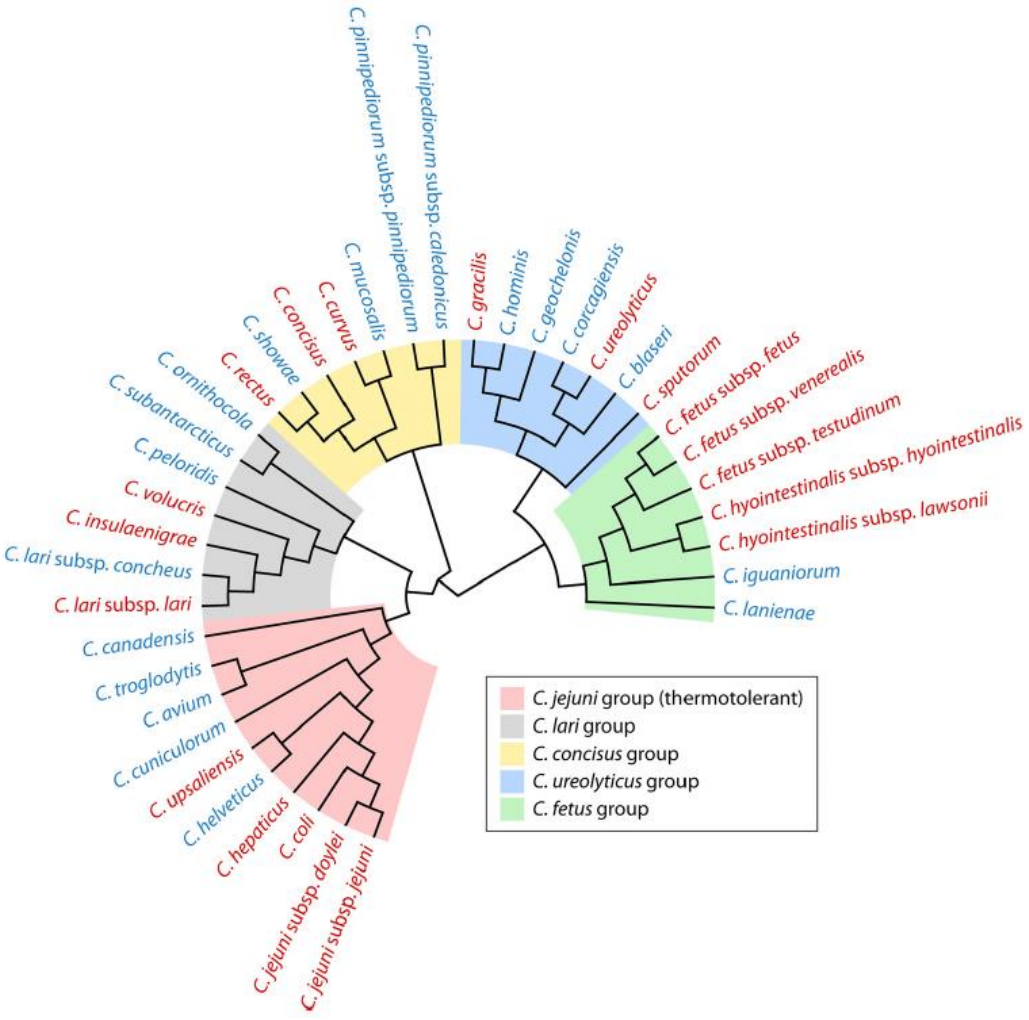

Figure 1. Phylogenetic relationships among described *Campylobacter* species (Costa and Iraola, 2019). Group names were assigned considering the most clinically relevant species within each group.

Cells of most *Campylobacter* species are slender, S-shaped, or spirally curved Gram-negative and non-spore forming rods with variable dimensions of 0.2-0.8 µm wide and 0.5-5 µm long, but in old cultures cells may form spherical or coccoid bodies. Most species are motile with a characteristic corkscrew-like motion by means of a single, polar, unsheathed flagellum at one or both ends of the cell. The flagella may be two or three times the length of the cell. There are many exceptions, such as the non-motile

bacteria of *Campylobacter gracilis* specie, or with multiple flagella of *Campylobacter showae* specie. The genus has a circular chromosome of around 1.6-1.7 Mbp with a G+C content of 27-31% (Facciolà et al., 2017; Garrity et al., 2005).

Campylobacter is considered a *fastidious bacterium* that requires specific conditions for its optimal growth. This bacterium is thermophilic, being able to grow at temperatures between 37 and 42 °C with an optimal growth temperature of 41.5 °C, but unable to grow below 30 °C (Silva et al., 2011). However, as it cannot grow at 55 °C or higher, it does not exhibit a real thermophilic behaviour. For this reason, Levin (2007) suggested referring to it as "thermotolerant". *Campylobacter* is microaerobic, requiring low oxygen concentrations to grow (3-15%) while its multiplication is inhibited at the atmospheric 21% oxygen (Osimani et al., 2017). This genus is also considered capnophilic, because requires CO_2 concentrations of between 1 to 10% to grow (Bolton and Coates, 1983; Mihowich et al., 1998). Occasionally, some strains grow under aerobic conditions and could even be aerotolerant (Vandamme and Deley, 1991). *Campylobacter* is chemoorganotroph, using amino acids and intermediate compounds of the tricarboxylic acid cycle as a source of carbon and, not oxidizing or fermenting carbohydrates. The optimal pH to grow is between 6.5 and 7.5 and is not able to survive neither in acid (pH below 4.9) nor in basic (pH above 9) environments. The growth of *Campylobacter* is affected by the water activity (a_w). The optimal a_w is 0.997 (0.5% w/v sodium chloride, NaCl) and it is unable to grow in conditions of a_w below 0.987. This bacterium is sensitive to concentrations of NaCl greater than 2% w/v (Facciolà et al., 2017; Silva et al., 2011).

Under adverse conditions, the switch to viable but non-culturable cells (VBNC) has been described for *Campylobacter* among other several bacteria species. In this state

cells are non-cultivable on conventional culture media but are metabolically active and could potentially influence human health. VBNC is considered an active survival strategy in which control pathways participate and cell differentiation responses occurs. These cells have higher physical and chemical resistance than cultivable ones. VBNC stage is reversible; cells in this stage are able to recover the cultivability under appropriate conditions. Then, this tendency for *Campylobacter* to enter a VBNC state could be considered another threat for food safety (Li et al., 2014; McDougald et al., 1998; Nascutiu, 2010).

2. Campylobacteriosis

2.1. Epidemiology

Campylobacter is the most commonly reported gastrointestinal bacterial pathogen in the European Union (EU) since 2005, with 246,571 reported confirmed cases of human campylobacteriosis and a notification rate of 64.1 per 100,000 population in 2018 (EFSA, 2019a). There was a significantly increasing trend over the 2008-2018 period. However, between 2014 and 2018, no statistically significant increase or decrease was shown. In Spain, 18,411 confirmed cases of campylobacteriosis were reported in 2018, showing the stabilization in the alarming upward trend of confirmed cases from 2012 to 2017 (EFSA, 2019a) (Figure 2).

Despite the high number of human campylobacteriosis cases, their severity in terms of reported case fatality is low, with 60 deaths (0.03%) in the entire EU in 2018. Nevertheless, this was the fourth most common cause of mortality among the zoonotic diseases (EFSA, 2019a). Additionally, the high number of reported cases only represents a small proportion of all clinical cases as it has been estimated that there are

approximately nine million cases of campylobacteriosis per year in the EU, with a total annual cost of 2.4 billion € (EFSA, 2011). Different *Campylobacter* species are involved in campylobacteriosis cases, being *C. jejuni* and *C coli*, responsible for the 83.9 and 10.3% of the confirmed cases, respectively, in 2018. The remaining cases are associated with *C. lari* (0.1%), *C. fetus* (0.1%) and *C. upsaliensis* (0.1%), while 'other' *Campylobacter* species accounted for 5.5%, the majority of them reported as '*C. jejuni*/*C. coli*/*C. lari* not differentiated (EFSA, 2019a). These five species are described as the main responsible of human campylobacteriosis (Patrick et al., 2018; van Vliet and Ketley, 2001).

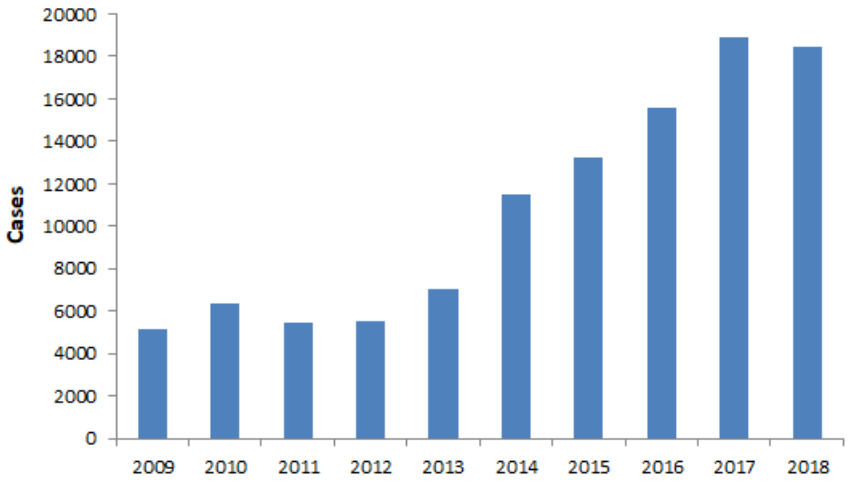

Figure 2. Reported confirmed campylobacteriosis cases in Spain from 2009 to 2018 according to the latest EFSA report (EFSA, 2015, 2019a).

2.2. Infection and transmission sources

Campylobacteriosis is considered a zoonoses, a disease transmitted to humans from animals or animal products. Only few cells, among 500 to 800 microorganisms, are needed to cause toxi-infection (Igwaran and Okoh, 2019). The main route of transmission is generally foodborne, via undercooked meat and meat products, as well

as raw or contaminated milk (Torralbo et al., 2014). Contaminated water or ice is also a source of infection. Moreover, a proportion of cases occur following contact with contaminated water during recreational activities (Mughini-Gras et al., 2016). The relative contribution of each of the above sources to the overall burden of disease is unclear but consumption of raw or undercooked contaminated poultry is believed to be a major contributor. The vast majority (99%) of human campylobacteriosis cases occur as isolated, sporadic events and not as part of recognized outbreaks (Hansson et al., 2018).

2.3. Pathogenesis and virulence factors

Specific virulence mechanisms of *Campylobacter* spp. have not been clearly elucidated yet, likely due to pathogenesis differences between this and other pathogens, or to the lack of an appropriate small animal model that reproduces the human disease (Young et al., 2007). Adhesion and invasion of the intestinal epithelium occur in early events, prior to the onset of inflammation and the development of diarrhoea (Bolton, 2015). The 'invasiveness' of *Campylobacter* strains is commonly used as a bacterial virulence measure, which reflects that multiple bacterial structures and mechanisms are involved in this process (Zilbauer et al., 2008). It is well known that this pathogen firstly colonizes the small intestine and moves then to the colon, its target organ. This invasion causes a cellular inflammation that reduces the absorptive capacity of the intestine. The severity of the disease depends on both the virulence of the strain and the host's immune condition (Zilbauer et al., 2008).

The virulence factors involved in host cell invasion and disease pathogenesis are mainly capsular polysaccharide, flagellar apparatus, cytolethal distending toxin and post-

translational glycosylation. Biofilm formation is also an important strategy to survive under unfavourable environmental conditions (Bolton, 2015).

2.4. Disease: symptoms and treatment

The onset of disease symptoms usually occurs two to five days after infection, although the incubation period varies from one to 10 days. The most common symptoms of infection include diarrhoea (frequently bloody), abdominal pain, fever, headache, nausea, and/or vomiting. They usually last up to six days (WHO).

Occasionally, *Campylobacter* infections may be followed by major complications such as reactive arthritis, irritable bowel syndrome and neurological disorders such as Guillain-Barré (GBS) and Miller Fisher (MFS) syndromes. Campylobacteriosis rarely causes death but such cases are usually confined to very young children, elderly patients or immunosuppressed (WHO).

It is generally a self-limited infection; most patients will recover with electrolyte replacement and rehydration. However, the use of antibiotics is recommended for the most serious cases, such as severe and/or prolonged enteritis, septicaemia or extraintestinal complications such as those mentioned above (reactive arthritis, GBS and FMS). The first- and second-choice drugs for the treatment of these cases are erythromycin, an antibiotic of the macrolide family, and ciprofloxacin, within the fluoroquinolone's family, although tetracyclines can also be prescribed. Resistance to antimicrobials in *Campylobacter* is of concern because of the large number of human infection cases and that some of them require treatment (Sifre et al., 2015).

2.5. Antimicrobial susceptibility

The use of antibiotics in animal production for control, prevention or treatment of diseases, as well as in food producing animals has led to the emergence and spread of antibiotic resistant *Campylobacter* strains (Kaakoush et al., 2015). The resistance to fluoroquinolones, macrolides and tetracyclines could compromise the effectiveness of the human treatments (EFSA, 2019b; Gibreel and Taylor, 2006; Payot et al., 2006). This warrying situation led the World Health Organization (WHO) to include *Campylobacter* in its global priority list of antibiotic resistant pathogens (WHO, 2017).

As summarized in Table 2, high to extremely high proportions of *Campylobacter* isolates from humans, fattening swine, broilers and broiler meat, have been found to be resistant to ciprofloxacin and tetracyclines in the EU in 2018. This proportion has been registered higher among *C. coli* isolates than among *C. jejuni* ones. For erythromycin, on the other hand, the proportion of resistant isolates has been low to moderate and also more frequent in *C. coli* species (EFSA, 2019b).

Table 2. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* in the EU (EFSA, 2019b).

Source	**Humans**		**Broilers**		**Broiler meat**		**Swine**	
Antibiotic	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*
CIP	57.7%	63.5%	66.9%	87.7%	64.9%	81.1%	NR	52.3%
ERY	2.0%	12.8%	1.3%	1.2%	2.2%	13.1%	NR	15.6%
TET	45.4%	68.3%	50.7%	61.7%	48.6%	73.0%	NR	51.5%
GEN	0.5%	1.8%	0.1%	0.6%	0.7%	0%	NR	7.7%
MDR	0.9%	10.3%	1.1%	1.9%	NR	NR	NR	21.2%

CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; GEN, gentamycin; MDR, multidrug resistance; NR, not reported.

The antimicrobial resistance mechanisms developed by *Campylobacter* depend on the antibiotic family. Mutations in genes such as 23S rRNA gene for macrolides, DNA

gyrase for quinolones and fluoroquinolones or the presence of the *tet*(*O*) gene for tetracycline are the main responsible mechanisms of the resistance to these agents (Wieczorek and Osek, 2013).

3. Prevalence of *Campylobacter* in animal production

3.1. Prevalence in food producing animals

Campylobacter species are ubiquitous in nature. They are prevalent in wildlife and a wide range of domesticated livestock, being avian populations the natural environmental reservoir par excellence. These species are also commensals in the gastrointestinal tract of food-producing animals, such as cattle, swine, sheep and poultry (Shange et al. 2019; Rossler et al. 2018), which are frequently asymptomatic (Newell and Fearnley, 2003; Silva et al., 2011). Ruminants, such as sheep, bovine and goats, are the food producing animals with the lowest *Campylobacter* prevalence, whereas poultry (broiler, hens and other farm birds) and swine show the higher prevalence (Rossler et al., 2019). *Campylobacter jejuni* is especially prevalent in poultry while *C. coli* predominates in swine (Rossler et al., 2019).

Vertical transmission at the farm level is not as well recognized as horizontal transmission, which has been reported to be the most effective way in transferring *Campylobacter* from vectors such as domestic pets, pests (insects, rodents and migratory birds), farm equipments, transport vehicles, feed, farm workers, litter and/or water (Costa and Iraola, 2019; Hald et al., 2016; Johnson et al., 2017; Umar et al., 2016).

3.2. Prevalence in poultry

Campylobacter spp. is rarely detected during the first three weeks of age of commercial flocks (Umar et al., 2016). However, natural colonization of broilers by single or multiple species rapidly occurs through horizontal transmission, as described for other food producing animals (Hald et al., 2016; Hermans et al., 2011; Johnson et al., 2017; Umar et al., 2016). Most of the birds in a flock are colonized within only a few days after the first chick is infected, reaching between 10^6 and 10^8 CFU/g in their intestinal tract, and they remain colonized until slaughter (Marotta et al., 2015). In poultry, especially in broiler, *C. jejuni* is the predominant species colonizing the flocks, followed by *C. coli* and occasionally by other species (Rossler et al., 2019; Umar et al., 2016). Prevalence of *Campylobacter* positive poultry flocks is, therefore, generally high, but it varies for multiple factors such as geographical area, seasons or production systems (conventional, free range, organic, etc.), ranging from 2% to 100% infection rates (Umar et al., 2016).

Colonization of broilers with *Campylobacter* during rearing is responsible for the contamination of the poultry meat after processing. During the slaughter process, cross-contamination of carcasses and poultry meat may happen from *Campylobacter* positive to *Campylobacter* negative flocks (Shange et al., 2019; Umar et al., 2016) due to the leaking of contaminated faeces from visceral rupture (Garcia-Sanchez et al., 2019; Hermans et al., 2011). Therefore, improper hygienic practices can ultimately allow the contamination of finished/retail products intended for human consumption (Shange et al., 2019).

4. Detection, identification and characterization of *Campylobacter*

The study of *Campylobacter* is performed both by culture-dependent and culture-independent techniques. The culture-dependent techniques require a previous step of cultivation of microorganisms onto agar plates and subsequent analysis by immunological or molecular techniques. On the other hand, culture-independent techniques are based on the study of the microorganisms directly from the natural matrix without any previous cultivation step. Several methods have been reported for detection, identification, characterization and enumeration of *Campylobacter* spp (Josefsen et al., 2015).

4.1. Culture-dependent methods

Campylobacter is fastidious microorganism in its growth requirements and very sensitive to stresses, thus its isolation is challenging. The use of selective media containing different combinations of selective agents is recommended for *Campylobacter* isolation from different samples. Supplements such as blood, charcoal, ferrous sulfate, sodium metabisulfite, sodium pyruvate, and hemin added to the culture media protect *Campylobacter* cells from the damage caused by oxygen. Supplementation with antimicrobials inhibits the growth of undesirable microorganisms (Vaz et al., 2014). Chromogenic media in which a synthetic chromogenic substrate specifically targets the species based on their enzymatic activity have also been designed for the isolation of *Campylobacter*. These media are mostly both selective and differential, inhibiting non target microorganisms through the use of antibiotics, among others, and enabling the expected microorganism to grow as colored colonies (Perry, 2017).

Nowadays, several culture media have been tested to improve the recovery of *Campylobacter* colonies and their later maintenance in pure culture (Josefsen et al., 2015; Kim et al., 2019; Vaz et al., 2014). Several of the most commonly used media are Bolton broth, Preston broth and agar, Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA), Campy-Line agar, RAPID'*Campylobacter*, R & F® *Campylobacter* chromogenic plating medium (CCPM), Brilliance™ CampyCount agar, CampyFood ID, *Campylobacter* selective agar (CASA) (Josefsen et al., 2015; Kim et al., 2019; Vaz et al., 2014).

4.1.1. *Campylobacter* detection and identification methods

After the plate recovery and isolation of *Campylobacter*, different techniques have been used to detect and identify *Campylobacter* isolates at species level. Conventional methods to identify *Campylobacter* are based on phenotypic characteristics such as morphology, motility and biochemical reactions under different growth conditions or latex agglutination. Limitations of these methods have led to the development of alternative molecular methods (Duarte et al., 2016; Hansson et al., 2018). Among these latter a number of molecular techniques such as single PCR (Polymerase Chain Reaction), multiplex PCR, nested PCR, sequencing or microarray PCR have been used for *Campylobacter* detection and identification (Divsalar et al., 2019; Josefsen et al., 2015).

Multiplex PCR: The simultaneous detection of some species-specific genes or fragments of them could be amplified in one PCR reaction. Wang et al. (2002) developed and optimized a multiplex PCR protocol capable of identifying reference strains and clinical isolates from five species with a high degree of specificity in one reaction. It

simultaneously identifies the 23S rRNA from *Campylobacter* spp., the *hip*O gene (hippuricase) from *C. jejuni* subsp. *jejuni*, the *gly*A gene (serine hydroxymethyl transferase) from *C. coli*, *C. lari*, and *C. upsaliensis* and the *sap*B2 gene (surface layer protein) from *C. fetus* subsp. *fetus* with six specific primer pairs. This multiplex PCR method has been successfully applied by several authors (Elbrissi et al., 2017; Garcia-Sanchez et al., 2018; Ge et al., 2018; Lazou et al., 2014). Recently, other protocols of multiplex PCR to identify *C. jejuni* and *C.coli* have been also used (Han et al., 2019; Melo et al., 2019).

Sequencing: This technique has proven to be one of the most powerful approaches for the classification and identification of microorganisms and the analysis of 16S rRNA gene is widely utilized. The portability and ease of use of 16S rRNA gene sequencing, and the continually growing and evolving data in public databases have made this approach the most widespread DNA-based method for prokaryotic identification (On, 2013). Gorkiewicz et al. (2003) described that the limitation of the 16S rDNA analysis is the inability to differentiate the species *C. jejuni* and *C. coli* and atypical *C. lari* strains. As these species are significant pathogens and their differentiation is important in clinical cases, they suggest the use of PCR assays for the accurate discrimination and identification of the respective species.

4.1.2. *Campylobacter* characterization: genotyping methods

Fingerprinting analysis allows the characterization at strain level, in which the step of culture cannot be bypassed at this moment. *Campylobaacter* recovery onto culture media is essential for classifying strains or isolates according to their phylogenetic relationship, clustering clonally and epidemiologically related isolates and

differentiating them from those unrelated. This provides appropriate discriminatory analysis to allow the detection of outbreaks, transmission routes and the relatedness of isolates. Both phenotypic and genotypic methods have been used in *Campylobacter* typing.

Phenotypic methods such as biotyping, serotyping (Penner and Lior schemes) or antimicrobial resistance profiling are based on the metabolic or biological activities expressed by the bacteria. Compared to these phenotypic methods, genotypic methods are much more discriminatory. Genotyping can analyse a part of the bacterium genome, a single locus or multiple loci, using techniques such as PCR-RFLP (PCR-restriction fragment length polymorphism) or PCR and sequencing like MLST (multilocus sequence typing). Whole genomes can also be compared by techniques such as s-AFLP (single amplified fragment length polymorphism), PFGE (Pulsed-Field Gel Electrophoresis), REP-PCR (repetitive extragenic palindromic-PCR), RAPD (random amplified polymorphic DNA) or WGS (whole genome sequencing) (Abay et al., 2014; Duarte et al., 2016; Garcia-Sanchez et al., 2019).

Single locus typing, flaA typing: The flagellin gene locus of *Campylobacter* contains *flaA* and *flaB* genes and is characterized by the coexistence of highly conserved and variable regions. Different techniques that analyse the variable region of *flaA* gene have been suitable for *Campylobacter* typing.

The PCR-RFLP technique is based on the amplification of an approximately 1.7 kb fragment of the *flaA* gene that is subsequently digested by a restriction endonucleases such as *Dde*I or *HpyF3*I. The restriction fragments are then separated by conventional electrophoresis (Nachamkin et al., 1993; Wassenaar and Newell, 2000). However, *Campylobacter* has a great capacity for transformation by naturally absorbing

exogenous DNA, due to recombination and intra-species DNA transfer, then *flaA* gene locus are not very stable and are not species-specific (Behringer et al., 2011). Despite, high discriminatory power was reported for both *C. jejuni* and *C.coli* isolates with the combination of *flaA*-RFLP with MLST (sequencing of short fragments of seven housekeeping loci and genotype assigned by PubMLST database) technique (Duarte et al., 2016).

Sequencing of short variable regions products of the *fla*A gene (*fla*A-SVR) based on the amplification followed by sequencing of a 321 bp fragment was considered very useful for discriminating among *C. jejuni* isolates (Taboada et al., 2013). Whole *fla*A gene locus sequencing has also been used as fingerprinting analysis (Meinersmann et al., 1997).

Whole genome typing, PFGE: This technique allows the separation of large DNA molecules in an agarose gel by applying an electric field that periodically changes its direction. In order to avoid random breaks, chromosomal DNA is protected by immobilizing a bacterial suspension in agarose, before the cells are lysed. The following steps in which cellular material is eliminated, with the exception of DNA, are carried out by passive diffusion of different buffers within the agarose blocks. The purified DNA inside the blocks is digested mainly by *Sma*I or *Kpn*I restriction endonucleases in a small number of large fragments that will be electrophoretically separated depending on their size (Taboada et al., 2013).

This technique has a high discriminatory power and is very useful to differentiate phylogenetically closely related strains of different species such as *C. jejuni, C. coli*, *C. lari, C. fetus, C. upsaliensis* or *C. hyointestinalis* (Wassenaar and Newell, 2000). However, PFGE is time-consuming because the samples preparation for electrophoresis

is lengthy and labour-intensive, which renders the method impracticable in routine analysis or assays with a large number of isolates (Behringer et al., 2011).

4.2 Culture-independent methods

Standardized reference culture-based methods issued by national and international standardization organizations are useful and suitable for *Campylobacter* detection in reference laboratories (Gharst et al., 2013). However, they are time-consuming and tedious with large numbers of samples. Nowadays, the use of culture independent methods, DNA or immunochemical-based ones, for the detection of *Campylobacter* is very common.

Several culture independent approaches have been developed such as miniaturized biosensors, chromatographic techniques or techniques based on PCR. Among this latter, real-time PCR technique provide a reliable tool to detect and to quantify *Campylobacter* in complex substrates such as food products or faecal samples (Josefsen et al., 2015). Targeting, for example, the *C. jejuni hipO* and the *C. coli glyA* genes, several authors have been able to simultaneously quantify and identify *Campylobacter* isolates (de Boer et al., 2015; Leblanc-Maridor et al., 2011).

In the application of culture-independent techniques, the sample preparation step is of great importance. Either the target bacteria or its DNA have to be concentrated and separated from the inhibitory compounds of the sample matrix. DNA based methods require and effective and reproducible DNA retrieving from the target organism. A low yielding DNA extraction can increase the risk of false negative results. Insufficient amounts of DNA and inferior DNA quality can also increase errors in the subsequent amplification (Josefsen et al., 2015). This aspect also applies to techniques such as RT-

PCR (reverse transcriptase PCR) or NASBA (nucleic acid sequence-based amplification) in which mRNA or rRNA of *Campylobacter* is the molecular target. Several of these techniques have also been described for *C. jejuni* and *C. coli* (Churruca et al., 2007; Kurakawa et al., 2012).

5. Control of *Campylobacter* in poultry

The prevention and control of *Campylobacter* in poultry is a food safety issue of high priority since it is widely accepted as a significant risk factor of human campylobacteriosis. In fact, reducing the load of *Campylobacter* in the intestines by three log_{10}-units at slaughter would reduce the public health risk by at least 90%. Reducing the numbers of *Campylobacter* on the carcasses by one log_{10}-unit, would reduce the public health risk by between 50 and 90%, and, reducing counts by more than two log_{10}-units would reduce the public health risk by more than 90% (EFSA, 2011).

With the aim of reducing those levels, the EU has recently developed the Commission Regulation (EU) 2017/1495 amending Regulation (EC) No 2073/2005 as regards *Campylobacter* in broiler carcasses. This allows carcasses complied with a limit of 1,000 CFU/g, a number that should gradually decrease over the seven years following the publication of this regulation (The European Comission, 2017). However, to date, there is no effective, reliable and practical intervention measure available in poultry. Significant advancement has been made during the past years in the research and development of pre- and post-harvest intervention strategies of *Campylobacter* for the Poultry Sector (Figure 3).

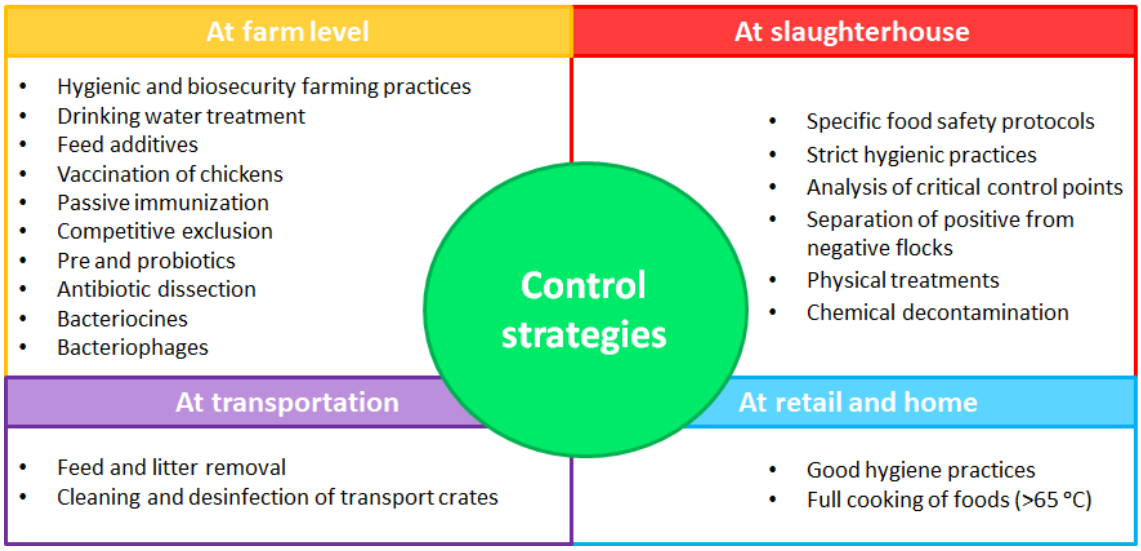

Figure 3. Current interventions measures for *Campylobacter* control in poultry (Hermans et al., 2011; Meunier et al., 2016; Umar et al., 2016).

5.1. At farm level

Several intervention strategies have been proposed to prevent *Campylobacter* introduction and transmission in poultry flocks or to reduce *Campylobacter* colonization on poultry farms (Hermans et al., 2011; Umar et al., 2016). Several measures, resumed in figure 3, consisting of:

(1) The reduction of environmental exposure, such as the application of strict biosecurity measures (including insects, rodents and other animals' control) and hygienic practices (e.g. washing and sanitizing of hands, changing boots and coveralls), drinking water treatment, litter and waste treatment or cleaning and disinfection of the whole plant, equipment and tools.

(2) The increase in host resistance to reduce carriage in the gut, such as the utilization of feed additives, vaccination, passive immunization, application of pre- and pro-biotics, competitive exclusion microbiota and host genetics selection.

(3) The use of antimicrobial alternatives to reduce and even eliminate the bacteria from colonized chickens, like the application of antibiotics, bacteriophages or bacteriocins. The use of bacteriocins and bacteriophages is highly promising and possibly commercially applicable, since safety concerns should not be a main obstacle and their use is ergonomic since they can be easily and efficiently administered through the feed or drinking water.

5.2. At slaughter

Different intervention strategies have been proposed to control *Campylobacter* at slaughter, dressing and processing:

(1) The application of specific food safety protocols and strict hygienic practices, and the systematic analysis of critical control points along the slaughter process (such as scalding, defeathering and evisceration) helps to identify the most appropriate measures to control contamination of carcasses (Osimani et al., 2017).

(2) Separation of *Campylobacter*-positive from negative flocks and slaughtering of the positive flocks has proved to be an effective method of reducing the spread of contamination (Silva et al., 2011).

(3) Physical treatments such as scalding and chilling of carcasses proved to be effective to reduce the contamination level of *Campylobacter* (Osimani et al., 2017). However, they may change the organoleptic properties of the food, which would make them less desirable to the consumer.

(4) Chemical decontamination of carcasses including the use of chlorine compounds or chlorine-based antimicrobials has also been successful. However, despite

being allowed in the United States, this practice is not permitted in EU (Osimani et al., 2017).

5.3. At retail and home

As with any raw product, good hygiene practices are very important to avoid cross contaminations during the preparation, storage and distribution of broiler meat. These practices include hands washing before and after handling raw and cooked food products, separating raw from cooked or ready to eat foods, avoiding the use of the same tools to handle raw meats and other foods and washing and disinfecting all surfaces and tools that have been in contact with raw meat. Not washing poultry products under running water is also recommended to avoid the spread of *Campylobacter* in working surfaces (Facciolà et al., 2017). Freezing chicken carcasses for up to three weeks has been observed to reduce the *Campylobacter* infection risks but it does not eliminate the bacteria (Silva et al., 2011). Finally, the full cooking of foods at temperatures above 65 °C will minimize the risk of contracting the infection (Silva et al., 2011).

5.4. Promising strategies

Despite extensive efforts, it is extremely difficult to reduce the prevalence of *Campylobacter* from the farm to the fork process. As the incidence of antibiotic resistant *Campylobacter* strains is increasing, the development of novel non-antibiotic anti-*Campylobacter* treatments is becoming more critical (Johnson et al., 2017). Treatment strategies that have shown highly promising results for *Campylobacter* control in poultry are currently under development. Among these strategies, the use of pre- and pro-biotics, competitive exclusion and application of vaccines and bacteriocins

are included (Facciolà et al., 2017; Johnson et al., 2017; Silva et al., 2011); or even a combination of them (Hermans et al., 2011). Several studies have also reported, with successful results, the use of *Campylobacter* specific bacteriophages as biocontrol agents, considering them one of the most promising strategies to reduce the prevalence of *Campylobacter* within the farm-to-fork process (Atterbury et al., 2003a; Carvalho, Gannon, et al., 2010; El-Shibiny et al., 2009; Fischer et al., 2013; Hammerl et al., 2014).

6. Bacteriophages as a food safety strategy

6.1. Definition and historical background

Bacteriophages, informally known as phages, are viruses able to specifically infect and replicate within target bacteria. The term was derived from "bacteria" and from the Greek φαγεῖν (*phagein*) meaning "to devour".

They are ubiquitous and the most abundant organisms on Earth with an estimated total number of 10^{32} bacteriophages (Hanlon, 2007). They play major ecological roles (Ofir and Sorek, 2018) and are often consumed in our diet as natural microbiota present in a wide variety of foods, including poultry products (Furuta et al., 2017; Nowaczek et al., 2019).

Bacteriophages were independently discovered by William Twort in 1915 and Felix d´Herelle in 1917. However, d´Herelle was likely the first considering their therapeutic potential. They were used for therapeutic purposes in the pre-antibiotic era, but the development of antibiotics in the 1940s caused the drop out of bacteriophage research in the western countries (Ofir and Sorek, 2018). However, bacteriophage therapy remained an active research topic in different parts of the former Soviet Union and Poland.

Nowadays, with the advent of biotechnology and the alarming emergence of antibiotic resistant bacteria, bacteriophages are once again being considered as potential antimicrobials for the treatment of bacterial diseases and their complications in humans, animals and plants (Jamal et al., 2019).

6.2. Bacteriophages classification

The International Committee on Taxonomy of Viruses (ICTV) takes a comprehensive approach to classifying bacteriophages according to their nucleic acid type and morphology, although other criteria such as host specificity or mode of infection are also considered (Sharma et al., 2017).

Attending to their genetic material, bacteriophages can be divided into four groups (Figure 4): single stranded DNA bacteriophages (ssDNA), double stranded DNA bacteriophages (dsDNA), single stranded RNA bacteriophages (ssRNA), and double stranded RNA bacteriophages (dsRNA) (Harada et al., 2018).

Morphologically, bacteriophages are small viruses ranging in size from 24 to 400 nm (Jamal et al., 2019). They exhibit a three-dimensional structure with a proteinaceous capsid (head) that encapsulates their genetic material and/or a tail attached to the capsid through a connector. Bacteriophage tails are key determinants of the host specificity and infection since they specifically recognize bacterial host cells, penetrate the cell envelope and deliver the genetic material from the capsid into the host cell (Nobrega et al., 2018). At the distal end of the tail, the receptor-binding proteins (RBPs), such as tail fibers and tail spikes, are responsible for recognizing specific receptors at the surface of the bacterial membrane (Harada et al., 2018).

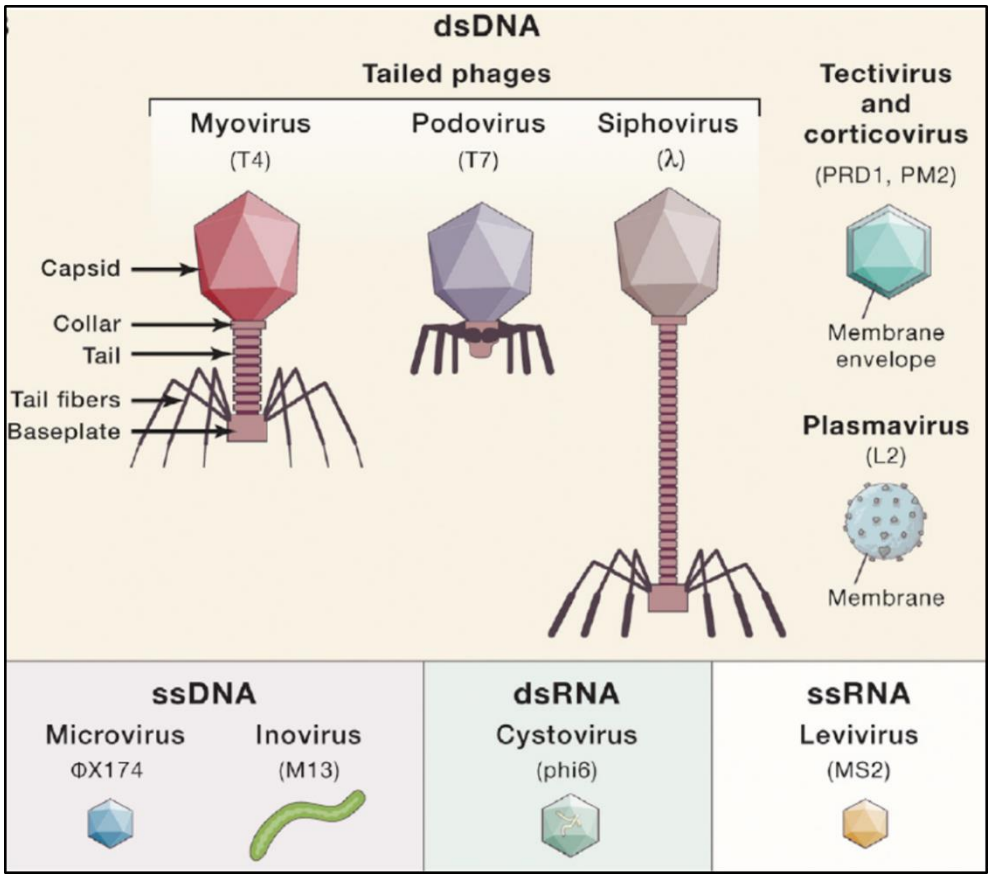

Figure 4. Bacteriophages classification attending to their nucleic acid type and their morphology. A representative type bacteriophage for each taxonomical group is in parenthesis (Ofir and Sorek, 2018).

More than the 96% of bacteriophages belong to the order of *Caudovirales* with an icosahedral capsid containing dsDNA and a tail (Sharma et al., 2017). According to the morphological characteristics of the tail, they have been classified into three families: *Myoviridae* (long and contractile tail), *Siphoviridae* (long and non-contractile tail) and *Podoviridae* (extremely short and non-contractile tail) (Ackermann, 2007; Nobrega et al., 2018) (Figure 5). Both *Myoviridae* and *Siphoviridae* bacteriophages have a baseplate at the distal end of the tail to which RBPs are attached. *Podoviridae* bacteriophages, however, have no baseplate so the RBPs directly attach to the tail. Additionally, all *Caudovirales* bacteriophages have a central tail fiber or spike that

extends from the distal end of the tail or baseplate. The rest of bacteriophages are cubic, filamentous or pleomorphic (Ackermann, 2007).

Bacteriophages have also been classified depending on their life cycle into virulent and temperate bacteriophages displaying lytic or lysogenic life cycles, respectively (Figure 6).

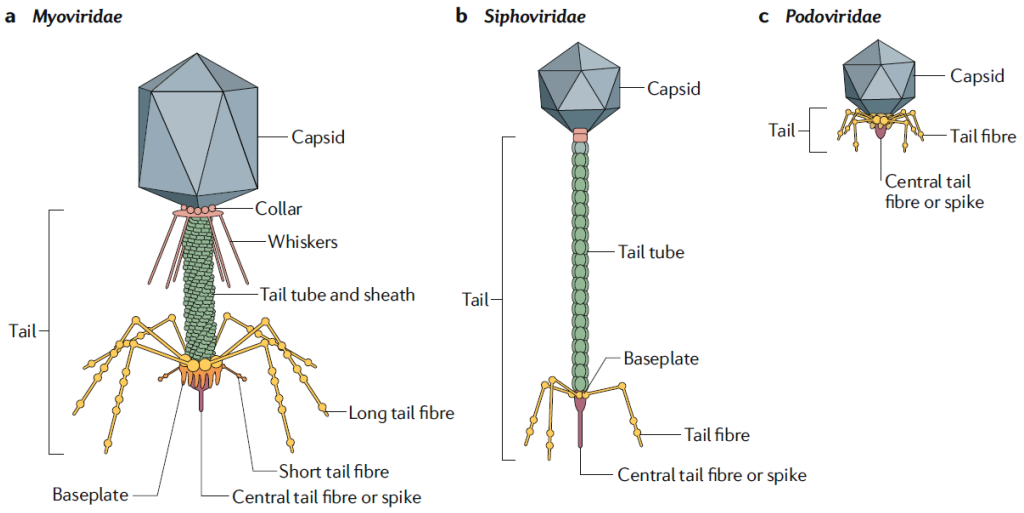

Figure 5. Representative structures of *Caudovirales* order bacteriophages (Nobrega et al., 2018).

Lytic cycle consists of the attachment of the RBPs to the bacterial cell wall receptors. An initial reversible attachment is followed by irreversible adsorption involving the aperture of a pore in the bacterial wall and injection of the genetic material into the host cell (Nobrega et al., 2018). After, the eclipse phase starts, where bacteriophage early genes are expressed, redirecting the bacterial machinery to the reproduction of its nucleic acids and proteins while replication of the bacterial DNA is inhibited (Wernicki et al., 2017). Then, bacteriophages are assembled and packaged, followed by the lysis of the bacterial cell and the release of the bacteriophage progeny capable of infecting other host cells. In few cases, host chromosome gets packed into the capsid causing the

horizontal gene transfer within the bacterial population via transduction (Sharma et al., 2017).

Lysogenic cycle involves the integration of the genetic material of temperate bacteriophages with the bacterial chromosome (prophage) and its subsequent replication as a part of the bacterial genome (Wernicki et al., 2017). The prophage is vertically transmitted along with the whole bacterial genome to its progeny. This cycle can be stable for thousands of generations until the lytic cycle is induced, either spontaneously or as a result of activation by external agents such as sunlight, UV radiation, alkylating agents or antibiotics (such as mitomycin C) (Sharma et al., 2017). During induction, lysogenic bacteriophage can sometimes transfer host genetic material adjacent to its insertion site on the chromosome of another bacterium, in a phenomenon called transduction. This process can promote the transfer of genes that are of selective advantage for bacterial host including antibiotic resistance genes.

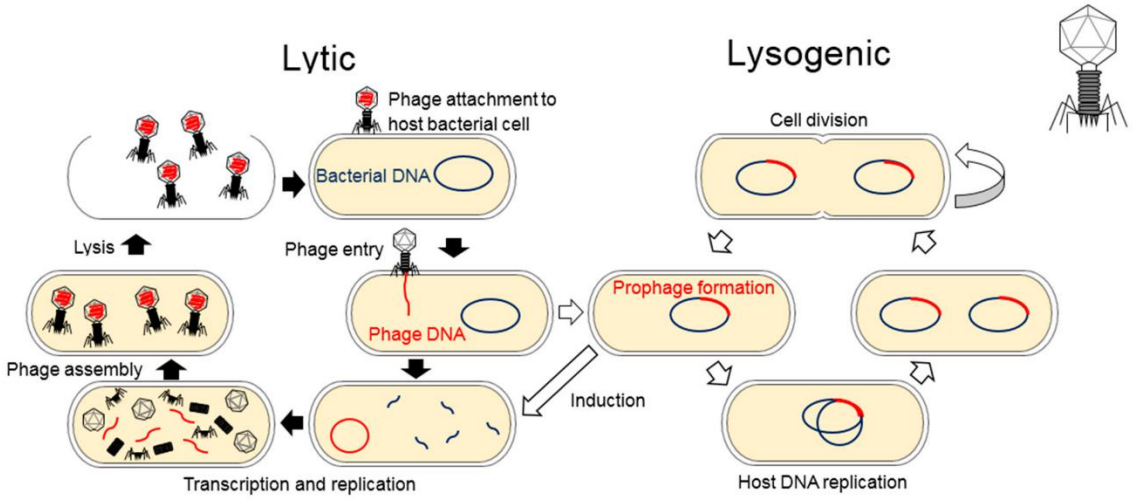

Figure 6. Lytic and lysogenic cycles of bacteriophages (Batinovic et al., 2019).

More rarely, bacteriophages can also display pseudo-lysogenic or chronic life cycles within their hosts.

Pseudo lysogeny occurs when a bacteriophage has entered a bacterial cell but does not integrate in a stable way. The bacteriophage remains in this mode until there is some conditions which triggers it to enter the lytic or lysogenic life cycles (Clokie et al., 2011).

Finally, during *chronic infection*, found only in some filamentous bacteriophages, they are slowly shed from the cell over a long time period without obvious cell death. The genome is injected into the host bacterium cytoplasm as single-stranded circular DNA and the virion is assembled at the bacterial cell envelope, with the maturing bacteriophage being actively secreted through the cell wall in a nonlytic wat (Hay and Lithgow, 2019).

6.3. Desirable properties of bacteriophages as biocontrol tools

Bacteriophages are promising natural antimicrobials and should fulfil several requirement to be used for biocontrol purposes. One of them is the ability to infect a wide range of different strains within a bacterial species or even different species within a genus. Achieving a broad host range or lytic spectrum with a single bacteriophage is very difficult. Bacteriophages present high specificity of infection towards a limited range of strains of the pathogen of concern (Harada et al., 2018; Romero-Calle et al., 2019). Therefore, they are harmless to humans, animals and plants, and do not affect the existing commensal microbiota of the host, food and/or the environment (Harada et al., 2018). Thus, bacteriophage cocktails has been proposed to obtain a higher specificity (Romero-Calle et al., 2019). . In these cocktails the presence of bacteriophages targeting different host receptors reduces the emergence of bacteriophage resistant bacteria (Nobrega et al., 2015).

Bacteriophage with a lytic cycle are appropriate and safe for biocontrol purposes (Nobrega et al., 2015). Bacteriophages with a lysogenic life cycle may contribute to the horizontal gene transfer, which is involved, among others, in the spread of antimicrobial resistance and virulence genes. However, the analysis of the whole genome sequence of bacteriophages is mandatory in order to ensure the absence of genes coding for virulence factors and/or lysogenic properties, as well as, the study that no transduction of bacterial DNA occur (Fernandez et al., 2019).

The stability at different storage and application conditions is another important aspect for biocontrol bacteriophages. They should be able to withstand the production, storage and intended administration conditions, being able to reach and infect their target bacteria. Therefore, they should be stable against a wide range of pH values, temperatures, NaCl contents, etc. (Fernandez et al., 2019).

Finally, and from the economical point of view, bacteriophages should be able to be produced at large commercial scale.

6.4. Commercial bacteriophage products for food applications

Bacteriophage biocontrol is increasingly accepted as natural and green technology for safely reducing specific target pathogenic bacteria in food products. Bacteriophages can be applied at multiple points within the farm-to-fork process; they can be used in both pre-harvest, and/or post-harvest food products and/or for disinfection of food-contact surfaces. The administration of bacteriophages could be performed by spraying orally or through water and feed in livestock animals or even in agriculture. They could be also applied directly to food surfaces by dipping or spraying as a liquid to a large volume of food material, via packaging materials for example.

Nowadays, several commercial products based on bacteriophages are available (Table 3). These products are typically water-based solutions with low levels of salts and no additives or preservatives, containing natural purified bacteriophages such as those isolated from the environment and not genetically modified (Moye et al., 2018). Bacteriophage biocontrol products against some of the most relevant food-borne pathogens worldwide, including *Salmonella*, *Listeria monocytogenes* or *Escherichia coli* are on the market. However, there is not yet any commercially available bacteriophage product to eliminate *Campylobacter*.

Concerning the regulation to use bacteriophages for food safety applications, the number of regulatory approvals issued (e.g. GRAS designation by the FDA) has been progressively increasing in recent years (Table 3). This started when FDA first authorised the use of the cocktail of *Listeria monocytogenes* specific bacteriophages "ListShield"as a food additive, in 2006. Following the lead of regulators in the USA, relevant health authorities of other non-European countries like Israel, Canada, Switzerland, Australia, New Zealand, the Netherlands or Brazil, have also approved bacteriophage applications on foods. At the European Union different favourable opinions have been published from the EFSA (European Food Safety Authority) recommending the use of bacteriophages as a promising natural antimicrobial alternative to antibiotics with high potential for food safety applications. However, no specific European regulation exists concerning bacteriophage application in food production yet. Several bacteriophage preparations exist on the market certified by Kosher and Halal and are available for use in organic foods (OMRI-listed in USA; SKAL in EU) (Table 3).

Table 3: Some of the commercially available bacteriophage-based products for food-safety applications.

Company	Product	Target bacteria	Regulatory	Certifications
Intralytix, Inc. -USA	ListShield	*Listeria monocytogenes*	FDA, 21 CFR172785; FDA, GRN528; EPA Reg. No.74234-1; Israel Ministry of Health, Health Canada	Kosher, Halal, OMRI
	EcoShield	*Escherichia coli* O157:H7	USDA, FCN1018, Israel Ministry of Health, Health Canada	Kosher, Halal
	ShigaShield	*Shigella* spp.		
	SalmoFresh	*Salmonella* spp.	FDA, GRN672	Kosher, Halal, OMRI
			FDA, GRN435, USDA, FSIS Directive7120.1, Israel Ministry of Health, Health Canada	
Elanco -USA	Finalyse	*Escherichia coli* O157:H7	USDA, FSIS Directive 7120.1	
Phagelux - China & Canada	Agriphage	*Xanthomonas campestris* *Pseudomonas syringae*	EPA Reg. No.67986-1 for use in agriculture	
	SalmoPro	*Salmonella spp.*	FDA, GRN603	
Micreos Food Safety -Netherlands	PhageGuard Listex	*Listeria monocytogenes*	FDA, GRN198/218, FSANZ, EFSA, Swiss BAG, Israel Ministry of Health, Health Canada, Ministerio da Saude Brasil	Kosher, Halal, OMRI, SKAL
	PhageGuard S (Salmonelex)	*Salmonella* spp.		Kosher, Halal, OMRI, SKAL
	PhageGuard E	*Escherichia coli* 0157:H7	FDA, GRN468, FSANZ, Swiss BAG, Israel Ministry of Health, Health Canada	
			FDA, GRN757	
APS Biocontrol Ltd.- Scotland	Biolyse	Soft rot *Enterobacteriacea*	Buscar info NO hay nada	Buscar info
FINK TEC GmbH - Germany	Secure Shield E1	*Escherichia coli*	FDA, GRN724 USDA, FSIS	
OmniLytics Inc.- USA	AgriPhage™	*Xanthomonas campestris* *Clavibacter michiganensis* *Erwinia amylovora* *Xanthomonas citri*	EPA, USDA's National Organic Program	For organic production

Adapted and modified from Moye et al. (2018). This is not meant to be an exhaustive list of bacteriophage products or approvals and listings. Some of the information was obtained from companies webpages and has not been independently verified. BAG: Bundesat Für Gesundheit; CFR: Code of Federal Regulations; FSIS: Food Safety and Inspection Service; GRN: GRAS Notice; FSANZ: Food Standards Australia New Zealand.

7. *Campylobacter* specific bacteriophages

7.1. Description and classification

Overall, *Campylobacter* specific bacteriophages, campylophages, are lytic and with double-stranded DNA. Morphologically, they are mostly tailed with icosahedral heads and belonging to *Myoviridae* family although few ones belong to the *Shiphoviridae* family. According to their morphology and genome size Sails et al. (1998) classified these bacteriophages into three groups and recently further characteristics of each campylophages group have been reported (O'Sullivan et al., 2018).

Campylophages group I contain two bacteriophages with head diameters of 140.6 and 143.8 nm and large genomes of about 320 kb that seem to be rare (Connerton et al., 2011). They have not been described in more detail and none of them have been sequenced to date or used for applications yet.

Campylophages group II have an average head diameter of 83-99 nm and genome sizes of approximately 180 kb. They are also uncommon but have been sometimes used, and their ability to infect both *C. jejuni* and *C. coli* strains has been revealed.

Campylophages group III have a head diameter that ranges from 100 to 130 nm and their genomes have around 140 kb size. They are very frequently isolated, especially from poultry sources. This group has been described in depth and used for applications and is able to infect *C. jejuni* strains, even more and with a stronger lytic activity than those of group II. Javed et al. (2014) classified campylophages group II and group III in the genera *Cp220likevirus* and *Cp8unalikevirus*, respectively. Later, in 2016, the ICTV renamed these genera to *Cp220virus* and *Cp8virus*, respectively (Adams et al., 2016).

Different host cell receptors have been reported for campylophage groups. For groups I and II, as resistance to these bacteriophages has been associated with motility defects, their receptor has been reported to be located somewhere in the flagellum. Resistance to campylophages group III has been associated with capsular polysaccharide modifications (Coward et al., 2006). Thus, these campylophages can also be classified as flagellotropic and capsular dependent bacteriophages, respectively (Sørensen et al., 2015). Furthermore, both group II and III have many common features such as a low burst size, a 26 to 27% of GC content in their DNA and resistance to cleavage by many restriction endonucleases (Jackel et al., 2019). Table 4 summarizes the most important characteristics of each campylophages group.

Table 4. Characteristics of each group of *Campylobacter* specific bacteriophages.

Group	Genus	Approx. genome size (kb)	Average head diameter (nm)	Isolation frequencies	Receptor	Species infected
I	NR	320	143	Two known isolates	Flagellum	NR
II	*Cp220virus*	180	83–99	Uncommon	Flagellum	*C. jejuni*
III	*Cp8virus*	140	100	Frequent	Capsule	*C. jejuni*, *C. coli*

NR, not reported.

7.2. Isolation of *Campylobacter* bacteriophages

Campylobacter specific bacteriophages have been isolated wherever their bacteria hosts are present, including retail poultry, chicken and duck faeces and intestines, abattoir effluents, human faeces, pig and poultry manure and sewage (Janež and Loc-Carrillo, 2013). However, the occurrence of campylophages in these samples is low (Nowaczek et al., 2019).

Different campylophage isolation methods have been proposed in the literature but there is no standardized one. Most of described methods consist on the elution of the bacteriophages from the samples followed by filtration and supernatant application onto

different *Campylobacter* strain lawns by spot assay (Atterbury et al., 2003; Loc-Carrillo et al., 2007; Nowaczek et al., 2019; Owens et al., 2013). However, the addition of an enrichment step with different *Campylobacter* strains seems more effective (Carvalho et al., 2010; Furuta et al., 2017).

A multiplex PCR based pre-screening of samples has been developed to detect *Campylobacter* bacteriophages of both group II and group III (Jackel et al., 2017). After the screening, both positive and negative samples should be examined for lytic activity since PCR negative samples may contain unusual *Campylobacter* bacteriophages such as campylophages group I (Jackel et al., 2019).

7.3. Characterization of *Campylobacter* bacteriophages

Bacteriophages should be deeply characterized before used for biocontrol purposes. Characterization allows bacteriophage classification and grouping, but also the selection of the most suitable candidates to be used as biocontrol agents (Kakasis and Panitsa, 2019; Sorensen et al., 2017). Characterization approaches include genome size determination, genomic profiling and bacteriophage morphology as the most common tests for their classification. For bacteriophage selection, on the other hand, the most important criteria are based on their specificity, efficacy and the avoidance of adverse effects (Kakasis and Panitsa, 2019). These characteristics are mainly analyzed by lytic spectra and technological properties, one step growth curve and whole genome sequencing.

Lytic spectra characterization: Determining the lytic activity of a bacteriophage is the most common and often the first characterization test and helps to determine if further analysis is required. Bacteriophages possessing broad lytic spectra and those capable of

lysing bacterial strains that are less susceptible to a wide variety of bacteriophages are highly desirable (Janež and Loc-Carrillo, 2013). Lytic activity of a campylophage should be assessed against a panel of wild-type *Campylobacter* strains of different species that reflect the environment under study (Hansen et al., 2007; Hyman, 2019). These *Campylobacter* strains should ideally be well characterized by genomic molecular methods (PFGE, MLST or *fla*A-RFLP) in order to obtain a panel as diversified as possible.

Genetic characterization: The genome size of *Campylobacter* bacteriophages is usually determined by PFGE technique in order to classify new isolated bacteriophages within campylophages group I, II or III (Sorensen et al., 2017). Whole genome sequencing is also an approach to know the genome size and even in a more accurate way.

Bacteriophage fingerprinting is also analyzed and allows knowing the bacteriophage diversity, clustering by their genome differences and similarities. Genomic profiling is commonly carried out by restriction digest analysis of bacteriophage genomes. Restriction endonucleases such as *Dra*I, *Vsp*I or *Smi*I are used in campylophages group II genotyping, visualizing their genomic profiles by conventional electrophoresis (Jackel et al., 2019; Jackel et al., 2015; Sørensen et al., 2015). Group III campylophages genome on the other hand, has been successfully digested with *Hha*I restriction endonuclease and subsequently separated by PFGE due to the long size of restriction fragments (Jackel et al., 2019; Janez et al., 2014; Sorensen et al., 2017).

Morphologic characterization: The determination of bacteriophage structure and morphology is also very commonly used for campylophages characterization. Bacteriophages are examined using transmission electron microscopy (TEM) to classify them within the different families (Ackermann, 2007). Although some studies reported

the existence of campylophages belonging to the *Shipoviridae* family (Nowaczek et al., 2019),the vast majority are into *Myoviridae* family (Janež and Loc-Carrillo, 2013) (Figure 7).

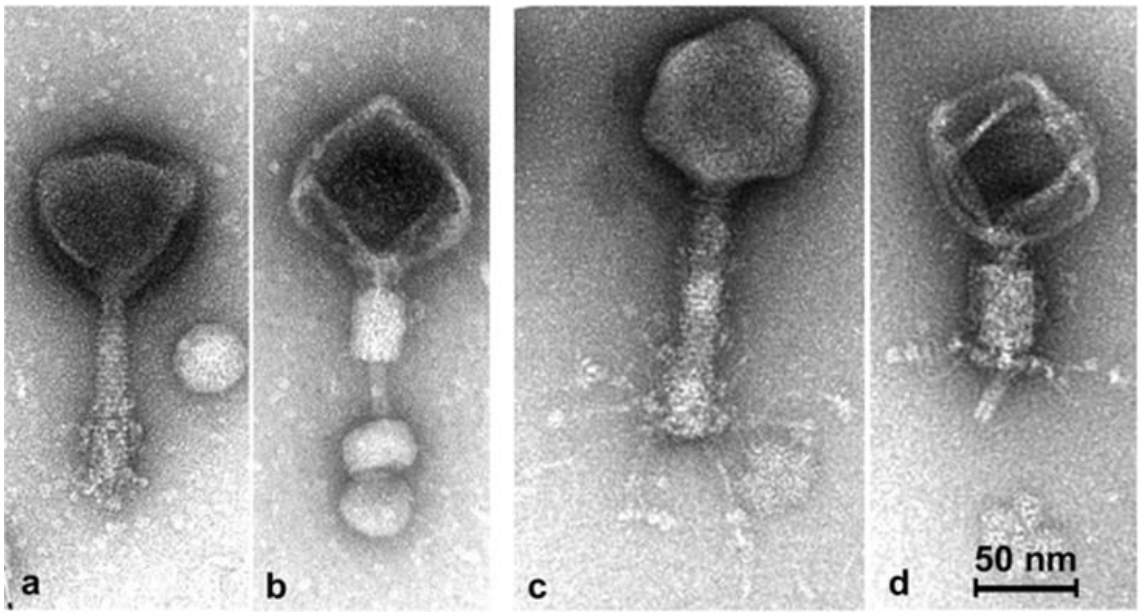

Figure 7. Electron micrographs of *C. jejuni* bacteriophages belonging to *Myoviridae* family (Sørensen et al., 2015).

Technological properties characterization: Bacteriophages are unstable at different environmental conditions such as temperature, pH, UV-light or salt concentrations. An in-depth analysis of bacteriophage stability is, therefore, a fundamental part of the characterization process. Indeed, the bacteriophage should remain infective from the moment of administration until it reaches the target pathogen (Fernandez et al., 2018). Moreover, *Campylobacter* bacteriophages may desirably be stable to temperatures approximately ranging from -20 °C (frozen products) to 42 ºC (poultry body temperature) but their stability at temperatures from 4 °C (chilled products) to 22 °C (room temperature) is also important. Regarding to the pH, *Campylobacter* bacteriophages should be active at the pH values that are in the digestive system of poultry (pH 2-8), which also cover those values found in the final product. To protect

them from the low pH, bacteriophage encapsulation or suspension in 30% $CaCO_3$ have been suggested (El-Shibiny et al., 2009).

One step growth curve: The one-step growth experiment is fundamental to the description of a new bacteriophage since it allows the determination of its latent period and burst size. The latent period is the time taken by a bacteriophage to reproduce inside an infected host cell. The burst size is the number of newly synthesized bacteriophages obtained from an infected cell (Sinha et al., 2018).

Whole genome sequencing: This is a mandatory analysis to perform in bacteriophages that present a high potential to be used for the pathogens control. As bacteriophages have to be safe for human health and for the environment, their whole genome should be verified to guarantee that there is no locus encoding for toxins or antimicrobial resistance determinants or to confirm that there are no genes involved in lysogeny (Fernandez et al., 2018).

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Study 1

Efficient isolation of *Campylobacter* bacteriophages from chicken skin, analysis of several isolation protocols

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Abstract

The application of *Campylobacter*-specific bacteriophages appears as a promising alternative for the biocontrol of this pathogen in poultry. However, there is no standardized method for the efficient isolation of *Campylobacter* bacteriophages, and their occurrence appears to be low. This work assessed the efficiency of seven protocols for recovering *Campylobacter* bacteriophages from broiler skin samples inoculated at bacteriophage loads from $5.0x10^1$ to $5.0x10^6$ PFU/g. The enrichment of broiler skin in selective Bolton broth containing target isolates was the most efficient procedure, showing a low detection limit of $5.0x10^1$ PFU/g and high recovery rates of up to 560%. This method´s effectiveness increased as bacteriophage concentration decreased, showing its suitability for bacteriophage isolation. When this method was applied to isolate new campylophages from retail broiler skin, a total of 280 bacteriophages were recovered achieving an isolation success rate of 257%. From the 109 samples 68 resulted bacteriophage positive (62%). Broiler skin could be, therefore, considered a rich source in *Campylobacter* specific bacteriophages. The enrichment of broiler samples with target hosts in a *Campylobacter* selective medium and the subsequent incubation under appropriate conditions supporting bacterial growth is a simple, reproducible and efficient method for successful isolation of both group II and III *Campylobacter* specific bacteriophages.

Key words: phage, campylophage, phage-therapy, biocontrol, isolation rate, poultry.

1. Introduction

Campylobacter is the most commonly reported gastrointestinal bacterial pathogen in the European Union (EU) since 2005, with 246,158 reported confirmed cases of human campylobacteriosis in 2017 (EFSA, 2018). However, the reported incidence only represents a small proportion of all clinical cases. It has been estimated that there are approximately nine million cases of campylobacteriosis per year in the EU, with a total annual cost of 2.4 billion € (EFSA, 2011). This underreporting can be attributed to the non-mandatory campylobacteriosis notification in some EU member states, as well as to its self-limiting nature, which presents as a severe gastrointestinal illness resulting in diarrhea, fever and abdominal cramps within six days (Janež and Loc-Carrillo, 2013). Although acute complications such as arthropathies, immune mediated neuropathies and septicemia might occasionally follow *Campylobacter* infection (Hansson et al., 2018), its fatality rate is low (0.04%) (EFSA, 2018). Majority of campylobacteriosis cases are associated with *Campylobacter jejuni* and *Campylobacter coli* species, responsible for the 84.4 and 9.2% of the confirmed cases, respectively, in 2017 (EFSA, 2018).

Poultry is considered the natural reservoir of *Campylobacter spp*. and raw and undercooked broiler meat the major source of human campylobacteriosis (EFSA, 2011; Silva et al., 2011). *Campylobacter* spp. are commensals in the avian gut and the cross contamination of carcasses at the slaughterhouse often leads to contamination of meat products at retail (Furuta et al., 2017). Different mitigation strategies, such as competitive exclusion, use of chemical additives or antibiotics, and strict hygiene protocols, have been used in the EU with variable success (Hwang et al., 2009). Additionally, there has been a worrying increase of antibiotic resistance in farm animals

in recent years (Van Boeckel et al., 2019). The World Health Organization (WHO) has included fluoroquinolone resistant *Campylobacter* in its global priority list of antibiotic resistant bacteria that pose the greatest threat to human health (WHO, 2017). The finding of new alternative methods to control *Campylobacter* by reducing the use of antibiotics in food-producing animals is, therefore, a challenge to global public health.

One promising alternative to reduce the prevalence of *Campylobacter* within the farm-to-fork process is the use of specific bacteriophages (phages) as biocontrol agents. Bacteriophages are viruses that specifically infect and kill bacteria, widely distributed in the environment and often consumed in our diet as natural microbiota present in foods, including poultry products (Atterbury et al., 2003; Connerton et al., 2011; Tsuei et al., 2007). The use of bacteriophages is an attractive food safety strategy because they are specific towards the pathogen of concern, harmless to humans, animals and plants, and do not affect the existing commensal microbiota or alter attractive food properties.

Campylobacter-infecting bacteriophages (campylophages) are natural specific enemies of this pathogen, being classified into three groups according to their genome size (Sails et al., 1998). Group I campylophages (320 kb) have been rarely isolated and have not been deeply described whereas campylophages of group II (180 kb; *Cp220virus*) and group III (140 kb; *Cp8virus*) are most common (Connerton et al., 2011). They have been isolated wherever their hosts are present, including both environmental samples such as pig, poultry, cattle and sheep faeces, abattoir effluent or sewage (Aprea et al., 2018; Connerton et al., 2011), as well as poultry food products (Atterbury et al., 2003; Tsuei et al., 2007). However, the occurrence of campylophages is very low even in these samples (Nowaczek et al., 2019). Furthermore, the reported isolation rate among published works is highly variable, which could be attributed to differences in the

isolation methods or the sample type and/or origin. For instance, campylophages isolation rate from broiler intestines and caecum samples varied between 3% (Hansen et al., 2007), 10% (Janez et al., 2014) and 20% (Atterbury et al., 2005; Hwang et al., 2009; Owens et al., 2013). Different isolation rates of 0% (Janez et al., 2014), 18% (Aprea et al., 2018), 40% (Loc-Carrillo et al., 2007) and 42% (Connerton et al., 2004) were also reached from broiler faeces. Remarkably, Carvalho et al. (2010) went from finding no bacteriophages to isolating 43 from six broiler intestine samples by including an enrichment step with *Campylobacter* isolates in their protocol. A great detection rate variety have also been observed from samples of food origin with reported isolation rates from broiler skin samples from 0% (Tsuei et al., 2007) to only 11% (Atterbury et al., 2003). Bacteriophage isolation success rates from broiler liver samples ranged from 3% (Firlieyanti et al., 2016) up to 169% after enrichment with different *Campylobacter* isolates (Furuta et al., 2017).

Different campylophage isolation methods have been proposed in the literature. However, no standardized isolation method is yet available. Therefore, to optimize the isolation of Campylophages, in this study seven new and previously reported isolation methods were evaluated on the basis of an efficient bacteriophage recovery. These seven protocols were tested in broiler skin matrices with known concentrations of campylophages, and the most successful procedure was then used for the isolation of new *Campylobacter* specific bacteriophages from broiler skin samples.

2. Materials and methods

2.1. *Campylobacter* isolation, identification and culture

Ten *Campylobacter* isolates were used as host bacteria in this study (Table 1). For their isolation, broiler skin or faeces samples were diluted (1:4 w/v) in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4$ and 0.01% w/v gelatin) and plated onto RAPID'*Campylobacter* Base (Bio Rad, Marmes la Coquete, France) plates combined with RAPID'*Campylobacter* Supplement (Bio Rad). Plates were allowed to dry at room temperature and incubated at 37 °C for 72 h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2). Presumptive brick-red colonies of *Campylobacter* Gram negative and oxidase positive were identified by multiplex PCR (Elbrissi et al., 2017). *Campylobacter* isolates were stored at -80 °C in Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, UK) supplemented with 20% glycerol.

Table 1. *Campylobacter* isolates used in this study.

Specie	Code	Origin
Campylobacter jejuni	CJE024	Broiler faeces
Campylobacter jejuni	CJE042	Broiler faeces
Campylobacter jejuni	CJE054	Broiler faeces
Campylobacter jejuni	CJE055	Broiler faeces
Campylobacter jejuni	CJE056	Broiler faeces
Campylobacter jejuni	CJE057	Broiler skin
Campylobacter coli	CCO044	Broiler skin
Campylobacter coli	CCO056	Broiler skin
Campylobacter coli	CCO059*	Broiler faeces
Campylobacter coli	CCO062*	Broiler faeces
Campylobacter coli	CCO066	Broiler skin
Campylobacter coli	CCO068	Broiler faeces

*Used for CAM294 and CAM287 bacteriophages propagation

For exponential phase cultures preparation, thawed stock cultures (200 µl) were cultivated on Columbia blood agar (Oxoid) with 5% (vol/vol) defibrinated sheep blood

(Oxoid) under microaerobic conditions at 37 °C. After overnight incubation, cells were harvested in BHI broth to an OD_{600} of 0.6 (10^8 CFU/ml) and incubated at 37 °C for 4 h under microaerobic conditions.

2.2. Bacteriophage lysates preparation and titration

Two campylophages of group II (180 kb; *Cp220virus*) and three of group III (140 kb; *Cp8virus*), isolated form pig and broiler faeces respectively, were used for broiler skin samples inoculation (Table 2). For bacteriophage lysates preparation, each bacteriophage was propagated in its corresponding host by the double agar layer method. Briefly, 600 µl of exponential phase bacterial cultures were supplemented with 1 mM $CaCl_2$ and 10 mM $MgSO_4$ and mixed with 400 µl bacteriophage stock suspension. Mixtures were incubated for 15 minutes at 37 ºC and added individually to 4 ml molten NZCYM soft agar (NZCYM broth (Pronadisa, Conda Laboratories, Madrid, Spain) supplemented with 0.7% Bacteriological Agar (Oxoid)) previously tempered at 50 ºC. The 5 ml of soft agar plus bacteria and bacteriophage was immediately poured onto NZCYM hard agar plates (NZCYM broth with 1.2% agar) and allowed to dry for 15 minutes before incubation at 37 ºC for 24 h under microaerobic conditions.

Table 2. *Campylobacter* specific bacteriophages used in this study.

Phage code	Propagating host	Origin	Genome size	Group
CAM1	CJE054	Broiler faeces	140 kb	III
CAM2	CJE024	Broiler faeces	140 kb	III
CAM3	CJE042	Broiler faeces	140 kb	III
CAM287	CCO062	Swine faeces	180 kb	II
CAM294	CCO059	Swine faeces	180 kb	II

Bacteriophages were recovered from plates presenting confluent lysis by adding 5 ml of SM buffer and incubating at 4 ºC for 24 h with gentle shaking. SM buffer with bacteriophages was then treated with 10% chloroform and kept at 4 ºC until use. Bacteriophage lysates titer was determined by spotting 20 µl of serially diluted suspensions onto NZCYM soft agar overlay plates.

2.3. Broiler skin samples inoculation

Raw skin-on whole broilers, thighs and wings were randomly purchased from different local supermarkets (pre-packaged raw broiler) and butcher´s shops (packaged at the point of sale) and processed within the 24 h of purchase. For experiments with artificially campylophage-inoculated samples, sections of 16 cm^2 (4 x 4 cm; 3.5 ± 0.92 g) were aseptically cut from previously confirmed bacteriophage negative broiler skins, using uninoculated sections as negative controls as indicated by Atterbury et al. (2003). Skin samples were surface inoculated with 100 µl of appropriate dilutions of the corresponding bacteriophage lysate in order to obtain bacteriophage loads from $5.0x10^1$ to $5.0x10^6$ PFU/g (approx. $1.1x10^1$ to $1.1x10^6$ PFU/cm^2, respectively). Inoculated samples were left to dry at room temperature for 90 min inside a laminar flow cabinet before processing.

2.4. Bacteriophage isolation protocols

In order to assess the effect of the methodology on campylophages recovery efficiency, inoculated broiler skin samples were processed by the seven methods summarized in Table 3. In all methods, inoculated broiler skin samples were initially stomached in filter sterile bags with different broth medium (1:4 w/v) for 2 minutes prior to

incubation under the different conditions corresponding to each method. These conditions are presented below:

Method 1: Samples were processed following the method described by Atterbury et al. (2003) but stomaching broiler skin sections in SM buffer (1:4 w/v).

Method 2: Samples were processed following the method described by Atterbury et al. (2005) for broiler faeces sampling and used later by Hwang et al. (2009) for skin samples. In this method, skin sections were stomached in SM buffer. The stomachate was then collected in sterile tubes and incubated at 4 °C overnight with gentle shaking.

Method 3: Broiler skin samples were placed in filter sterile bags with BHI broth and enriched with exponential phase cultures of the corresponding host *Campylobacter* isolate to a final concentration of 10^6 CFU/ml. After stomaching, mixtures were incubated at 37 °C for 48 h under microaerobic conditions.

Method 4: Broiler skin samples were processed and enriched as described for method 3 but incubated at 42 °C (instead of at 37 ºC).

Method 5: Samples were processed, enriched and incubated as described for method 4, but with gentle shaking during incubation.

Method 6: Broiler skin samples were processed following the method described by Carvalho et al. (2010) for campylophages isolation from broiler intestines with minor modifications. Skin sections were placed in filter sterile bags with *Campylobacter* selective Bolton broth (Oxoid) supplemented with selective supplement of antibiotics (Oxoid) and 5% of lysed horse blood (Oxoid), a selective media for food samples

Table 3. Description of the the seven methods used assayed for bacteriophage recovery.

Method	Medium	Enrichment[1]	Temperature	Atmosphere	Time	Shaking	Reference
1	SM buffer	no	-	-	-	-	Atterbury *et al*. 2003
2	SM buffer	no	4 ºC	Aerobic	24 h	250 rpm	Hwang *et al*. 2009
3	BHI broth	yes	37 ºC	Microaerobic	48 h	-	-
4	BHI broth	yes	42 ºC	Microaerobic	48 h	-	-
5	BHI broth	yes	42 ºC	Microaerobic	48 h	250 rpm	-
6	Bolton broth	yes	42 ºC	Microaerobic	48 h	-	Carvalho *et al*. 2010[2]
7	Bolton broth	no	42 ºC	Microaerobic	48 h	-	Carvalho *et al*. 2010[2]

[1] Addition of corresponding bacterial host to a final concentration of 10^6 CFU/ml.

[2] Method used for campylophages isolation from broiler intestines.

Campylobacter enrichment (UNE EN ISO 10272-1:2017). The preparation was enriched by inoculating exponential phase culture of each corresponding propagating host (Table 2) to a final concentration of 10^6 CFU/ml. Mixtures were stomached and incubated at 42 ºC for 48 h under microaerobic conditions.

In addition, in order to assess the effect of host bacteria load on bacteriophage isolation efficiency, this method 6 was evaluated using three concentrations of exponential phase culture of propagating host (10^4, 10^6 and 10^8 CFU/ml). Finally, this method was also used to assess the effect of the campylophage group on the isolation efficiency. Specifically, two group II (CAM287 and CAM294) and three group III (CAM1, CAM2 and CAM3) campylophages at titers between $5.0x10^1$ and $5.0x10^6$ PFU/g were assayed followed by the enrichment step with each of the corresponding hosts.

Method 7: Samples were processed and incubated as described for method 6, but with no host bacterial enrichment.

After incubation under each condition, all resultant solutions were collected in sterile tubes and centrifuged at 5,000 x g at 20 °C for 10 min. Chloroform was added (10% v/v) to recovered supernatants and the bacteriophage titer was determined as explained before to assess each method´s recovery efficiency. All tested conditions were repeated at least three times.

The limit of detection was considered the lowest inoculated bacteriophage titer at which bacteriophages were recovered in all replicates. Recovery efficiency data were calculated as the mean percentage of bacteriophage recovery ± standard deviation per broiler skin gram.

2.5. Isolation of *Campylobacter* bacteriophages from broiler skin

A total of 109 raw skin-on whole broilers, thighs or wings were randomly purchased from different local supermarkets and butcher´s shops. For campylophages isolation, three point five g of natural and non-inoculated broiler skin from wing, neck and thigh regions were processed within the 24 h from purchase. These pieces were mixed with *Campylobacter* selective Bolton broth as described in method 6 and the enrichment step was prepared by inoculating a mixture of exponential phase cultures of ten *Campylobacter* isolates (Table 1) to a final concentration of 10^6 CFU/ml of each isolate. Bacteriophage presence was evaluated by spotting 10 µl of the samples onto lawns of each of the ten *Campylobacter* isolates. Plates were incubated overnight at 37 °C under microaerobic atmosphere and then examined for bacteriophage plaques presence.

Bacteriophages detected from broiler skin samples were recovered and purified following the method described by Loc-Carrillo et al., (2007) with minor modifications. Briefly, single plaques were removed from the overlay agar using a sterile 1 ml pipette tip and resuspended in 900 µl SM buffer. Then, bacteriophages were propagated by the double agar layer method as described in section 2.2. Isolated bacteriophage plaques were picked and plated twice more to ensure purity. Fresh bacteriophage lysates were conserved at 4 °C in sterile tubes and at -80 °C in SM buffer supplemented with 20% glycerol.

3. Results and discussion

3.1. Evaluation of the efficiency of seven *Campylobacter* bacteriophage isolation protocols

Broiler skin samples were inoculated with campylophage CAM1 to titers between $5.0x10^1$ and $5.0x10^6$ PFU/g and then processed by seven different methods. As shown in Figure 1, method 6 was able to recover campylophage CAM1 from all broiler skin samples, including those inoculated at the lowest bacteriophage titer. Method 7 also recovered bacteriophages from samples with the lowest phage titer but, unlike the previous method, it showed high variability in the recovery efficiency rates. By contrast, the remaining methods were only effective for recovering campylophage CAM1 from samples containing more than $5.0x10^3$ or $5.0x10^4$ PFU/g.

For all the inoculated bacteriophage titers, recovery efficiency rates ranging from 116 to 338% and from 109 to 156% were achieved for methods 6 and 7, respectively, in comparison to the lower rates of between 0 and 84% reached by the other tested methods. These results suggest an enhanced bacteriophage multiplication during samples incubation in *Campylobacter* selective Bolton broth used in methods 6 and 7, which promoted the growth of both inoculated (method 6) and/or naturally existing (methods 6 and 7) *Campylobacter* isolates present in broiler skin. *Campylobacter* growth promotion increases the probability of bacteriophage attachment to the host cell surface, promoting thereby bacteriophage infection and replication.

The lowest detection limit, determined as the minimum titer at which bacteriophages were recovered from all replicates, was reached by method 6, being able to detect only $5.0x10^1$ PFU/g. Additionally, the recovery efficiency of these method was higher as the

inoculated bacteriophage titer decreased. The better recovery efficiency, showing rates up to 338%, was observed from samples containing only $5.0x10^1$ PFU/g. This could be consequence of the predator-prey relationship between bacteriophage and bacteria, revealing that there is a threshold beneath which susceptible bacteria are more abundant than bacteriophages, promoting thereby an active bacteriophage replication (Payne and Jansen, 2003). Above that threshold, the very high number of bacteriophages will clear the bacterial population without active replication in the phenomenon called lysis from without (Jackel et al., 2019). As *Campylobacter* replication was not improved by other methods, predator-prey relationship was probably high, and bacteriophages were not able to propagate.

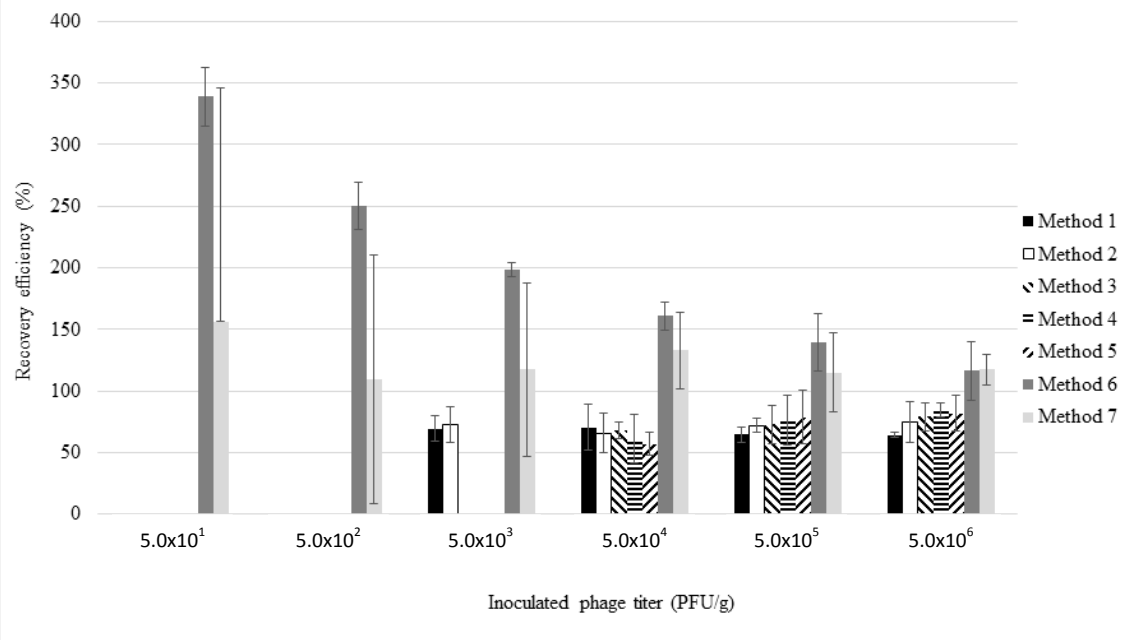

Figure 1. Recovery efficiency rates found after processing broiler skin pieces, previously inoculated with campylophage CAM1 between $5.0x10^1$ and $5.0x10^6$ PFU/g ($1.1x10^1$ and $1.1x10^6$ PFU/cm^2), by seven different isolation methods.

In the case of method 7, using Bolton broth without enrichment, a high variability in the recovery efficiency rates was observed. Although bacteriophages were isolated from broiler skin inoculated to $5.0x10^1$ PFU/g, it was not possible to isolate them in the three

replicates. Therefore, this method showed a bad limit of detection of $5.0x10^4$ PFU/g. This high variability could be attributed to sample-to-sample variation, that is, to the presence or absence of indigenous bacteriophage sensitive *Campylobacter* isolates on the surface of broiler skin. Thus, the presence and subsequent growth of sensitive isolates into Bolton broth would allow bacteriophage attachment and replication, resulting in high recovery rates. However, the lack of sensitive bacteria within the sample would cause a deficient bacteriophage-host interaction and a poor bacteriophage replication, resulting in low recovery rates. Therefore, this would be a non-reproducible method that should be avoided for campylophages isolation from broiler skin samples.

The detection limit of methods 1 and 2, that used SM buffer as rinsed broth, was $5.0x10^3$ PFU/g ($1.1x10^3$ PFU/cm^2). These results were in agreement with those reported by Tsuei et al. (2007) that used *Escherichia coli* broth to isolate campylophages, but higher than the $2.0x10^2$ PFU/cm^2 reported by Atterbury et al. (2003), who suggested that bacteriophage replication does not occur on the surface of broiler skin samples stored at 4 ºC since *Campylobacter* does not grow under these conditions and hence does not have an active metabolism. However, results obtained in the present work showed even higher detection limits of $5.0x10^4$ PFU/g with methods 3, 4 and 5 using BHI broth at 37 and 42 ºC for *Campylobacter* growth. This broth is not a selective media as Bolton broth then it could promote the growth of other bacteria naturally present onto the broiler skin samples. Moreover, the low multiplication rate of *Campylobacter* cells under these competitive conditions could limit both CAM 1 campylophage attachment and propagation, especially in samples with lower bacteriophage titers. Therefore, the enrichment step consisting on the addition of host bacteria to the rinsed broth could be a

key factor in bacteriophage recovery only when it is carried out with a *Campylobacter* selective media, such us Bolton broth, that benefit the *Campylobacter* growth.

Overall, method 6 was the most efficient in the isolation of campylophage CAM1, since it showed the best recovery efficiency rates (up to 338%) as well as the lowest detection limit ($5.0x10^1$ PFU/g). In addition, the low variability among the replicates of each trial demonstrates that it is a a reliable and reproducible method.

3.2. Effect of host bacteria load and bacteriophage group on isolation efficiency

Bacteriophage propagation is dependent on the host bacteria concentration since the probability of bacteriophage attachment to the surface of the bacteria depends on the available bacterial amount (Payne and Jansen, 2003). Therefore, the effect of the host bacteria burden as enrichment step of method 6 on the efficiency of bacteriophage recovery was studied. As shown in Figure 2, poor differences on bacteriophage recovery efficiencies were observed with the three host *Campylobacter* concentration analyzed (10^4, 10^6 and 10^8 CFU/ml) and, the same bacteriophage detection limit of $5.0x10^1$ PFU/g was established. Recovery efficiencies ranging from 110 to 355% were achieved for all inoculated bacteriophage titers with 10^4 and 10^6 CFU/ml of host bacteria and, unexpectedly, slightly lower recovery efficiencies, from 82 to 262%, were observed with a large amount of 10^8 CFU/ml. However, the suitability of this method 6 for *Campylobacter* multiplication, which is promoted by Bolton selective broth, makes none of the three tested bacterial concentrations a limiting factor for bacteriophage replication, resulting in similar recovery efficiencies.

The possible effect of campylophage group was also assessed on method 6 isolation efficiency. Broiler skin samples were inoculated with two group II (CAM287 and

CAM294) and three group III (CAM1, CAM2, CAM3) campylophages. The five campylophages were recovered from all broiler skin samples, including those inoculated to the lowest bacteriophage titer, showing a detection limit of $5.0x10^1$ PFU/g (Figure 3).

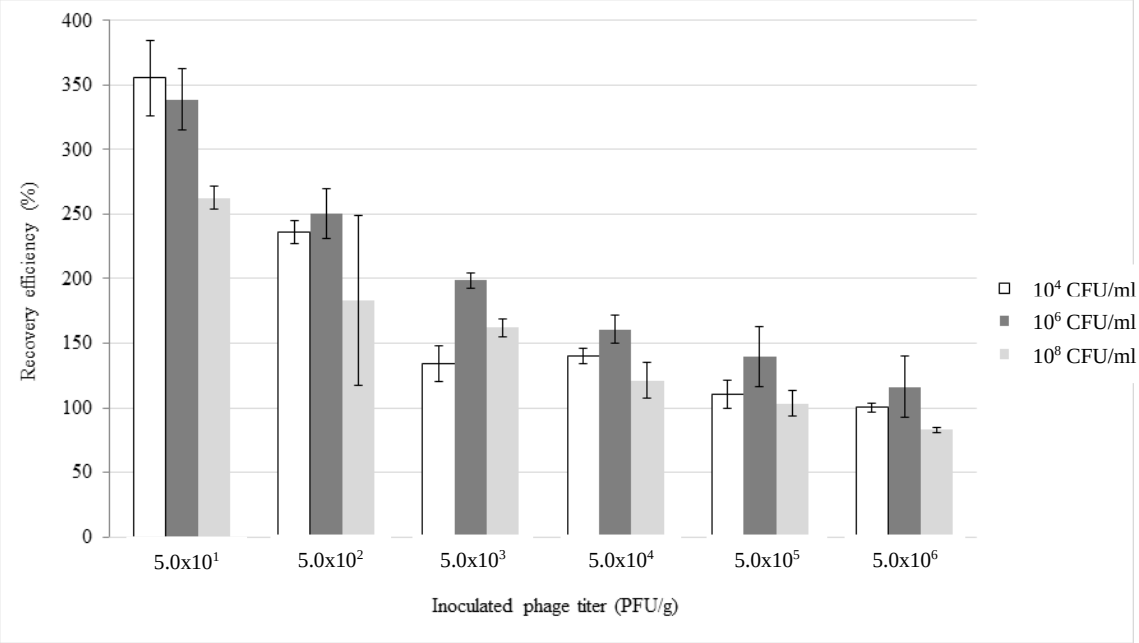

Figure 2. Effect of host population during the enrichment step of method 6 on campylophages recovery efficiency.

Recovery efficiencies were similar for all campylophages when inoculated titers varied between $5.0x10^2$ and $5.0x10^6$ PFU/g. As previously observed for CAM1, a clear increasing trend in the recovery efficiency was also detected for the remaining campylophages as the inoculated titer decreased, showing higher recovery rates in samples inoculated to the lowest bacteriophage titer ($5.0x10^1$ PFU/g). Specifically, campylophages of group II (CAM294 and CAM287) showed recovery efficiencies of 526 and 568%, respectively, and those of group III (CAM1, CAM2 and CAM3) from 330 to 414%.

Therefore, it could be concluded that this method 6, including enrichment with host bacteria in *Campylobacter* selective Bolton broth and subsequent incubation at 37 ºC under microaerophilic conditions, is an efficient method for both group II and group III campylophages isolation, mainly from those samples with low amount of bacteriophage.

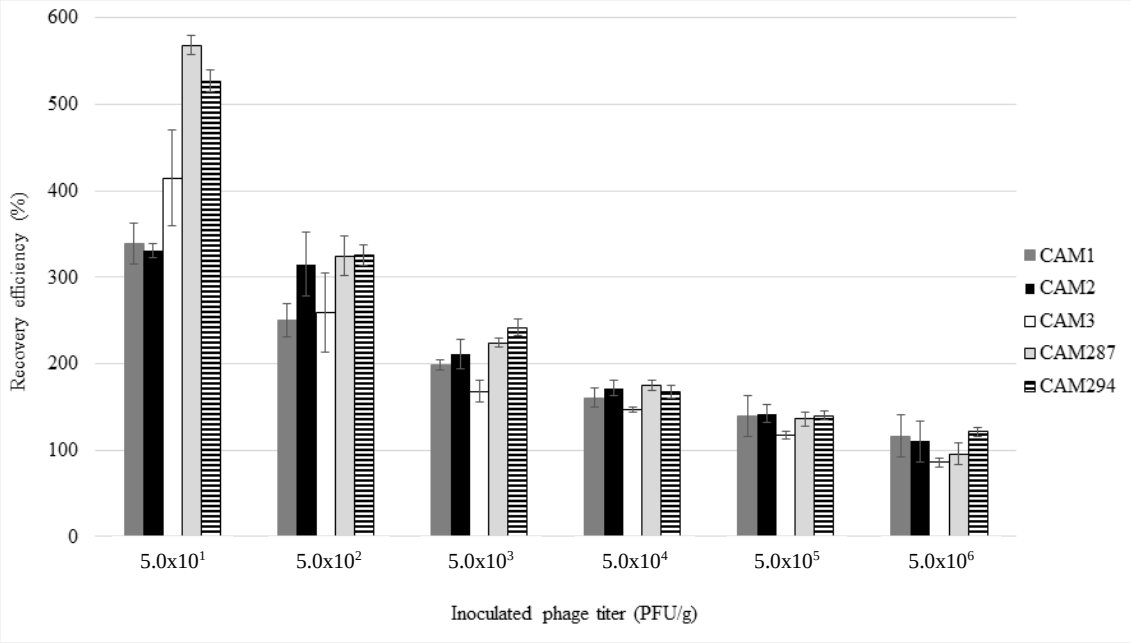

Figure 3. Recovery efficiency rates found after processing by method 6 broiler skin pieces previously inoculated with campylophages CAM1, CAM2, CAM3, CAM287 and CAM294 between $5.0x10^1$ and $5.0x10^6$ PFU/g ($1.1x10^1$ and $1.1x10^6$ PFU/cm^2).

3.3. Isolation of *Campylobacter* bacteriophages from broiler skin

A total of 109 raw whole broiler, thigh and wing skin samples were enriched with a mixture of ten *Campylobacter* isolates and processed following method 6. A high proportion of campylophage positive samples was observed since bacteriophages were isolated from 68 of the 109 processed samples (62%). A total of 280 *Campylobacter* specific bacteriophages were isolated which means a high bacteriophage isolation success rate of 257% (280/109). In this sense, Carvalho et al. (2010) and Furuta et al.

(2017) showed also high success rates of 717% and 177% by performing enrichments in Bolton and Preston selective media for campylophage isolation from broiler intestines and broiler livers, respectively. By contrast, Tsuei et al. (2007) did not isolate any campylophage after the enrichment of 42 broiler meat and offal samples in selective Exeter broth, evidencing that the type of sample would also affect campylophage isolation rate. Since bacteriophages can be found wherever their hosts are present, the choice of a type of sample known to hold high levels of *Campylobacter*, such as broiler skin, could increase the campylophage isolation success compared to samples with a lower prevalence in *Campylobacter*.

Low percentages of *Campylobacter* specific bacteriophages have been isolated from broiler skin samples in previous studies. Atterbury et al. (2003), following a methodology based on phage elution in SM buffer, isolated 34 campylophages from 300 fresh broiler skin samples (11%) and none from 150 frozen samples. Tsuei et al. (2007) also followed a bacteriophage elution protocol but they were not able to isolate any campylophage from broiler skin samples. Therefore, the high *Campylobacter* specific bacteriophage isolation rate observed in this study confirms the suitability of method 6 for campylophages isolation from broiler skin.

Furthermore, considering the high percentage of bacteriophage positive samples (62%), it could be said that broiler skin is a rich source in *Campylobacter* specific bacteriophages, which are able to survive on retail poultry products after processing and packaging under commercial storage conditions. These findings confirm the fact that poultry products are a rich source of campylophages and that, as previously stated by other authors (Fischer et al., 2013; Sillankorva et al., 2012), bacteriophages are often consumed in our diet. Therefore, the application of bacteriophages against

Campylobacter at any point in the poultry production chain would not imply the addition of any foreign element in our diet, which could ease the authorization of the use of bacteriophages as *Campylobacter* biocontrol tool.

Regarding the bacterial host, all the 280 campylophages were isolated using the six isolates of the specie *C. jejuni* as propagative hosts (Table 4). These were not surprising results because campylophages are usually present everywhere with high *Campylobacter* presence (Carvalho et al., 2010), and poultry meat is known to be a prominent source of *Campylobacter* with a significantly higher occurrence of *C. jejuni* than *C. coli* (EFSA, 2018; Garcia-Sanchez et al., 2018).

Table 4. Distribution of the number of new phages recovered from broiler skin samples using isolates of *Campylobacter* as propagative hosts.

Code of the propagating host	No. of isolated bacteriophages
CJE024	66
CJE042	67
CJE054	66
CJE055	6
CJE056	58
CJE057	17
CCO044	-
CCO056	-
CCO066	-
CCO068	-
Total	280

4. Conclusions

Broiler products and, particularly, broiler skin can be considered a rich source in *Campylobacter* specific bacteriophages. The enrichment of broiler skin samples with different *Campylobacter* isolates in a selective medium such as Bolton broth, and the subsequent incubation under appropriate conditions supporting *Campylobacter* growth is a simple, reproducible and efficient method for both group II and III campylophages

isolation. The use of a *Campylobacter* selective medium in the enrichment step is a key factor since it enhances *Campylobacter* growth and, consequently, bacteriophage replication, allowing the achievement of really high recovery efficiencies up to 560% and low detection limits of $5.0x10^1$ PFU/g. Furthermore, the effectiveness of this method increases as bacteriophage concentration decreases, being therefore a consistent method for the isolation of campylophages at the low bacteriophage concentrations naturally occurring in foods.

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Study 2

Molecular typing and antimicrobial susceptibility of *Campylobacter* spp. isolates in northern Spain

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Abstract

Campylobacter is one of the most common zoonotic pathogens responsible for foodborne diseases worldwide and antibiotic resistance of this bacterium to clinically relevant antibiotics is in constant increase. This study provides new information on genetic diversity and antimicrobial susceptibility of *Campylobacter* spp. from human, retail broiler samples and faecal droppings of broiler and swine in northern Spain. The prevalence of *Campylobacter* in broiler products was 35.4%, while it was higher in broiler and swine faeces, 62% and 42.8%, respectively. *C. jejuni* or *C. coli* were the only species identified from samples of meat and faeces while *C. fetus* was also detected in human clinical isolates by multiplex-PCR. The fingerprinting analysis by *fla*A-RFLP (restriction fragment length polymorphism) and PFGE (pulsed field gel electrophoresis) revealed 68 different genotypes from the total of 89 isolates analyzed. Most of the *Campylobacter* isolates were resistant to ciprofloxacin (77 out of 89 isolates, 86.5%) and tetracycline (76 out of 89 isolates, 85.4%) and almost half of the isolates to erythromycin (44 out of 89 isolates, 49.4%), with high levels of multidrug resistance (40.4%). All the *C. coli* isolates and two of the three *C. fetus* isolates were resistant to one or more antimicrobial agents. This alarming level of resistance detected among the high number of *Campylobacter* isolates analysed encourages efforts to reduce this bacterium through innovative control strategies.

Key words: broiler, swine, human, C. *fetus*, *fla*A-RFLP, PFGE.

1. Introduction

Campylobacter is a foodborne pathogen, widespread in the environment and frequent colonizer of the natural intestinal microbiota of wild and domesticated animals, such as birds, ruminants or swine (Silva et al., 2011). This bacterium can be spread to human through handling and consuming raw or undercooked contaminated food, such as raw milk or contaminated water, with poultry products being the major source of infection and broiler skin the most contaminated part of the poultry (Davis and Connert, 2007; Josefsen et al., 2015; Stella et al., 2017).

The last European Union (EU) summary report confirmed campylobacteriosis as the most commonly reported zoonosis in the EU which, accounting 246,571 reported infections, it represented almost 70% of all the reported cases in 2018. Most of these infections are associated with *Campylobacter jejuni* which accounted for the 83.9% of the cases, and *Campylobacter coli*, responsible for the 10.3% (European Food Safety Authority (EFSA), 2019b). However, other species such as *Campylobacter fetus*, *Campylobacter upsaliensis* or *Campylobacter lari* could also be implicated (Patrick et al., 2018; Sinulingga et al., 2019).

Symptoms of campylobacteriosis include diarrhoea, headache, fever, abdominal pain and nausea. Sometimes this disease can be associated with serious complications such as Guillain–Barré syndrome, acute transverse myelitis or reactive arthritis (EFSA, 2019). Overall, it is self-limiting and no antibiotic treatment is required but in severe infections quinolones, macrolides and tetracyclines are the drugs of choice (Sifre et al., 2015). However, the alarming scenario for the public health caused by the high incidence of antimicrobial resistance in *Campylobacter* is well known. In recent years, *C. jejuni* and *C. coli* resistance to antibiotics has increased throughout the world (Di

Giannatale et al., 2019; Divsalar et al., 2019; Elhadidy et al., 2019; Han et al., 2019; Wieczorek et al., 2019). Resistance to fluoroquinolones, such as ciprofloxacin, seems to be associated with the use of these antimicrobials to treat poultry. The use of macrolides in animals is also significantly associated with the resistance to this drug in *C. coli* isolates from humans, and the use of tetracyclines in food producing animals with the resistance to tetracyclines in *C. jejuni* isolates from food-producing animals and humans (ECDC/EFSA/EMA, 2017). The high to very high mean values of resistance to ciprofloxacin and tetracycline and the low levels of resistance to erythromycin reported by the EU member states, and even more, the extremely high levels to ciprofloxacin and tetracycline and low to moderate to erythromycin reported by Spain (EFSA, 2019), describe a disturbing situation.

The study of the genetic relatedness of the isolates is essential for the epidemiological research of foodborne outbreaks or to trace a foodborne pathogen throughout the food chain. Epidemiological studies can be performed sampling poultry and the surrounding environment in the primary production or taking faecal droppings in farm (Josefsen et al., 2015). A wide diversity of *Campylobacter* populations from human, environment and specially poultry meat has been reported from different countries (Behringer et al., 2011; Di Giannatale et al., 2019; Du et al,. 2018; Duarte et al., 2019; Elhadidy et al., 2019; Garcia-Sanchez et al., 2018; Guyard-Nicodeme et al., 2015). In these studies, several molecular techniques have been proposed for typing *Campylobacter* isolates being flagellin A restriction fragment length polymorphism (*fla*A-RFLP), pulsed field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) the most widespread.

Nowadays, much of the studies available are related to *C. jejuni* or *C. coli* and poultry as the main human infection source but the more updated information there is on these

and other species of *Campylobacter* as well as on their reservoirs is essential for knowing and understanding the current state of this pathogen.

In this study, a collection of *Campylobacter* isolates obtained from different samples from north of Spain was analysed with the aim of achieving new information on genetic diversity and antimicrobial resistance of *Campylobacter* spp. and assessing the correlations between specific genotypes and antibiotic susceptibility/resistance profiles.

2. Materials and methods

2.1. Samples and *Campylobacter* isolation conditions

During 2016 to 2018 pre-packaged raw broiler samples were purchased from different local supermarkets in the Basque Country, northern Spain, by performing two samplings per year. A total of 82 samples were analysed, including thighs (n=32), necks (n=28) and wings (n=22). The wings and thighs were packed in plastic containers sealed under modified atmosphere while necks were obtained from whole broilers wrapped in a simple plastic film. Samples were transported under refrigeration to the laboratory as quickly as possible and processed within 24 h from purchase. Skin sections of three point five grams of each broiler sample were aseptically cut and introduced in sterile plastic bags.

Additionally, during this period, fresh faecal droppings were collected from seven chicken and one swine farms in the Basque Country. Five grams from 50 chicken and seven swine faeces samples were introduced into sterile tubes, transported under refrigeration and processed within 24 h from collection.

Once in the laboratory, all samples were diluted 1:4 (w/v) in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM MgSO4 and 0.01% w/v gelatin) and manually

homogenized for two minutes. Then, samples were spread with sterile cotton swabs onto plates of RAPID'*Campylobacter* Base with RAPID'*Campylobacter* Supplement (Bio Rad, Marmes la Coquette, France). Plates were dried at room temperature and incubated at 37 °C for 72 h in a microaerobic atmosphere (5% O2, 10% CO2 and 85% N2) using an INVIVO2 400 hypoxia workstation (Ruskinn Technology Ltd, Bridgend, UK). Brick-red colonies were picked and streaked on Tryptone Soy Agar plates (TSA; Oxoid, Basingstoke, UK), and subjected to Gram stain and to oxidase test. The colonies that resulted Gram negative bacilli and oxidase positive were considered as putative *Campylobacter* isolates, and a single colony per sample was analysed by molecular methods.

Furthermore, 26 *Campylobacter* isolates were obtained from human clinical samples; eight from the University Hospital of Donostia (Donostia, Basque Country) and 18 isolates from the University Hospital of Vall d'Hebron (Barcelona, north-eastern Spain). These isolates were sent to AZTI for further analysis.

All *Campylobacter* isolates were stored at -80 °C in vials containing Brain Heart Infusion broth (BHI; Oxoid) supplemented with 20% glycerol until their use. For the different analysis, fresh cultures were obtained thawing 200 µl of stock cultures and plating onto Columbia blood agar plates with 5% (vol/vol) defibrinated sheep blood (Oxoid). After incubation of plates at 37 °C overnight in a microaerobic atmosphere, cells were recovered in BHI broth or 0.85% NaCl.

2.2. *Campylobacter* identification and typing

Bacterial DNA was extracted using the commercial DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The DNA

concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.2.1. Multiplex PCR

Three genes, *hipO, glyA* and, *sapB2*, were amplified to identify the species *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* subsp. *fetus* using the method described by Wang et al. (2002). Briefly, a mixture including 50–100 ng of DNA, BioMix™ Red (Bioline, London, UK), five pairs of primers and primers for 23S rRNA gene as internal control (Table 1) was adjusted in a total volume of 25 µl. The amplification reactions were performed on a BioRad C1000TM Thermal Cycler and the products were visualized by electrophoresis of 30 min at 50 V on 1.5% agarose gel; stained with GelRed (Biotium, Fremont, CA, USA) and observed in ChemiDoc™ imaging system (Bio Rad).

2.2.2. *fla*A-restriction fragment length polymorphism (*fla*A-RFLP)

A 1.7 kb fragment of *flaA* gene was amplified with A1 and A2 primers (Table 1) and digested with *Dde*I restriction enzyme as proposed Nachamkin et al. (1993).

Amplifications were performed in a mixture of 100 µl containing BioMix™ Red (Bioline), 2 µl of each primer (50 µM) and 5 µl bacterial DNA (50–100 ng). PCR were performed on a BioRad C1000TM Thermal Cycler using a denaturation step for 60 s at 94 °C, followed by 35 cycles of denaturation during 15 s at 94 °C, annealing 45 s at 45 °C and extension of 105 s at 72 °C; and a final extension for 5 min at 72 °C.

The correct amplification was checked by electrophoresis for 30 minutes at 100 V on a 1% agarose gel in 1X TBE buffer and GelRed. A volume of 5 µl of amplified product was digested with 0,2 µl *Dde*I (10 U/µl) restriction enzyme (New England Biolabs

Ipswich, MA, USA) and 3 µl of PCR Buffer (10X, New England Biolabs) in a final volume of 30 µl. Restriction fragments generated after an incubation at 37 °C for 3 h were detected by electrophoresis on 2.5% agarose gel in 1X TBE buffer and GelRed for 90 min at 90 V. Lengths of restriction fragments were assigned by comparison with a 100 bp ladder (Promega, Madison, WI, USA).

2.2.3. Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis was based on the CDC's PulseNet protocol (CDC, 2017), with minimal modifications. Briefly, *Campylobacter* spp. cell suspensions prepared in 0.85% NaCl were adjusted to 0.4 optical density at 610 nm wavelength. Four hundred µl of the bacterial suspensions containing 20 µl Proteinase K (20 mg/ml) were mixed with 400 µl of 1% molten Pulsed Field Certified agarose (Bio-Rad), prepared in TE buffer (10 mM Tris:1 mM EDTA, pH 8.0), and dispensed into four plug molds each. Plugs were let solidify and introduced in 5 ml tubes with 2 ml Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0, 1% Sarcosyl and 10 µl Proteinase K (20 mg/ml)). Tubes were incubated in a shaking water bath at 56 °C overnight for cell lysis. After this step, these agarose plugs were washed three times with sterile ultrapure water and six times more with TE buffer. Two mm plug slices were digested with *Sma*I (Thermo Scientific) and DNA fragments were separated on 1% Pulsed Field Certified agarose (Bio Rad) gel in 0,5X TBE buffer and GelRed using a CHEF-DR III PFGE system (Bio Rad). The electrophoresis conditions included an initial switch time of 6.8 s and final switch time of 35.4 s for 18 h at 6 V/cm and 14 °C. Sizes of the fragments were determined by comparison with Lambda Ladder PFG Marker (New England BioLabs).

Table 1. Primers used for *Campylobacter* identification and *flaA*-RFLP typing.

Name	Sequence (5'→ 3')	PCR product	Target gene	References
CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	323 bp	*hipO* gene (*Campylobacter jejuni*)	(Wang et al., 2002)
CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	126 bp	*glyA* gene (*Campylobacter coli*)	
CLF CLR	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	251 bp	*glyA* gene (*Campylobacter lari*)	
CUF CUR	AATTGAAACTCTTGCTATCC TCATACATTTTACCCGAGCT	204 bp	*glyA* gene (*Campylobacter upsaliensis*)	
CFF CFR	GCAAATATAAATGTAAGCGGAGAG TGCAGCGGCCCCACCTAT	435 bp	*sapB2*gene (*Campylobacter fetus*)	
23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	650 bp	23S rRNA gene (*Campylobacter* spp.)	
A1 A2	GGATTTCGTATTAACACAAATGGTGC CTGTAGTAATCTTAAAACATTTTG	1725 bp	*flaA* gene (typing)	(Nachamkin,et al., 1993)

2.3. Antimicrobial susceptibility

The antimicrobial susceptibility of the *Campylobacter* isolates was evaluated by disk diffusion using discs of fluoroquinolones (ciprofloxacin, 5 µg), macrolides (erythromycin, 15 µg) and tetracyclines (tetracycline, 30 µg) (Oxoid), and following the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019a). Cells were initially cultured as above mentioned and harvested in BHI broth. Bacterial cell suspensions for each isolate were prepared with an optical density of 0.6 at 600 nm wavelength (1×10^8 CFU/ml, approximately). Inoculums were spread using sterile swabs onto Mueller-Hinton agar plates supplemented with 5% mechanically defibrinated horse blood and 20 mg/l β-NAD (Bio-Rad). Discs were antiseptically dispensed onto the surface of the inoculated plates and after 48 h incubation at 37°C under microaerobic conditions, inhibition zone diameters were measured.

The susceptibility of isolates was categorized according to EUCAST breakpoints (EUCAST, 2019b): (a) ciprofloxacin ≥ 26 mm, susceptible and < 26 mm, resistant; (b) erythromycin ≥ 20 mm, susceptible and < 20 mm, resistant for *C. jejuni* and ≥ 24 mm, susceptible and < 24 mm, resistant for *C. coli*; (c) tetracycline ≥ 30 mm, susceptible and < 30 mm, resistant. The *C. jejuni* strain ATCC 33560 from American Type Culture Collection was used as control. Isolates exhibiting phenotypic resistance to the three classes of antibiotics were considered multidrug resistant (ECDC et al., 2017). It should be noted that the suggested *C. jejuni* breakpoints were used to interpret the results for *C. fetus*, since no such criteria are available for this specie.

2.4. Data analysis

Gels of PFGE and *fla*A-RFLP were photographed with a ChemiDoc™ imaging system (Bio-Rad) and phylogenetic relationships were determined using a temporary

BioNumerics software version 7.6 evaluation license (Applied Maths, Sint-Martens-Latem, Belgium) (permission to publish received). Cluster analysis was performed using Dice similarity coefficient with the unweighted pair group method with arithmetic mean (UPGMA) and a tolerance of 1.5% (An et al., 2018). All band profiles were carefully checked by visual inspection to be correctly marked, and those profiles clustered in the dendrogram with more than 90% similarity were assigned to the same genotypic profile. Dendrograms, combining PFGE and *fla*A-RFLP gels and antimicrobial susceptibility data, were created with BioNumerics software evaluation license using the Composite data sets.

Diversity was calculated using the Simpson's Index of Diversity (SID), which shows the probability that two randomly selected isolates are different genotypes. The index was calculated using $1-\Sigma pi2$, where pi is equal to the number of isolates of the same genotype divided by the total number of isolates.

3. Results

3.1. *Campylobacter* identification

Campylobacter positive isolates were detected in both meat and faeces samples and all of them were identified as *C. jejuni* or *C. coli* species.

From the 82 broiler meat samples processed, a total of 29 putative *Campylobacter* isolates (35.4%) were recovered and analyzed by multiplex PCR. Among these, 25 isolates were identified as *C. coli* (25 out of 29 isolates, 86.2%) and four as *C. jejuni* (4 out of 37 isolates, 13.8%). Wings and thighs products were more contaminated than necks (Table 2).

Table 2. Prevalence of *Campylobacter jejuni* and *Campylobacter coli* in broiler meat and faecal droppings of broiler and swine.

Sample	No. of samples analyzed	No. of *Campylobacter* positive samples (%)	No. of *C. jejuni* isolates	No. of *C. coli* isolates
Broiler meat	82	29 (35.4%)	4	25
Wings	22	9 (40.7%)	-	9
Necks	28	9 (31.8%)	-	9
Thighs	32	11 (34.5%)	4	7
Broiler faeces	50	31 (62%)	19	12
Swine faeces	7	3 (42.8%)	-	3

(-) Not detected

Putative *Campylobacter* colonies were isolated from six of the seven farms analysed (85.7%). Among the 50 broiler faecal samples 31 colonies (62%) were identified as *Campylobacter* positive isolates by multiplex PCR. *C. jejuni* was the species most frequently recovered (19 out of 31 isolates, 61.3%), and *C. coli* was identified in the remaining isolates (12 out of 31 isolates, 38.7%). In the only swine farm analysed, *Campylobacter* was isolated from three of the seven swine faeces samples (42.8%). The three isolates were identified as *C. coli*.

Regarding the 26 *Campylobacter* isolates obtained from human clinical samples, the predominant species identified was *C. coli* (15 out of 26 isolates, 57.7%) followed by *C. jejuni* and *C. fetus*, detected in eight (30.7%) and three (11.6%) of the isolates, respectively (Table 3). These three species were identified in both hospitals.

Table 3.- Identification and *in vitro* activity of antimicrobial agents against *Campylobacter* isolates (n = 89). The antimicrobial agents are indicated by abbreviation: ciprofloxacin (CIP), erythromycin (E) and tetracycline (TE).

Sample origin	**No. of isolates studied**	**Species (no. of isolates)**	**Antimicrobial susceptibility (no. of isolates)**							
			Resistant							**Susceptible to the three agents**
			CIP	**E**	**TE**	**CIP+E**	**CIP+TE**	**E+TE**	**CIP+E+TE**	
Human	26	*Campylobacter jejuni* (8)	-	-	-	1	3	-	4	-
		Campylobacter coli (15)	-	-	-	2	5	1	7	-
		Campylobacter fetus (3)	-	1	-	-	1	-	-	1
Broiler meat	29	*Campylobacter jejuni* (4)	-	-	-	-	4	-	-	-
		Campylobacter coli (25)	-	-	2	-	8	2	13	-
Broiler faeces	31	*Campylobacter jejuni* (19)	2	-	-	1	9	-	2	5
		Campylobacter coli (12)	-	-	-	-	3	-	9	-
Swine faeces	3	*Campylobacter coli* (3)	-	-	-	-	2	-	1	-
Total	89	*Campylobacter jejuni* (31) *Campylobacter coli* (55) *Campylobacter fetus* (3)	**2**	**1**	**2**	**4**	**35**	**3**	**36**	**6**

(-) Not detected

Overall, the multiplex PCR protocol identified three *Campylobacter* species from the 89 *Campylobacter* positive isolates: *C. coli* (55 out of 89 isolates, 61.8%), *C. jejuni* (31 out of 89 isolates, 34.8%) and *C. fetus* (3 out of 89 isolates, 3.4%) (Table 3). These isolates were subsequently analysed to study their genetic relatedness and *in vitro* antimicrobial susceptibility.

3.2. *Campylobacter* typing and diversity

Fig. 1 shows the dendrogram with the different profiles obtained by *fla*A-RFLP and PFGE methods, and Table 4 the distribution of the genotypes in relation to the *Campylobacter* species and the samples origin. A total of 68 genotypes from 89 isolates resulted from the combination of both techniques.

The number of profiles obtained with each technique differed. PFGE was, in general, more discriminatory than *fla*A-RFLP, generating different profiles from isolates that the *fla*A-RFLP technique considered equal. Moreover, PFGE was able to group the isolates of different species into different profiles while *fla*A-RFLP genotypes were not species specific.

*Fla*A-RFLP typing grouped 86 of the isolates into 30 different genotypes; and the PCR amplification failed in the three *C. fetus* isolates from human clinical samples. The typing profiles contained up to 11 bands of sizes ranging from 50 to 1,500 bp.

The PFGE technique grouped 82 of the isolates into 47 different genotypes; the remaining seven isolates were not typable by this technique despite made more than three testing attempts, and included two isolates of *C. jejuni* and five of *C. coli* from human and broiler faeces samples. PFGE profiles contained from four to 22 bands with sizes up to 388 kbp.

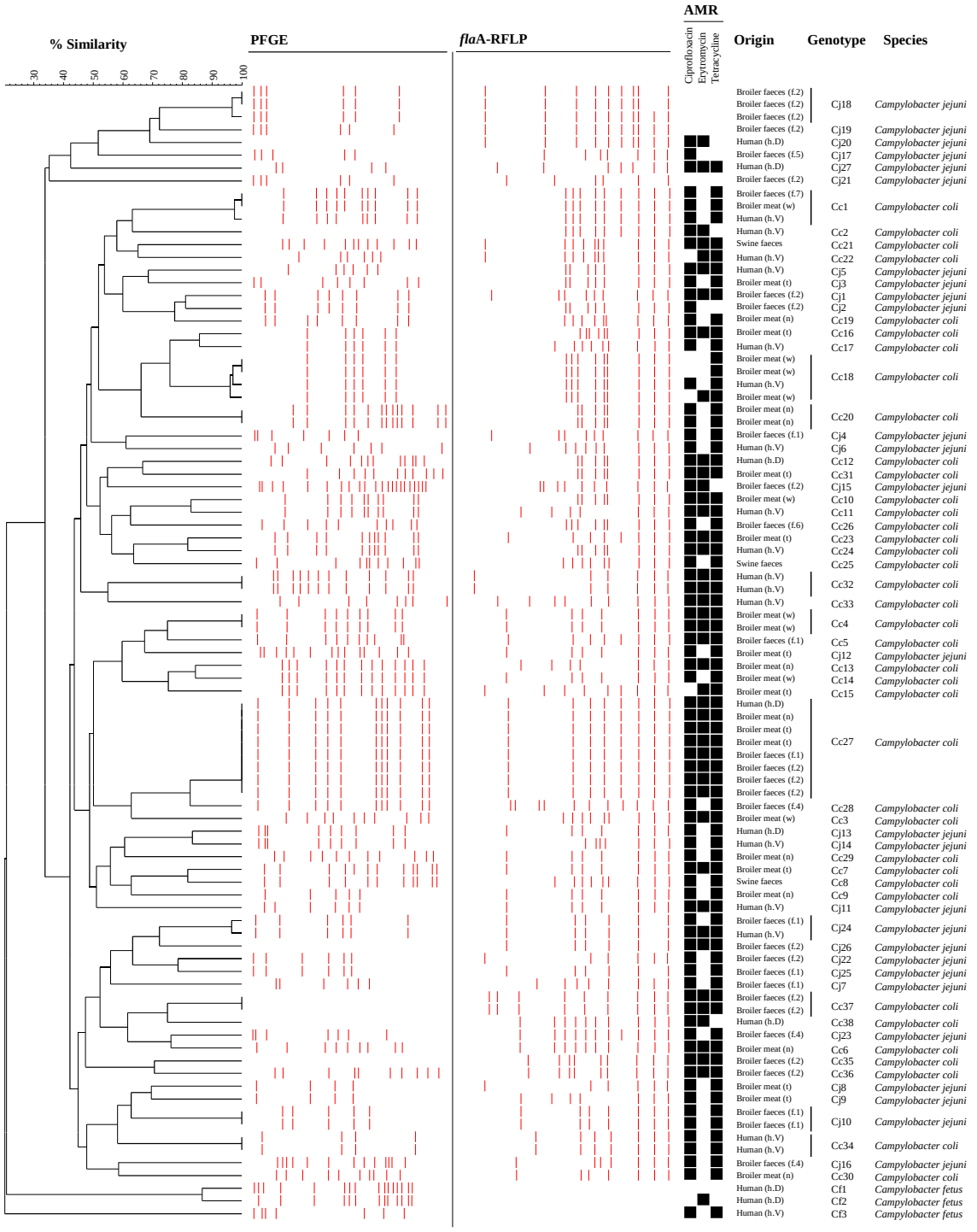

Figure 1. Dendrogram obtained by the combination of *fla*A-RFLP and PFGE profiles and antimicrobial susceptibility data from *Campylobacter* isolates (n = 89). In the antimicrobial resistance profiling (AMR) the black squares indicate resistance to that antimicrobial agent. The dendrogram was derived from UPGMA linkage of Dice correlation coefficients. The origin of the isolates is indicated in brackets (Broiler farms analysed (f) and 1, 2, 4, 5, 6, 7 numbers of farms. Broiler meat samples; t: thigh; n: neck; w: wing. Hospitals from the human clinical isolates; h.D: University Hospital of Donostia; h.V: University Hospital of Vall d'Hebron).

Table 4.- Typing of *Campylobacter* isolates (n = 89). The genotyping profiles were indicated by species abbreviation followed by the assigned profile number obtained by combination of *fla*A-RFLP and PFGE techniques. The number in brackets indicates the number of isolates detected.

	***fla*A-RFLP/PFGE profiles (no. of isolates)**				
	Human clinical isolates	**Broiler meat**	**Broiler faeces**	**Swine faeces**	**Total**
Campylobacter jejuni	Cj5(1), Cj6(1), Cj11(1), Cj13(1), Cj14(1), Cj20(1), Cj24*(1), Cj27(1)	Cj3(1), Cj8(1), Cj9(1), Cj12(1)	Cj1(1), Cj2(1), Cj4(1), Cj7(1), Cj10(2), Cj15(1), Cj16(1), Cj17(1), Cj18(3), Cj19(1), Cj21(1), Cj22(1), Cj23(1), Cj24*(1), Cj25(1), Cj26(1)		27
Campylobacter coli	Cc1*(1), Cc2(1), Cc11(1), Cc12(1), Cc17(1), Cc18*(1), Cc22(1), Cc24(1), Cc27*(1), Cc32(2), Cc33(1), Cc34(2), Cc38(1)	Cc1*(1), Cc3(1), Cc4(2), Cc6(1), Cc7(1), Cc9(1), Cc10(1), Cc13(1), Cc14(1), Cc15(1), Cc16(1), Cc18*(3), Cc19(1), Cc20(2), Cc23(1), Cc27*(3), Cc29(1), Cc30(1), Cc31(1)	Cc1*(1), Cc5(1), Cc26(1), Cc27*(4), Cc28(1), Cc35(1), Cc36(1), Cc37(2)	Cc8(1), Cc21(1), Cc25(1)	38
Campylobacter fetus	Cf1(1), Cf2(1), Cf3(1)				3
No. of different genotypes	24	23	24	3	68
Biodiversity Simpson's index	0.948	0.915	0.908	0.667	

* Genotyping profiles recovered from different origins

A total of four *fla*A-RFLP profiles grouped 18 isolates of *C. jejuni* and *C. coli*. The PFGE technique was able to differentiate these four profiles into 13. Taking into account the combination of the results obtained by both techniques, of the 68 genotypes detected in the dendrogram, 38 resulted for *C. coli*, 27 for *C. jejuni* and three for *C. fetus*. The main genotype (Cc27) included eight *C. coli* isolates followed by another one (Cc18) with four *C. coli* isolates. There were also two genotypes with three isolates, seven with two isolates, and the remaining 59 genotypes with a single isolate (Table 4).

A total of four genotypes clustered isolates from two or three origins. Three genotypes included *C. coli* isolates: genotype Cc1 and Cc27 with three and eight isolates, respectively, obtained from broiler faeces, broiler meat and human samples; and genotype Cc18 with four isolates obtained from broiler meat and human samples. The remaining genotype, Cj24, was composed by one *C. jejuni* isolate each from broiler faeces and human origin (Table 3).

3.3. Antimicrobial susceptibility testing

The *in vitro* antimicrobial susceptibility of the 89 isolates is shown in Table 3. Only six out of 89 *Campylobacter* isolates (6.7%) were susceptible to the three antimicrobial agents tested. Most of the *Campylobacter* isolates were resistant to ciprofloxacin (77 out of 89 isolates, 86.5%) and tetracycline (76 out of 89 isolates, 85.4%) and almost half of the isolates to erythromycin (44 out of 89 isolates, 49.4%). Only five out of 89 isolates (5.6%) exhibited resistance to a single antimicrobial agent.

Resistance to the three antibiotics was detected in 36 out of 89 isolates (40.4%), which were considered multidrug resistant: 11 human clinical isolates (12.3%), 13 isolates from broiler meat (14.6%) and 12 isolates from faeces samples (13.5%). Simultaneous

resistance to two antibiotics was observed in 42 out of 89 isolates (47.2%): 35 isolates were resistant to ciprofloxacin and tetracycline, four isolates to ciprofloxacin and erythromycin and three isolates to tetracycline and erythromycin. These isolates were obtained from samples of all origins: 13 out of 26 from human clinical samples (50%), 14 out of 29 broiler meat samples (48.3%) and 15 out of 34 broiler and swine faeces samples (44.1%)

The most resistant species was *C. coli*, as all the isolates were resistant to one or more antimicrobial agents. A total of 30 out of 55 isolates (54.5%) were multidrug resistant and were detected from all kind of samples (human, broiler meat and faeces samples) also maintaining this percentage per origin. Moreover, 18 out of 55 isolates (32.7%) were simultaneously resistant to ciprofloxacin and tetracycline.

C. jejuni isolates also showed high antibiotic resistance levels, with all the isolates resistant to any drug except for five of them (26 out of 31 isolates, 83.9%). Multidrug resistant isolates were detected from human and broiler faeces samples (6 out of 31 isolates, 19.4%); and isolates resistant to ciprofloxacin and tetracycline were observed in all the origins in which this species was present (16 out of 31 isolates, 51.6%).

Among the three *C. fetus* isolates, two (66.7%) were resistant to one or two antimicrobial agents and the remaining one was susceptible to the three antibioticS tested.

The antimicrobial susceptibility results did not modify the number of genetic profiles when those were taken into account in the fingerprinting analysis. However, two exceptions were observed in genotypes Cj24 and Cc18, which included isolates with different antimicrobial resistance profiles among them. The two isolates belonging to the genotype Cj24 from human and broiler faeces samples were multidrug resistant and

resistant to both ciprofloxacin and tetracycline, respectively. The three broiler meat isolates grouped into the genotype Cc18 were two of them resistant to tetracycline, and the other one resistant to erythromycin and tetracycline. The isolate from human sample also included in this genotype showed resistance to ciprofloxacin and tetracycline (Figure 1).

4. Discussion

Campylobacter is included in the global priority list of antibiotic resistant bacteria of the World Health Organization (WHO, 2017). Species of *C. jejuni* and *C. coli* among other *Campylobacter* thermophilic species colonize the intestines of most warm-blooded hosts including humans, and poultry meat is the most common transmission vehicle for human infection (Silva et al., 2011). Monitoring studies are essential to understand the diversity and prevalence of *Campylobacter* from farm to food chain that constitute the major potential risk to human health (Josefsen et al., 2015). Within this framework, the present study provides updated information on the genetic diversity and antimicrobial susceptibility of *Campylobacter* spp. isolates from different sources in northern Spain.

Campylobacter isolates were obtained from raw broiler samples and faecal droppings of broiler and swine and higher presence of the pathogen was observed in faecal droppings than in meat. *Campylobacter* species are very frequently isolated from broiler meat, being the skin on products the most contaminated ones (Davis and Connert, 2007; Guyard-Nicodeme et al., 2015; Stella et al., 2017). The reported *Campylobacter* prevalence in broiler meat is very variable worldwide. In Malaysia or Italy the values (26.6% and 34.1%, respectively) were lower compared with those detected in Czech Republic (56%), France (76%) or in Brazil (91.7%) (Bardoň et al., 2011; Guyard-Nicodeme et al., 2015; Sinulingga et al., 2019; Stella et al., 2017; Wurfel et al., 2019).

Positive *Campylobacter* percentage detected in the present study (35.4%) was very similar to that reported by EFSA from fresh broiler meat (37.5%) (EFSA 2019b). This prevalence was also in accordance with other recent study performed in the North of Spain (39.4%) (García-Sanchez et al., 2018). Moreover, these authors described the ability to form biofilm and other survival strategies of *Campylobacter* to persist longer in the environment and to resist packaging conditions in a modified atmosphere (García-Sanchez et al., 2018; García-Sanchez et al., 2019). In our study, similar percentages of *Campylobacter* were observed between the samples packed and unpacked in these conditions, which supports this idea of developing the ability to survive under unfavourable environmental conditions.

The study of faecal material from animal reservoirs going into the environment provides information of interest in understanding the environmental sources of *Campylobacter* infection, which is also important in order to prevent and control this pathogen (Asakura et al., 2019; Karikari et al., 2017; Kim et al., 2019; Torralbo et al., 2014). *Campylobacter* was detected in all except for one (85.7%) of the broiler farms analysed in the Basque country area. This data is slightly higher than that previously reported by Esteban et al. (2008) that isolated *Campylobacter* from the 70.6% of farms of Basque Country, and equal to that detected in a work performed in broiler farms from Malaysia, with percentages of 85.7% (Sinulingga et al., 2019). However, the prevalence of *Campylobacter* in the processed broiler faeces samples (62%) was in agreement with these last studies (Esteban et al., 2008; Sinulingga et al., 2019) and in southern Spain (Torralbo et al., 2014), but lower than the prevalence reported in Iran (80%) (Divsalar et al., 2019). In the single swine farm analysed, *Campylobacter* was isolated from faecal droppings, in the 42.8% of the samples processed. Although the last report of EFSA showed just an overall *Campylobacter* prevalence of 2% in swine faeces of EU (EFSA,

2019b), it is with cattle or sheep, other source of *Campylobacter* contamination in humans (Asakura et al., 2019). Similar value of prevalence (52.9%) was observed in a previous study carried out in the Basque Country area (Oporto et al., 2007). However, a variable *Campylobacter* prevalence was reported in other countries such as Ghana, Japan or Malaysia, 28.7%, 47.2% and 50.9%, respectively (Haruna et al., 2012; Karikari et al., 2017; Sinulingga et al., 2019).

In swine samples higher proportion of *C. coli* than *C. jejuni* was reported in several studies as well as in the present one (Asakura et al., 2019; Karikari et al., 2017; Oporto et al., 2007; Patrick et al., 2018). However, the presence of *C. jejuni* in broiler is more common than *C. coli* (Bardoň et al., 2011; Garcia-Sanchez et al., 2018; Patrick et al., 2018; Sinulingga et al., 2019; Torralbo et al., 2014) with reported percentages of 76.3% for *C. jejuni* and 23.5% for *C. coli* in broiler meat (EFSA 2019b). Although we detected higher presence of *C. jejuni* in broiler faeces samples, *C. coli* was the predominant species in broiler meat. Pezzotti et al. (2003) connected the high presence of *C. coli* in broilers with the higher antimicrobial resistance observed in *C. coli*, which can have a selective effect on *Campylobacter* population. However, and although other authors also detected a higher proportion of *C. coli* isolates in broiler meat from Italy (Pergola et al., 2017; Stella et al., 2017), or even in broiler faecal material from farms of Basque Country (Esteban et al., 2008), this higher proportion of *C. coli* isolates should be confirmed by further sampling. The reasons for the variation of the results among published studies have been attributed to differences in sampling or testing methods, since there is no standard method (Kim et al., 2019), and variations in geographical and seasonal factors, but above all, to sanitary conditions at farm and slaughterhouse level (Divsalar et al., 2019).

The improvement of different approaches to detect *Campylobacter* allows for the identification of *Campylobacter* species, distinct from *C. jejuni* and *C. coli*, as responsible of bacterial gastroenteritis in humans (Josefsen et al., 2015). Among the human isolates analysed in this study, *C. fetus* was identified besides *C. coli* and *C. jejuni*. The presence of this emerging species as foodborne pathogen was also detected in human samples (Patrick et al., 2018). The gastrointestinal tract of cattle and sheep is considered the primary reservoir of *C. fetus* (Escher et al., 2016; Wagenaar et al., 2014), although this species was also detected in poultry products (Sinulingga et al., 2019).

Fingerprinting analysis revealed a high heterogeneity. From the 89 isolates of *Campylobacter* spp. analysed, 68 genotypes were detected by the combination of PFGE and *fla*A-RFLP. However, the discriminatory power of both typing methods was different since PFGE allowed obtaining a highest number of different genotypes, 47 vs the 30 obtained by *fla*A-RFLP. This former technique was described as one the most useful methods for *Campylobacter* typing and for differentiating closely related isolates (Di Giannatale et al., 2019; Du et al., 2018; Garcia-Sanchez et al., 2018; Wurfel et al., 2019). *Fla*A-RFLP was also reported to give good discrimination for *Campylobacter* isolates, but as we detected in our findings, it is not specie specific (Behringer et al., 2011; Duarte et al., 2019; Elhadidy et al., 2019). Among the different genotypes obtained by the combination of the two techniques, four of them included isolates of different origins, overlapping human clinical isolates, broiler faeces and meat. The clustering of isolates from different origin as well as the prevalence of the same genotypes throughout the time was also reported by many other authors (Behringer et al., 2011; Duarte et al., 2019; Du et al., 2018). It was attributed to a possible random distribution of genotypes with no relationship to the source of the isolates (Behringer et al., 2011). However, studies in which MLST or *fla*A-approaches were applied indicated

that the spread and transfer of the bacteria between different sources could occur (Elhadidy et al., 2019; Wieczorek et al., 2019). It is worth noting that the transmission of isolates from farm and broiler meat to humans suggests a possible source of human infection. This incident could remark the importance to continue implementing the strategies to control *Campylobacter* all over the farm to fork process.

The community biodiversity indicated by Simpson index showed high values in human, broiler faeces and meat isolates. It suggests a notable diversity of *Campylobacter* isolates in these niches. As it has been previously described, *Campylobacter* is a genetically diverse microorganism and shows significant intraspecies biodiversity as a result of intra- and interspecies genetic recombination (Asakura et al., 2019; Duarte et al., 2019; Wieczorek et al., 2019; Wurfel et al., 2019).

The inclusion of antimicrobial resistance/susceptibility profiles did not modify the number of genotypes obtained by the fingerprinting analysis. However, several isolates clustered in the same genotype (Cj24 and Cc18 genotypes) showed different antimicrobial resistance profile, irrespective of its origin. An extremely high level of resistance to ciprofloxacin (86.5%) and tetracycline (85.4%) and a high level of resistance to erythromycin (49.4%) was detected in *Campylobacter* spp. isolates from different origins. It was more widespread in *C. coli* than in *C. jejuni*, being all *C. coli* isolates resistant to at least one antibiotic. An overall higher resistance in *C. coli* than in *C. jejuni* was also reported by other authors (Elhadidy et al., 2019; Han et al., 2019; Ocejo et al., 2019; Pergola et al., 2017). The resistance to three antibiotics simultaneously was the most common (40.4%) followed by resistance to ciprofloxacin and tetracycline that was also extremely high (>80%) for both *C. jejuni* and *C. coli*. The resistance percentages to these two antibiotics were in agreement with those reported in Spain; however, in the case of erythromycin, the resistance levels in this study were

higher (EFSA 2018a, 2019). Furthermore, resistance levels obtained in this study for the three antibiotics were higher than those reported in the Basque Country area (Ocejo et al., 2019; Oporto et al., 2009) and also in EU member states (EFSA, 2018a, 2019). However, awful percentages of resistant and multidrug resistant isolates were also reported in *Campylobacter* isolates from domesticated livestock such as poultry, ducks, cattle or sheep in Belgium (Elhadidy et al., 2019), China (Du et al., 2018; Han et al., 2019), Iran (Divsalar et al., 2019), Italy (Di Giannatale et al., 2019; Pergola et al., 2017) or Poland (Wieczorek et al., 2019).

Most of the studies regarding *Campylobacter* spp. antimicrobial resistance are focused on *C. jejuni*, and *C. coli* while the information about other *Campylobacter* species is poorly reported. In this study, two of the three *C. fetus* isolates showed resistance to at least one antibiotic. One of them resulted resistant to erythromycin while the other showed combined resistance to ciprofloxacin and tetracycline. Isolates of *C. fetus* from human patients in Belgium were susceptible to ciprofloxacin, erythromycin and tetracycline (Vandenberg et al., 2006). However, this specie showed low resistance to ciprofloxacin and erythromycin (0.6% and 0.1% respectively) but resistance to tetracycline reached an alarming 39% in beef cattle isolates from Canada (Inglis et al., 2006). Furthermore, in the past few years, a worrying increase of *C. fetus* resistance to fluoroquinolones has been observed in France, reaching a high resistance level of 26.9% (Benejat et al., 2018). Tetracycline and aminoglycoside resistance genes within a transferable pathogenicity island were identified in this specie (Abril et al., 2010). Even though this species is only isolated in the 0.1% of the cases (EFSA, 2019b), the worldwide high incidence of campylobacteriosis suggests that *C. fetus* infections are not uncommon and may be a threat to public health. Moreover, this infection is most commonly produced in patients with immunodeficiency problems or in those with

underlying diseases (Wagenaar et al., 2014). In these cases, treatments with antimicrobials are often followed, which could also spread antimicrobial resistance among *C, fetus* isolates. Thereby the propagation of antimicrobial resistant *C. fetus* is a matter that should be further studied.

The unregulated and widespread use of antibiotics in the growth promotion, disease prevention or the treatment of infections in both food producing animals and humans, has promoted the selection and spread of antimicrobial-resistant *Campylobacter* isolates (Divsalar et al., 2019; Han et al., 2019; Karikari et al., 2017; Pergola et al., 2017; Wieczorek et al., 2019). Moreover, tetracycline resistance has been reported to persist for years even in the absence of antibiotic selection pressure (Luangtongkum et al., 2009). Our finding shows a very worrying scheme of antimicrobial resistance of *Campylobacter* spp. in the Basque Country which is especially concerning because these antibiotics are the drugs of choice for campylobacteriosis treatment. Due to the high occurrence of acquired antimicrobial resistances in *Campylobacter* however, these drugs could not be effective in an early future for the treatment of *Campylobacter* infections in countries such as Spain. This critical situation highlights that although several strategies have been employed for reducing the presence of *Campylobacter,* it is mandatory to develop new alternative tools for the control of this pathogen within the field of food industry, mainly in poultry processing plants since poultry and poultry products are considered the most important sources of human infections.

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CAPITULO 3 SUJETO A CONFIDENCIALIDAD POR EL AUTOR

Study 4

Promising *Campylobacter* specific bacteriophage candidates for biocontrol applications

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Abstract

This work characterized ten *Campylobacter*-specific bacteriophages in order to study their suitability as biocontrol agents in the poultry production chain. Bacteriophage susceptible *Campylobacter* strains included antibiotic resistant and multi-resistant strains from different countries, Spain, Italy and Poland. Host range analyses found a high host specificity and similar lytic spectrum among the five campylophages of each group. Group II campylophages infected strains of *C. jejuni*, *C. coli* and *C. fetus* species, whereas the lytic activity of group III campylophages was restricted to *C. jejuni* species. All bacteriophages remained stable and active at ranges of temperatures from 4 to 42 °C and pH values from 2 to 9. Campylophages of both groups showed complementary host ranges, so, their combination in a cocktail could achieve a collective broader lytic spectrum. Considering the results of host range, one step growth experiments and stability assays five *Campylobacter* specific bacteriophages were selected for its high potential as biocontrol agents.

Keywords: phage-therapy, biocontrol, lytic spectrum, stability, cocktail, burst size, latent period

1. Introduction

Campylobacter is the main zoonotic pathogen, responsible for almost the 70% of all the reported zoonotic cases in the European Union (UE) and, despite its low fatality rate (0.03%), is the fourth most common mortality cause among the zoonotic pathogens (EFSA, 2019a). *Campylobacter jejuni* (83.9%) is the major etiological agent of campylobacteriosis, followed by *Campylobacter coli* (10.3%) (European Food Safety Authority (EFSA, 2019a)) and species such as *Campylobacter fetus*, *Campylobacter upsaliensis* or *Campylobacter lari* can also be isolated from human (Patrick et al., 2018; Sinulingga et al., 2019).

Poultry is considered the natural reservoir of *Campylobacter*, and raw and undercooked chicken the major source of human campylobacteriosis (EFSA, 2011; Silva et al., 2011). *Campylobacter* spp. are commensals in the avian gut at farm level and the posterior cross contamination of carcasses at the slaughterhouse often leads to contamination of poultry meat products at retail (Furuta et al., 2017). Significant advancement has been made during the past years in the research and development of pre- and post-harvest intervention strategies of *Campylobacter* for the Poultry Sector (Hansson et al., 2018; Meunier et al., 2016; Wagenaar et al., 2013). At farm level, the application of strict biosecurity measures and hygienic practices, drinking water treatment, utilization of feed additives, vaccination or the use of pre-/pro-biotics and other antimicrobial alternatives have not entirely solved the problem (Hermans et al., 2011; Umar et al., 2016). At slaughter, dressing and processing level, the separation of *Campylobacter*-positive from negative flocks has helped to control the spread of contamination of carcasses (Silva et al., 2011). However, the use of physical and chemical decontamination processes, such us scalding or chilling of carcasses (negative

effect on organoleptic properties of meat products) and the use of chlorine compounds or chlorine-based antimicrobials (not authorized in the EU) have neither been the solution (Osimani et al., 2017). Moreover, the development and spread of antimicrobial resistant *Campylobacter* throughout the world has become a serious threat to public health. Several studies have reported a warrying increase of *C. jejuni* and *C. coli* resistance to erythromycin and ciprofloxacin, the first- and second- choice drugs to treat campylobacteriosis among others (Divsalar et al., 2019; Elhadidy et al., 2019; Han et al., 2019; Wieczorek et al., 2019). It is, therefore, critical to develop innovative non-antibiotic based strategies to reduce the burden of *Campylobacter* contamination within the farm-to-fork.

The application of specific *Campylobacter* bacteriophages, also called campylophages, is highly promising as a complement to currently existing measures (Atterbury et al., 2003; Carvalho et al., 2010; El-Shibiny et al., 2009; Fischer et al., 2013). Bacteriophages are viruses that specifically infect target bacteria, widely distributed in the environment and often consumed in our diet as they are present in the natural microbiota of several food products, including poultry products (Atterbury et al., 2003; Connerton et al., 2011; Tsuei et al., 2007). The use of bacteriophages as a food safety strategy is desirable as they are eco-friendly antimicrobials, specific towards the pathogen of concern, without affecting humans, animals, plants or the existing commensal microbiota, and they do neither alter food organoleptic properties. Other positive attributes of bacteriophages include their natural origin, their ability to self-replication and their effectiveness against antibiotic-resistant bacteria. However, bacteriophages intended to be used for biocontrol purposes need to fulfill a number of requirements. They should ideally have the broadest host range as possible against the target bacteria (Merabishvili et al., 2018). In this regard, campylophages have been

generally reported as highly specific with a narrow host range (Hammerl et al., 2014; Hwang et al., 2009). Therefore, the combination of different complementary campylophages in cocktails should be considered as the best approach to broaden the lytic spectrum against target *Campylobacter*. Candidate bacteriophages should also have high target pathogen clearance rates, displaying short latent period (time from bacteriophage entry into the bacteria until the first progeny is released), large burst size (number of newly synthesized bacteriophage particles from an infected bacterium) and even short rise period (the time over which a simultaneously infected population of bacteria lyse) (Merabishvili et al., 2018). Finally, biocontrol bacteriophages should also be safe, being free from genes involved in lysogeny or encoding for toxins, virulence factors and/or antibiotic-resistance. Good candidates should also be stable and remain infective not only during the production and storage stages, but also during their application (Fernandez et al., 2019).

In this study, ten *Campylobacter* specific bacteriophages, previously isolated from Northern Spain, were characterized in order to select the most promising candidates for their use in a cocktail as pre- and post-harvest biocontrol agents in poultry. For this purpose, host range analysis against several strains of different *Campylobacter* species from Spain, Italy and Poland were analyzed. Moreover, their one step growth curve as well as their stability and effectiveness under different pH and temperature conditions were also tested.

2. Materials and methods

2.1. *Campylobacter* strains and growth conditions

Campylobacter strains used in this study were selected from the C-SNIPER Strain Collection established within the EIT-Food C-SNIPER project (Table 1). The 110 strains were stored at -80 ºC in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) supplemented with 20% glycerol. For their culture, strains were plated onto Columbia blood agar with 5% (vol/vol) defibrinated sheep blood plates (Oxoid) and incubated at 37 °C overnight, under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) using an INVIVO$_2$ 400 hypoxia workstation (Ruskinn Technology Ltd, Bridgend, UK).

Table 1. *Campylobacter* strains used in the host-range analysis.

***Campylobacter* species**	**No. of strains from different origins***			
	SP	**IT**	**PO**	**Total**
Campylobacter jejuni	24	26	35	**85**
Campylobacter coli	15	0	5	**20**
Campylobacter lari	1	2	0	**3**
Campylobacter fetus	1	0	0	**1**
***Campylobacter* spp.**	0	1	0	**1**
Total	**41**	**29**	**40**	**110**

* Strains provided by C-SNIPER project partners: AZTI, Spain (SP), University of Torino, Italy (IT) and Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Poland (PO).

For *Campylobacter* lawns preparation bacterial cells were harvested in BHI broth to an OD_{600} of 0.6 (approximately 10^8 CFU/ml), cation supplemented with 1 mM $CaCl_2$ and 10 mM $MgSO_4$ and incubated at 37 °C for 4 h under microaerobic conditions. Then,

600 µl of exponential growth phase cultures were added to four ml of molten NZCYM soft agar (NZCYM broth (Pronadisa, Conda Laboratories, Madrid, Spain) supplemented with 0.7% Bacteriological Agar (Oxoid) tempered at 50 ºC and poured onto NZCYM hard agar plates (NZCYM broth with 1.2% agar).

2.2. rep-PCR typing of *Campylobacter*

Fingerprinting analysis by the repetitive element sequence-based PCR (rep-PCR) was performed with bacterial DNA extracted using the commercial DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The DNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then, DNA amplification reactions were carried out with the primer $(GTG)_5$ as described by (Markiewicz et al., 2010). Briefly, reaction mixtures consisting of 2 µl of 10x reaction buffer supplied with 0.5 U of Taq DNA polymerase (Fermentas, Vilnus, Lithuania), 2.5 mM $MgCl_2$, 0.5 µM of the primer, 250 µM of dNTP mix, 1.0 µL of DNA and PCR water up to 20 µl were prepared. Amplification reactions were performed in a TProffesional thermocycler (Biometra GmbH, Göttingen, Germany) in following conditions: an initial step of denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 42 °C for 30 s and extension at 72 °C for 30 s; and a final step of extension of 4 min at 72 °C. Amplification products were separated in GelRed (Biotium, Fremont, CA, USA) stained 1.5 % agarose gel in 0.5x TBE buffer at 80 V for 1 h 20 min. Gels were photographed with a GelDoc™ EZ Imager (Bio-Rad) and phylogenetic relationships determined with a temporary BioNumerics software version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Profiles with a similarity above a 95% were considered identical (Markiewicz et al., 2010).

2.3. Antimicrobial susceptibility testing of *Campylobacter*

Antimicrobial susceptibility testing of the all *Campylobacter* strains was performed using the disk diffusion susceptibility method, following the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019). Three antibiotics were tested: ciprofloxacin (5 μg), erythromycin (15 μg) and tetracycline (30 μg) (Oxoid).

2.4. Bacteriophages, propagation and titration

Ten *Campylobacter* bacteriophages previously isolated, purified and conserved as detailed in studies 1 and 3 were used in this work (Table 2). They were selected from a panel of 304 campylophages based on their lytic spectra, genome size and genetic diversity (study 3).

Propagation of bacteriophages was performed by the double agar layer method on their corresponding host *Campylobacter* strains (Table 2). Briefly, 600 µl of exponential phase bacterial cultures were supplemented with 1 mM $CaCl_2$ and 10 mM $MgSO_4$ and mixed with 400 µl bacteriophage lysate suspension. After 15 minutes incubation at 37 °C this mixture was individually added to 4 ml molten NZCYM soft agar previously tempered at 50 ºC and poured onto NZCYM hard agar plates. Plates were dried for 15 minutes before overnight incubation at 37 ºC under microaerobic conditions. New bacteriophages were recovered from plates presenting confluent lysis by adding 5 ml of SM buffer and incubating at 4 ºC for 24 h with 250 rpm orbital shaking. Bacteriophages in SM buffer were then collected in sterile tubes, treated with 10% chloroform and kept at 4 ºC until use. Bacteriophage lysates titer was determined by spotting 20 µl of serially

diluted suspensions onto bacterial lawns of the corresponding hosts on NZCYM soft agar overlay plates.

Table 2. *Campylobacter* specific bacteriophages used in this study.

Group	Campylophage code	Genotype[a]	Origin	Genome size[b] (kb)	Propagative host strain
II	CAM304	II-1	Swine faeces	180	CCO 059
	CAM302	II-5	Swine faeces	180	CCO 059
	CAM297	II-2	Swine faeces	180	CCO 059
	CAM296	II-1	Swine faeces	180	CCO 059
	CAM289	II-1	Swine faeces	180	CCO 059
III	CAM165	III-2	Broiler skin	140	CJE 079
	CAM79	III-1	Broiler skin	140	CJE 079
	CAM62	III-1	Broiler skin	140	CJE 079
	CAM30	III-3	Broiler skin	140	CJE 079
	CAM26	III-2	Broiler skin	140	CJE 079

[a] Genotype determined by *Smi*I-RFLP and RAPD-PCR for group II campylophages and by *Hha*I-RFLP for group III campylophages (study 3).
[b] Genome sizes determined by PFGE.

2.5. Host range analysis

To determine the host range, bacteriophage suspensions of 10^6 PFU/ml were prepared, and tenfold serial dilutions performed in SM buffer. A volume of 10 µl of each dilution was spotted onto bacterial lawns of the different 110 target *Campylobacter* strains (Table 1). After allowing the plates to dry for 10 min at room temperature, they were incubated at 37 °C overnight under microaerobic conditions. Then, plates were checked for the appearance of single plaques in the spotting zones.

2.6. One step growth curve

Single-step growth experiments were performed in order to assess the latent period, rise period and burst size of the bacteriophages in a single round of replication. A wild type strain of *C. jejuni* (CJE 079) and *C. coli* (CCO 059), isolated from broiler faecal droppings and meat respectively, were used as propagative hosts of the bacteriophages

(Table 2). Host cells were grown to early exponential phase (OD_{600} nm = 0.4) in 8 ml BHI broth and incubated with shaking at 37 °C for 4 h under microaerobic conditions. They were then infected with the bacteriophage at a multiplicity of infection (MOI) of 0.001. Samples were taken every 15 min for 4 h and the titer determined immediately by the spot test method in NZCYM agar. The burst size was determined by dividing the number of lysis plaques at the stationary phase by the number of plaques at the latent phase (Silva et al., 2014).

2.7. pH and thermal stability

The stability of *Campylobacter* bacteriophages was investigated at different pH values (2-9) and temperatures (4 °C - 70 °C) (Table 3). After propagation, bacteriophages were harvested in SM buffer previously adjusted to the different pH values, 2, 3.5, 5.5 and 9. For thermal stability testing, bacteriophage solutions of the different pH values were maintained at the corresponding temperatures. Thereafter, aliquots of the buffered lysates were taken at different times and were serially diluted to determine the bacteriophage titer by spotting appropriate dilutions onto NZCYM bacterial lawns of the corresponding host strain.

Table 3. Conditions tested in the stability assay of *Campylobacter* specific bacteriophages.

pH	Temperature (°C)			
	4	**22**	**42**	**70**
2.0	√		√	
3.5	√		√	
5.5	√		√	
7.5	√	√	√	√
9.0	√		√	

3. Results

3.1. Host range analysis

For the host range analysis of the ten *Campylobacter* specific bacteriophages belonging to campylophage groups II (n=5) and III (n=5), 110 *Campylobacter* strains of *C. jejuni*, *C. coli*, *C. lari* and *C. fetus* recovered from Spain, Italy and Poland were used.

As shown in Figure 1, the rep-PCR fingerprinting analysis distinguished 109 different genotypes from the total of 110 analyzed strains. Moreover, the results of the 41 Spanish strains were comparable to those previously revealed by *fla*A-RFLP/PFGE fingerprinting (study 2).

Concerning their antibiotic resistance profiles (Table 4 and Figure 1), 18 out of 110 *Campylobacter* strains (16.4%) were susceptible to the three antimicrobial agents tested. Most of the *Campylobacter* strains were resistant to ciprofloxacin (76 out of 110 strains, 69.1%) and tetracycline (79 out of 110 strains, 71.8%) and almost half of the strains to erythromycin (50 out of 110 strains, 45.5%), with high levels of multidrug resistance (32.7%). Interestingly, Italian strains showed the highest levels of multidrug resistance (18/29, 62.1%) followed by Spanish (14/41, 34.1%) and Polish (4/40, 10.0%) strains. Similarly, only the 6.9% (2/29) of Italian strains were susceptible to all antibiotics, in contrast to the 17.1 % (7/41) and the 22.5% (9/40) of Spanish and Polish strains.

Table 4. In vitro antimicrobial activity of ciprofloxacin (CIP), erythromycin (E) and tetracycline (TE) against the 110 *Campylobacter* strains.

***Campylobacter* strains from different origins**	**No. of strains resistant**							**No, of strains susceptible**
	CIP	**E**	**TE**	**CIP+E**	**CIP+TE**	**E+TE**	**CIP+E+TE**	
Spain (n=41)	2	0	0	2	16	0	14	7
Italy (n=29)	0	2	0	0	0	7	18	2
Poland (n=40)	6	1	4	0	14	2	4	9
Total (n=110)	8	3	4	2	30	9	36	18
Percentage	7.3%	2.7%	3.6%	1.8%	27.3%	8.2%	32.7%	16.4%

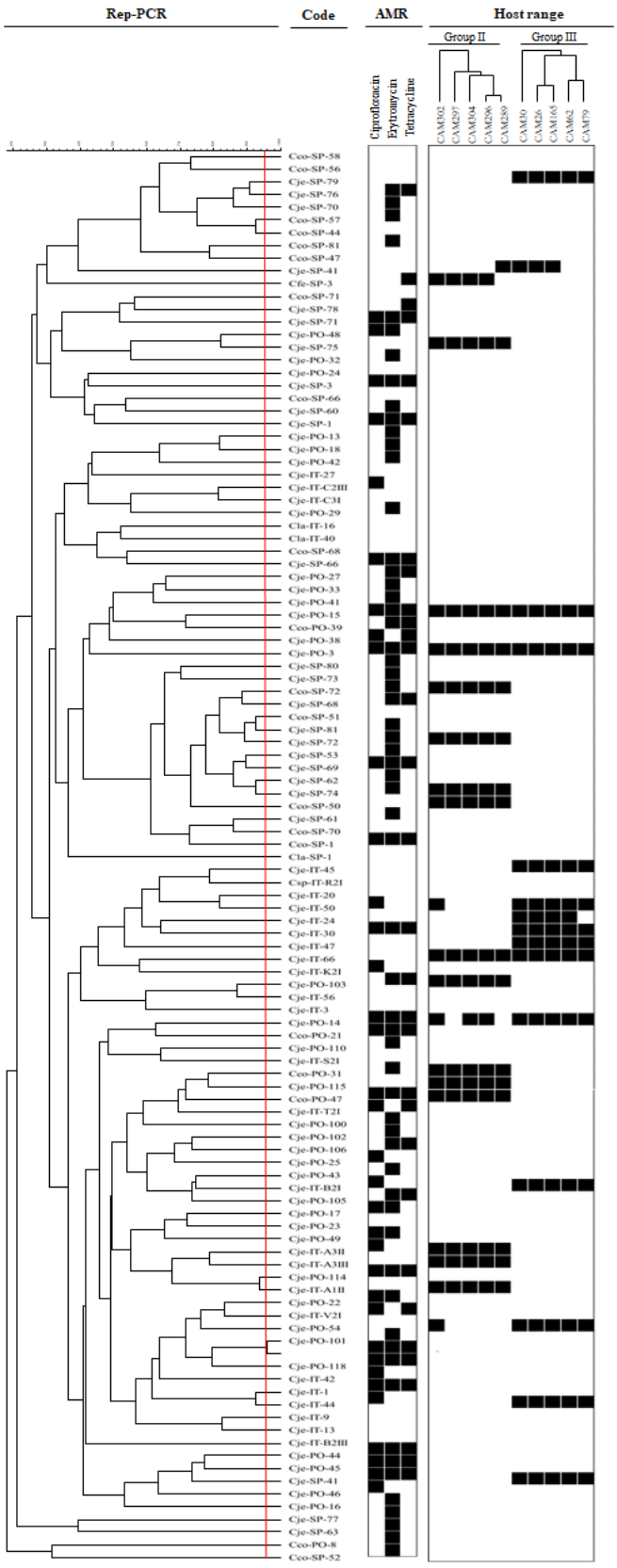

Figure 1. Host range analysis of the ten *Campylobacter* specific bacteriophages against the 110 *Campylobacter* strains.

Campylobacter susceptibility to the ten bacteriophages is shown in Table 5 and Figure All group II campylophages showed a similar host range by lysing between 11 and 14 out of 85 (12.9-16.5%) *C. jejuni* and four out of 20 (20.0%) *C. coli* strains. Four campylophages of the group II also infected a *C. fetus* strain but none of them infected any *C. lari* strain. Group III campylophages also showed a similar host range among them but different to that shown by group II campylophages. They infected between 13 and 15 out of 85 (15.3-17.6%) *C. jejuni* strains. Other *Campylobacter* species were not infected.

Concerning the bacteriophage-susceptibility of *Campylobacter* strains, only 28 out of the 110 (25.5%) were susceptible to bacteriophage infection, including 23 out of 85 (27.1%) *C. jejuni*, four out of 20 (20.0%) *C. coli* and the only *C. fetus* strains (100.0%). A total of seven *C. jejuni* strains resulted sensitive to both campylophages groups, finding that three of them were lysed by all the characterized bacteriophages (Figure 1). Similarly, the four bacteriophage-sensitive *C. coli* strains were lysed by all the group II campylophages.

As shown in Table 6, near the half of the bacteriophage-sensitive strains (12 out of 28, 42.9%) were multidrug resistant, showing resistance to the three different antimicrobial agents (ECDC/EFSA/EMA, 2017). Nine out of 28 (32.1%) were resistant to two antimicrobials and other one (3.6%) exhibited resistance to a single antimicrobial agent. By contrast, only six out of the 28 (21.4%) bacteriophage-sensitive strains were also sensitive to the three antibiotics.

Regarding the geographical origin, Italian strains were the most bacteriophage-sensitive ones being the 37.9% (11/29) infected by at least one campylophage, while Spanish and Polish strains showed similar bacteriophage-sensitivity with of the 21.9% (9/41) and 20% (8/40) bacteriophage sensitive strains, respectively.

3.2. One step growth curve

Figure 2 shows the one step growth curves obtained for each *Campylobacter* bacteriophage. All group II campylophages started a second replication cycle at 2.25-3.5 h of experiment, being CAM304 the first bacteriophage to start that second cycle (135 min). Group III campylophages on the contrary, do not start a second cycle of replication in the time the experiment lasts as, since the end of the rise period until the end of the experiment, bacteriophage titer remains almost constant.

As shown in Table 7, latent periods from 45 to 60 min, burst sizes of 16 to 64 PFU and rise periods of 60 to 90 min were observed for group II campylophages. Bacteriophage CAM296 showed the shortest latent period (45 min) and largest burst size (64 PFU) with an intermediate rise period value of 75 min. CAM297 on the other hand, was the one with longer latent period (60 min) and shorter burst size (16 PFU), with the same rise period. Group III campylophages showed slightly shorter latent periods (30 to 45 min), larger rise periods (75 to 135 min) and overall smaller burst sizes (nine to 24 PFU) than those of group II. In this case, campylophage CAM165 showed both the shortest latent period (30 min) and the largest burst size (24 PFU) with an intermediate rise period of 90 min.

Table 5. Activity of *Campylobacter* specific bacteriophages against the 110 *Campylobacter* strains.

Campylobacter **species from different origins**	**Group II campylophages**					**Group III campylophages**					**Total**
	CAM289	**CAM296**	**CAM297**	**CAM302**	**CAM304**	**CAM26**	**CAM30**	**CAM62**	**CAM79**	**CAM165**	
Campylobacter jejuni **(n=85)**	12	11	12	14	12	15	15	14	13	15	23
Percentage	14.1%	12.9%	14.1%	16.5%	14.1%	17.6%	17.6%	16.5%	15.3%	17.6%	27.1%
Positive: Spain (n=24)	4	3	3	3	3	3	3	2	2	3	6 (25.0%)
Italy (n=26)	4	4	4	5	4	8	8	8	7	8	11 (42.3%)
Poland (n=35)	4	4	5	6	5	4	4	4	4	4	6 (17.1%)
Campylobacter coli **(n= 20)**	4	4	4	4	4	0	0	0	0	0	4
Percentage	20.0%	20.0%	20.0%	20.0%	20.0%	0%	0%	0%	0%	0%	20.0%
Positive: Spain (n=15)	2	2	2	2	2	0	0	0	0	0	2 (13.3%)
Poland (n=5)	2	2	2	2	2	0	0	0	0	0	2 (40.0%)
Campylobacter fetus **(n= 1)**	0	1	1	1	1	0	0	0	0	0	1
Percentage	0%	100%	100%	100%	100%	0%	0%	0%	0%	0%	100%
Positive: Spain (n=1)	0	1	1	1	1	0	0	0	0	0	1 (100%)
Campylobacter lari **(n= 3)**	0	0	0	0	0	0	0	0	0	0	0
Percentage	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Positive: Spain (n=1)	0	0	0	0	0	0	0	0	0	0	0 (0.0%)
Italy (n=2)	0	0	0	0	0	0	0	0	0	0	0 (0.0%)
Campylobacter **spp. (n= 1)**	0	0	0	0	0	0	0	0	0	0	0
Percentage	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Positive: Italy (n=1)	0	0	0	0	0	0	0	0	0	0	0 (0.0%)
Total positive (n=110)	16	16	17	19	17	15	15	14	13	15	28
Percentage	14.5%	14.5%	15.5%	17.2%	15.5%	12.8%	12.8%	12.0%	11.1%	12.8%	25.5%

Table 6. In vitro antimicrobial activity of ciprofloxacin (CIP), erythromycin (E) and tetracycline (TE) against the bacteriophage-sensitive *Campylobacter* strains.

***Campylobacter* strains from different origins**	**No. of strains resistant**							**No, of strains susceptible**
	CIP	**E**	**TE**	**CIP+E**	**CIP+TE**	**E+TE**	**CIP+E+TE**	
Spain (n=9)	0	0	0	1	3	0	4	1
Italy (n=11)	0	0	0	0	0	4	6	1
Poland (n=8)	1	0	0	0	1	0	2	4
Total (n=28)	1	0	0	1	4	4	12	6
Percentage	3.6%	0.0%	0.0%	3.6%	14.3%	14.3%	42.9%	21.4%

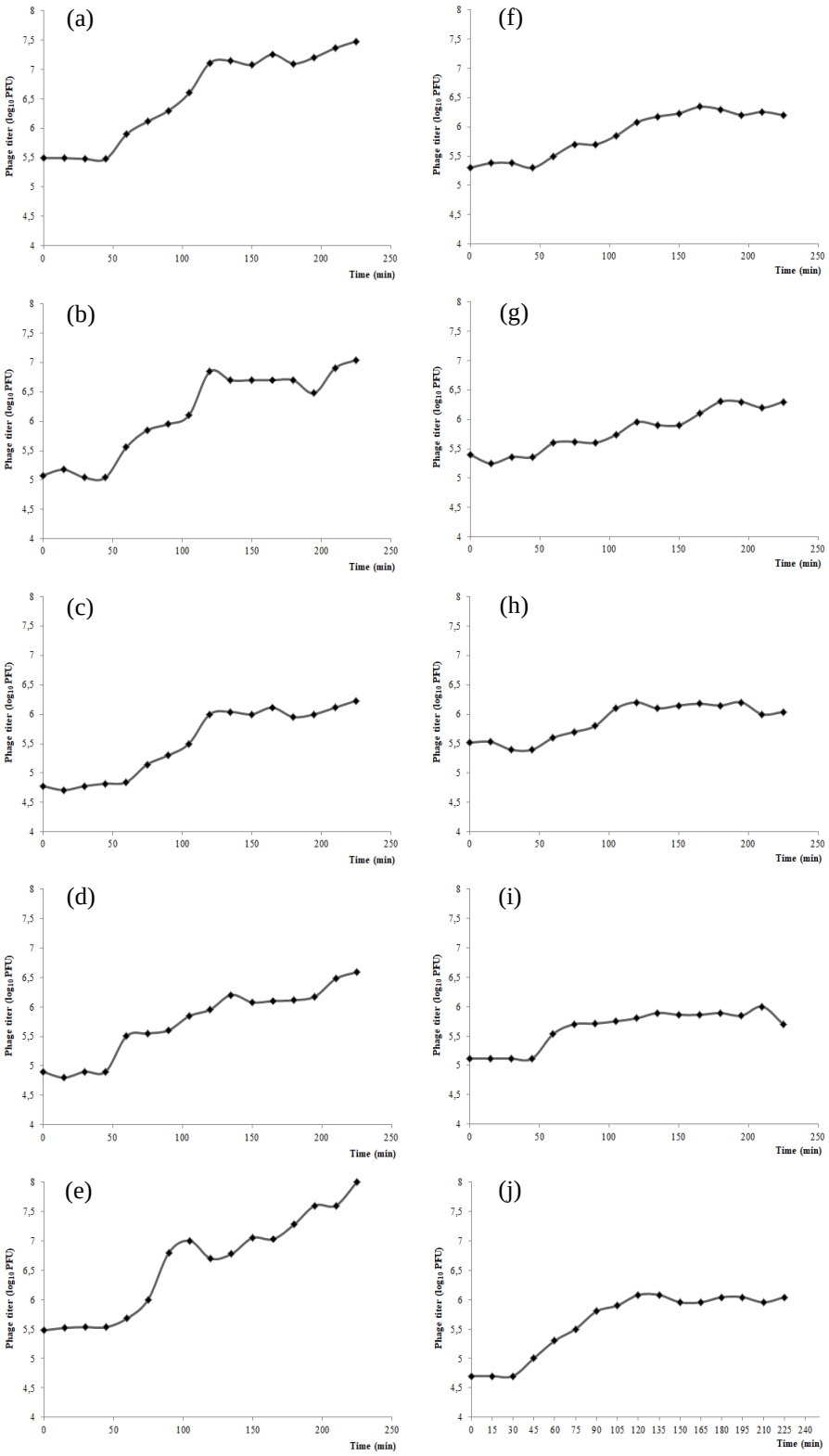

Figure 2. One step growth curves of *Campylobacter* specific bacteriophages of group II: CAM289 (a), CAM296 (b), CAM297 (c), CAM302 (d), CAM304 (e); and group III: CAM26 (f), CAM30 (g), CAM62 (h), CAM79 (i), CAM165 (j).

Table 7. Summary of the results of the one step growth curve experiments.

Group	Campylophage code	Genotype[a]	Latent period (min)	Burst size (PFU)	Rise period (min)
II	CAM304	II-1	45	11	120
	CAM302	II-5	45	9	135
	CAM297	II-2	45	6	75
	CAM296	II-1	45	6	90
	CAM289	II-1	30	24	90
III	CAM165	III-2	45	43	75
	CAM79	III-1	45	64	75
	CAM62	III-1	60	16	75
	CAM30	III-3	45	20	90
	CAM26	III-2	45	29	60

3.3. Stability assays

Monitoring the stability of tested bacteriophages during their storage at temperatures between 4 °C and 70 °C for 35 days revealed that, independently of their group, all of them presented similar behavior. As shown in figure 3, both group II CAM296 and group III CAM165 bacteriophages remained stable and active for at least 35 days of storage at 4 °C and 22 °C. By contrast, under storage at 42 °C, these campylophages maintained stable for 14 days but showed a great drop in phage titers after 35 days storage. Specifically, reductions from 3 to 3.5 and from 4 up to 8 $\log_{10}$ units were found for group II and group III campylophages, respectively (data not shown). At 70 °C, no bacteriophage from none of the groups was detected after 24 h of storage.

Regarding their stability under different pH conditions, all ten bacteriophages remained stable and active at pH values from 2 to 9 for, at least, 35 days of storage at 4 °C and for 14 days at 42 °C.

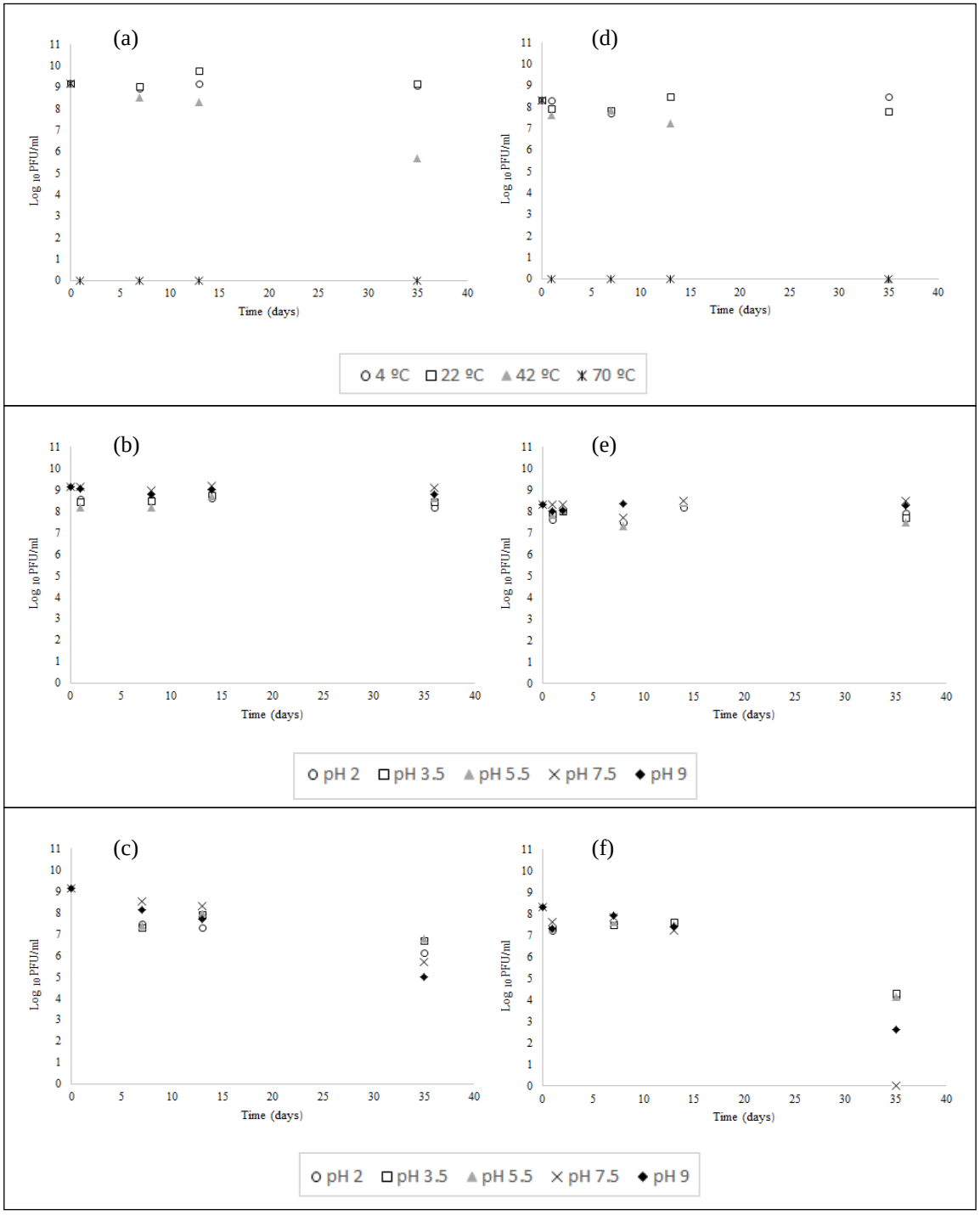

Figure 3. Temperature and pH stability of *Campylobacter* specific bacteriophages of group II CAM296 (a, b, c) and group III CAM165 (d, e, f). Temperature stability at pH 7.5 (a, c). pH stability at 4 ºC (b, d). pH stability at 42 ºC (c, f).

4. Discussion

The application of *Campylobacter*-specific bacteriophages has been proposed as an alternative natural intervention strategy to reduce the prevalence of this pathogen at both pre- and post-harvest stages of poultry production (EFSA, 2017). However, bacteriophages intended to be used for bacteriophage therapy and biocontrol applications need to fulfill several requirements. The present study characterized a selection of ten previously isolated *Campylobacter* specific bacteriophages in order to assess their potential as biocontrol agents.

All ten campylophages showed narrow individual host ranges that did not exceed the 20% of tested *Campylobacter* strains. These results are in concordance with the very high host specificity previously reported for other *Campylobacter* bacteriophages (El-Shibiny et al., 2009; Hammerl et al., 2014; Hwang et al., 2009). However, since good candidate bacteriophages should be able to infect a wide range of the target bacteria (Merabishvili et al., 2018), a mixture of different campylophages should be considered in order to achieve a complementary and collective broader lytic spectrum. The best combination of the bacteriophages characterized in this study would hypothetically cover the 25.5% of the *Campylobacter* strains tested (28 out of 110), including 27.1% of *C. jejuni*, 20% of *C. coli* and 100% of *C. fetus* strains. Even if this last species is only isolated in the 0.1% of the campylobacteriosis cases (EFSA, 2019a), the inclusion of campylophages able to lyse *C. fetus* strains in a cocktail could be of interest as it is also a relevant human and animal pathogen. It is worth noting that in combinations, bacteriophages could interfere with each other during the coinfection, causing additive or even synergetic or antagonistic effects (Merabishvili et al., 2018). Consequently, the affinity of bacteriophages in cocktails should be tested by facing them against the

Campylobacter strains panel. In addition to their complementary host range, the combination of group II and III campylophages in a cocktail could reduce or avoid the development of *Campylobacter* resistance since each campylophage group binds to different receptors on the bacterial surface (Coward et al., 2006; Jackel et al., 2019). Fernandez et al. (2019) stated that bacteriophages from a given reservoir or geographic location could be more effective at targeting strains from the same place. To our knowledge, this is the first study using a panel of environmental *Campylobacter* strains from different countries. Used *Campylobacter* strains were concretely isolated in Spain, Italy and Poland, considered among the ten leading EU member countries in poultry and broiler meat production and responsible of the 36% (10.7, 8.5 and 16.8%, respectively) of the total 15.2 million tons produced in the EU in 2018 (European Comission, 2019). Very interestingly, host range analyses showed that all ten campylophages were able to infect *Campylobacter* strains from all geographical areas, including locations near and far from the Basque Country (North of Spain), area in which campylophages were isolated. In fact, all bacteriophages showed even broader host range against Italian *C. jejuni* strains than against Spanish ones. These results make these campylophages suitable candidates for preparation of a bacteriophage cocktail to combat *Campylobacter* isolates from different geographical origins.

The antimicrobial resistance results shown in this study highlights the serious threat to public health of the antibiotic-resistant *Campylobacter* strains many times reported by EFSA (EFSA, 2019b). In fact, only the 16.8% of the strains were susceptible to the three antibiotics (17.1, 6.9 and 22.5% of the Spanish, Italian and Polish strains respectively), whereas a 32.7% of them resulted multi-resistant. In the current work, as shown by other authors (Aprea et al., 2018; Janez et al., 2014) antimicrobial resistance did not correlate with bacteriophage resistance and the tested bacteriophages showed

potential to infect *Campylobacter* strains resistant to the three antimicrobials used for campylobacteriosis treatment (erythromycin, ciprofloxacin and tetracycline). In fact, Italian strains showed the highest levels of antibiotic multi-resistance (62.1%) and bacteriophage susceptibility (37.9%), whereas Polish strains, on the contrary were the less antibiotic multi-resistant (10%) and also the less bacteriophage susceptible ones. Finally, Spanish strains showed intermediate values for both antibiotic and bacteriophage susceptibility.

The latent period, burst size and rise period are important factors to consider for bacteriophage selection (Abedon et al., 2001; Jariah and Hakim, 2019; Merabishvili et al., 2018; Sinha et al., 2018). Bacteriophages with short latent periods and large burst sizes are more effective inactivating the target bacteria, but usually large burst sizes are associated with long latent periods, making the selection more difficult (Silva et al., 2014). Few studies reporting the kinetics of *Campylobacter* bacteriophages are available. Carvalho et al. (2010) reported latent periods and burst sizes of 52.5-82.5 min and 9-24 PFU for group II campylophages. Similar latent periods were later reported by Hammerl et al. (2014) for both group II and III campylophages (67-82 min). Concerning the burst size, the latter showed higher values for group II campylophages than for those of group III (14 to 20 vs 4-7 PFU). Overall, the current work displays shorter latent periods and larger burst sizes for both group campylophages than those previously reported. As shown by Hammerl et al. (2014), the latent period of both groups was similar but the burst sizes were longer in group II campylophages.

Good candidate campylophages should also be stable and remain infective during the production, storage and intended administration conditions, being able to reach and infect their target bacteria (Fernandez et al., 2019). Bacteriophage production is usually carried out through 24 to 72 hours processes under the optimal temperature and pH

conditions for both bacterial growth and bacteriophage infection (Garcia et al., 2019). Thus, campylophages characterized in the present study would be adequate for their efficient production at industrial scale, since they have been proved to be stable at 42 ºC and neutral pH values for at least 14 days.

After their production, campylophages should also have a good stability under normal storage conditions at farm level (under refrigeration or at room temperature) as well as at slaughter, dressing and processing level (under refrigeration: 4 ºC). The campylophages characterized in the present study have been proved to be stable and fully active at temperature ranges from 4 to 22 ºC for at least 1 month, suggesting their good shelf life.

Finally, campylophages should be stable and remain effective during their application conditions. All the bacteriophages characterized in this study could be suitable for pre-harvest applications (e.g. in live animals administered orally or via animal feed or drinking water), as they are able to withstand the pH of the whole digestive system of poultry birds, including those acid proventriculus and gizzard pH (2.0-3.5), as well as their body temperature (42 ºC) for at least 14 days, long enough to reach their target host. Moreover, this shown high pH resistance suggests that they could be applied directly without the need of encapsulation or $CaCO_3$ administration, as recommended by El-Shibiny et al. (2009).

In the same way, these characterized bacteriophages would be also suitable for post-harvest applications (e.g. applied onto poultry carcasses or meat surfaces via spraying, packaging materials…), since they remain stable and effective under the chilling temperatures of poultry processing and at wide pH ranges for at least 30 days.

Findings on campylophages temperature stability described in the current study are similar to other previous works (Hammerl et al., 2014). However, these bacteriophages

retained maximum infectivity between pH 2.0 to 9.0 for at least a month under refrigeration temperatures, which differed from the findings of previous studies that reported campylophages being stable from pH 5.0 to 11.0 or pH 7.0 to 9.0 (El-Shibiny et al., 2009; Hammerl et al., 2014). Although *Campylobacter* specific bacteriophages resistant to pH values from 2.0 to 5.0 have not been previously described, authors such as Yin et al. (2019) have done so for *Vibrio parahaemolyticus* bacteriophages. The results shown in the current work indicate that the ten bacteriophages exhibit an enhanced tolerance to extreme acidic pH values. This broader pH stability could open a wider option of applications not only in livestock animals but also in food products.

At last, five *Campylobacter* specific bacteriophages were selected as the best candidates to be used as biocontrol agents. As the ten bacteriophages characterized during this study similarly responded to pH and temperature conditions, the criteria followed to select them included i) host range; ii) latent period; iii) burst size and iv) rise period. Although all the group II campylophages showed a similar host range, CAM302 presented the broadest one, infecting the same strains as the rest of group II campylophages, but also lysing *C. jejuni* strains that were only infected by group III campylophages. This is an interesting finding since the inclusion of bacteriophages able to lyse the same bacterium by binding to different cell surface receptors in a cocktail could enhance bacterial lysis and delay or prevent resistance development. This group bacteriophages also showed similar latent periods but the burst size of CAM296 was the largest one. Likewise, CAM304 showed the shortest rise period and was the quickest starting the second replication cycle. Regarding group III campylophages, CAM26 and CAM165 showed the broadest host ranges together with the shortest latent periods and largest burst sizes. Therefore, these five bacteriophages were selected as the most promising candidates to be combined in bacteriophage cocktails.

To confirm the suitability of these five bacteriophages for biocontrol purposes it is necessary to evaluate their whole genome sequence for the presence of genes encoding toxins and antibiotic resistance. Moreover, the effectiveness of the cocktails should be tested against different *Campylobacter* strains both *in vitro* and in the field, to evaluate the potential of this approach under environmental factors.

Conclusions

The ten bacteriophages characterized in the current study showed high host specificity. Group II campylophages infected strains of *C. jejuni*, *C. coli* and *C. fetus* species, whereas group III campylophages activity was restricted to *C. jejuni* species. Since both groups campylophages showed complementary host ranges, their combination in bacteriophage cocktails should be considered in order to achieve a collective broader lytic spectrum. Furthermore, both group bacteriophages were able to infect antibiotic resistant and multi-resistant *Campylobacter* strains from different geographical areas. The short latent periods and large burst sizes displayed by some of them, as well as their high temperature (4 °C to 42 °C) and pH (2 to 9) stability, makes them promising candidates for being included in *Campylobacter* specific cocktails for biocontrol applications. The bacteriophages CAM26, CAM165, CAM296, CAM302 and CAM304 were selected as the most promising candidates. However, further characterization of these bacteriophages is needed to ensure their safety and their potential as biocontrol agents.

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GENERAL DISCUSSION

Sujeto a confidencialidad por el autor

GENERAL CONCLUSIONS

1. The isolation method involving enrichment of the sample with different *Campylobacter* strains as target hosts in a *Campylobacter* selective medium was a simple, reproducible and efficient method for successful isolation of both group II and III *Campylobacter* specific bacteriophages.

2. Swine faecal droppings proved to be a rich source of *Campylobacter* specific group II bacteriophages and broiler skin a rich source of *Campylobacter* specific group III bacteriophages.

3. High diversity of *Campylobacter* spp. strains existed in human, retail broiler samples and faecal droppings of broiler and swine in northern Spain. Furthermore, these strains showed high resistance to ciprofloxacin, erythromycin and tetracycline, choice antibiotic drugs for campylobacteriosis treatment.

4. The technique of RFLP using *Smi*I and *Hha*I endonucleases was successfully applied to analyze the genetic diversity of both group II and III *Campylobacter* specific bacteriophages, respectively. The RAPD-PCR technique was effective in the genetic characterization of group II *Campylobacter* specific bacteriophages.

5. The lytic spectrum of *Campylobacter* specific bacteriophages was strain specific. Bacteriophages of group II showed lytic activity against strains of

Campylobacter jejuni, *Campylobacter coli* and *Campylobacter fetus* species, whereas bacteriophages of group III only infected *Campylobacter jejuni* strains. Both campylophages group II and III were able to infect antibiotic resistant and multi-resistant *Campylobacter* strains from different countries.

6. The *Campylobacter* specific bacteriophages of both groups selected according to their lytic spectrum were stable at a range of pH values from 2 to 9 and temperatures from 4 to 42 °C, comprising the usual conditions during the production, storage and administration of them.

7. The combination of the *Campylobacter* specific bacteriophages of both groups selected in this thesis according to their efficiency and stability is a promising strategy for *Campylobacter* biocontrol.

ANNEXES

ANNEX INDEX

ANNEX 1: Material & Methods

ANNEX 1

1. *Campylobacter* strains

In this study the 164 *Campylobacter* strains shown in Table 1 were used.

Table 1. *Campylobacter* strains used in this thesis work.

***Campylobacter* species**	**Strains origin**					
	Culture collection[1]	**Human[2]**	**Broiler and swine[2]**			
			Spain	**Italy**	**Poland**	**Total**
Campylobacter jejuni	3	8	23	26	35	95
Campylobacter coli	1	15	40	0	5	61
Campylobacter fetus	1	3	0	0	0	4
Campylobacter lari	1	0	0	2	0	3
Campylobacter upsaliensis	0	0	0	0	0	0
***Campylobacter* spp.**	0	0	0	1	0	1
Total	6	26	63	29	40	164

[1]Culture collection strains were purchased from CECT and CCUG collections. [2]Kindly provided by University Hospital of Donostia and University Hospital of Vall d'Hebron; [3] Provided by C-SNIPER project partners: AZTI, Spain, University of Torino, Italy and Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Poland.

1.1. Isolation

For *Campylobacter* field strains isolation, broiler skin and broiler and swine faecal dropping samples were analyzed. Briefly, three point five grams skin sections or five grams of faecal droppings were diluted (1:4 w/v) in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4$ and 0.01% w/v gelatin) and plated onto RAPID'*Campylobacter* Base (Bio Rad, Marmes la Coquete, France) plates combined with RAPID'*Campylobacter* Supplement (Bio Rad). Plates were allowed to dry at room

temperature and incubated at 37 °C for 72 h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), using an INVIVO$_2$ 400 hypoxia workstation (Ruskinn Technology Ltd, Bridgend, UK). Brick-red colonies were picked and streaked on Tryptone Soy Agar plates (TSA; Oxoid, Basingstoke, UK), and subjected to Gram stain and to oxidase test. The colonies that resulted Gram negative bacilli and oxidase positive were considered as putative *Campylobacter* isolates and were stored at -80 °C in Brain Heart Infusion broth (BHI; Oxoid,) supplemented with 20% glycerol.

1.2. Culture

For exponential phase cultures preparation, thawed stock cultures (200 µl) were cultivated on Columbia blood agar with 5% (vol/vol) defibrinated sheep blood (Oxoid) under microaerobic conditions at 37 °C. After overnight incubation, cells were harvested in BHI broth or 0.85% NaCl.

2. *Campylobacter* identification and typing

2.1. DNA extraction

Bacterial DNA was extracted using the commercial DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The DNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.2. Multiplex PCR

Three genes, *hipO*, *glyA* and, *sapB2*, were amplified to identify the species *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* subsp. *fetus* using the method described by

Wang et al. (2002). Briefly, a mixture including 50–100 ng of DNA, BioMix™ Red (Bioline, London, UK), five pairs of primers and primers for 23S rRNA gene as internal control (Table 2) was adjusted in a total volume of 25 µl. The amplification reactions were performed on a BioRad C1000TM Thermal Cycler and the products were visualized by electrophoresis of 30 min at 50 V on 1.5% agarose gel; stained with GelRed (Biotium, Fremont, CA, USA) and observed in ChemiDoc™ imaging system (Bio Rad).

2.3. *fla*A-restriction fragment length polymorphism (*fla*A-RFLP)

A 1.7 kb fragment of *flaA* gene was amplified with A1 and A2 primers (Table 2) and digested with *Dde*I restriction enzyme as proposed Nachamkin et al. (1993). Amplifications were performed in a mixture of 100 µl containing BioMix™ Red (Bioline), 2 µl of each primer (50 µM) and 5 µl bacterial DNA (50–100 ng). PCR were performed on a BioRad C1000TM Thermal Cycler using a denaturation step for 60 s at 94 °C, followed by 35 cycles of denaturation during 15 s at 94 °C, annealing 45 s at 45 °C and extension of 105 s at 72 °C; and a final extension for 5 min at 72 °C.

The correct amplification was checked by electrophoresis for 30 minutes at 100 V on a 1% agarose gel in 1X TBE buffer and GelRed. A volume of 5 µl of amplified product was digested with 0,2 µl *Dde*I (10 U/µl) restriction enzyme (New England Biolabs Ipswich, MA, USA) and 3 µl of PCR Buffer (10X, New England Biolabs) in a final volume of 30 µl. Restriction fragments generated after an incubation at 37 °C for 3 h were detected by electrophoresis on 2.5% agarose gel in 1X TBE buffer and GelRed for 90 min at 90 V. Lengths of restriction fragments were assigned by comparison with a 100 bp ladder (Promega, Madison, WI, USA).

Table 2.- Primers used for *Campylobacter* identification and *flaA*-RFLP typing.

Name	Sequence (5'→ 3')	PCR product	Target gene	References
CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	323 bp	*hipO* gene (*Campylobacter jejuni*)	(Wang et al., 2002)
CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	126 bp	*glyA* gene (*Campylobacter coli*)	
CLF CLR	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	251 bp	*glyA* gene (*Campylobacter lari*)	
CUF CUR	AATTGAAACTCTTGCTATCC TCATACATTTTACCCGAGCT	204 bp	*glyA* gene (*Campylobacter upsaliensis*)	
CFF CFR	GCAAATATAAATGTAAGCGGAGAG TGCAGCGGCCCCACCTAT	435 bp	*sapB2*gene (*Campylobacter fetus*)	
23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	650 bp	23S rRNA gene (*Campylobacter* spp.)	
A1	GGATTTCGTATTAACACAAATGGT GC		*flaA* gene (typing)	
A2	CTGTAGTAATCTTAAAACATTTTG	1725 bp		(Nachamkin,et al., 1993)

2.4. Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis was based on the CDC's PulseNet protocol (CDC, 2017), with minimal modifications. Briefly, *Campylobacter* spp. cell suspensions prepared in 0.85% NaCl were adjusted to 0.4 optical density at 610 nm wavelength. Four hundred µl of the bacterial suspensions containing 20 µl Proteinase K (20 mg/ml) were mixed with 400 µl of 1% molten Pulsed Field Certified agarose (Bio-Rad), prepared in TE buffer (10 mM Tris:1 mM EDTA, pH 8.0), and dispensed into four plug molds each. Plugs were let solidify and introduced in 5 ml tubes with 2 ml Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0, 1% Sarcosyl and 10 µl Proteinase K (20 mg/ml)). Tubes were incubated in a shaking water bath at 56 °C overnight for cell lysis. After this step, these agarose plugs were washed three times with sterile ultrapure water and six times more with TE buffer. Two mm plug slices were digested with *Sma*I (Thermo Scientific) and DNA fragments were separated on 1% Pulsed Field Certified agarose (Bio Rad) gel in 0,5X TBE buffer and GelRed using a CHEF-DR III PFGE system (Bio Rad). The electrophoresis conditions included an initial switch time of 6.8 s and final switch time of 35.4 s for 18 h at 6 V/cm and 14 °C. Sizes of the fragments were determined by comparison with Lambda Ladder PFG Marker (New England BioLabs).

3. Antimicrobial susceptibility of *Campylobacter* isolates

The antimicrobial susceptibility of the *Campylobacter* isolates was evaluated by disk diffusion using discs of fluoroquinolones (ciprofloxacin, 5 µg), macrolides (erythromycin, 15 µg) and tetracyclines (tetracycline, 30 µg) (Oxoid), and following the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2019a). Cells were initially cultured as above mentioned and harvested in BHI broth.

Bacterial cell suspensions for each isolate were prepared with an optical density of 0.6 at 600 nm wavelength (1×10^8 CFU/ml, approximately). Inoculums were spread using sterile swabs onto Mueller-Hinton agar plates supplemented with 5% mechanically defibrinated horse blood and 20 mg/l β-NAD (Bio-Rad). Discs were antiseptically dispensed onto the surface of the inoculated plates and after 48 h incubation at 37°C under microaerobic conditions, inhibition zone diameters were measured.

The susceptibility of isolates was categorized according to EUCAST breakpoints (EUCAST, 2019b): (a) ciprofloxacin ≥ 26 mm, susceptible and < 26 mm, resistant; (b) erythromycin ≥ 20 mm, susceptible and < 20 mm, resistant for *C. jejuni* and ≥ 24 mm, susceptible and < 24 mm, resistant for *C. coli*; (c) tetracycline ≥ 30 mm, susceptible and < 30 mm, resistant. The *C. jejuni* strain ATCC 33560 from American Type Culture Collection was used as control. Isolates exhibiting phenotypic resistance to the three classes of antibiotics were considered multidrug resistant (ECDC et al., 2017). It should be noted that the suggested *C. jejuni* breakpoints were used to interpret the results for *C. fetus*, since no such criteria are available for this specie.

4. Bacterial lawns preparation

For bacterial lawns preparation, *Campylobacter* cells grown in Columbia Agar were harvested in BHI broth to an optical density 0.6 at 600 nm wavelength (approximately 10^8 CFU/ml), cation supplemented with 1 mM $CaCl_2$ and 10 mM $MgSO_4$ and incubated at 37 °C for 4 h under microaerobic conditions. Then, 600 µl of exponential phase cultures were added to four ml of molten NZCYM soft agar (NZCYM broth (Pronadisa, Conda Laboratories, Madrid, Spain) supplemented with 0.7% Bacteriological Agar

tempered at 50 ºC and poured onto NZCYM hard agar plates (NZCYM broth with 1.2% agar).

5. Bacteriophages isolation

Three hundred and four bacteriophages were isolated and characterized in this study. Most of them (280) were isolated from broiler meat whereas the remaining ones from swine (18) and broiler (six) faecal droppings.

For bacteriophages isolation from broiler meat and swine faecal droppings, three point five grams of skin or five grams of faeces were diluted 1:4 (w/v) in Bolton broth supplemented with selective supplement of antibiotics and 5% of lysed horse blood (Oxoid). After stomacher-blending, samples were enriched with 100 µl of 16 exponential phase culture *Campylobacter* strains (Table 3) and incubated at 42 ºC for 48 h under microaerobic conditions. For the isolation of those from broiler faecal droppings, five grams of samples were diluted 1:4 (w/v) in SM buffer, and after stomacher-blending, samples were collected in sterile tubes and incubated at 4 ºC overnight in an orbital shaker at 250 rpm.

After incubation, all resultant solutions were collected in sterile tubes and centrifuged at 5,000 x g at 20 °C for 10 min. Chloroform was added (10% v/v) to recovered supernatants and phage presence evaluated by spotting 10 µl of onto lawns of each of the *Campylobacter* isolates. Plates were incubated overnight at 37 ºC under microaerobic atmosphere and then examined for phage plaques presence.

Table 3. *Campylobacter* strains used for bacteriophages isolation.

Campylobacter specie	Code	Origin
Campylobacter jejuni	CJE024	Broiler faeces
Campylobacter jejuni	CJE042	Broiler faeces
Campylobacter jejuni	CJE054	Broiler faeces
Campylobacter jejuni	CJE055	Broiler faeces
Campylobacter jejuni	CJE056	Broiler faeces
Campylobacter jejuni	CJE057	Broiler skin
Campylobacter coli	CCO044	Broiler skin
Campylobacter coli	CCO056	Broiler skin
Campylobacter coli	CCO066	Broiler skin
Campylobacter coli	CCO068	Broiler faeces
Campylobacter coli	CCO046*	Broiler faeces
Campylobacter coli	CCO050*	Broiler skin
Campylobacter coli	CCO059*	Broiler skin
Campylobacter coli	CCO062*	Broiler faeces
Campylobacter coli	CCO064*	Broiler skin
Campylobacter coli	CCO072*	Broiler skin

*Only used with swine faeces samples

6. Bacteriophage purification, propagation and titration

Single plaques were removed from the overlay agar using a sterile 1 ml pipette tip and resuspended in 900 µl SM buffer. For propagation, 600 µl of exponential phase bacterial cultures were supplemented with 1 mM $CaCl_2$ and 10 mM $MgSO_4$ and mixed with 400 µl phage stock suspension. Mixtures were incubated for 15 minutes at 37 ºC and added individually to 4 ml molten NZCYM soft agar previously tempered at 50 ºC. The 5 ml of soft agar plus bacteria and phage was immediately poured onto NZCYM hard agar plates and allowed to dry for 15 minutes before incubation at 37 ºC for 24 h under microaerobic conditions. For purification, single plaques pick and plating was repeated three times. Once purified, bacteriophages were propagated once again to amplify them. Then bacteriophages were recovered from plates presenting confluent lysis by adding 5 ml of SM buffer and incubating at 4 ºC for 24 h with gentle shaking.

SM buffer with bacteriophages was then treated with 10% chloroform and kept at 4 ºC until use. Titer was determined by spotting 20 µl of serially diluted suspensions onto NZCYM soft agar overlay plates.

7. Bacteriophage recovery assays

7.1. Broiler skin samples inoculation

Skin sections of 16 cm^2 (4 x 4 cm; 3.5 ± 0.92 g) were aseptically cut from previously confirmed phage negative chicken skins, using uninoculated sections as negative controls. Skin samples were surface inoculated with 100 µl of appropriate dilutions of the corresponding phage suspensions in order to obtain phage loads from $5.0x10^1$ to $5.0x10^6$ PFU/g (approx. $1.1x10^1$ to $1.1x10^6$ PFU/cm^2, respectively). Inoculated samples were left to dry at room temperature for 90 min inside a laminar flow cabinet before processing.

7.2. Isolation protocols

In all the methods described below, broiler skin samples were initially stomached in filter sterile bags with different broth medium (1:4 w/v) for 2 minutes prior to incubation under the different conditions corresponding to each method.

Method 1: Samples were just stomached in SM buffer.

Method 2: Samples were stomached in SM buffer. The stomachate was then collected in sterile tubes and incubated at 4 °C overnight with gentle shaking.

Method 3: Samples were placed in filter sterile bags with BHI broth and enriched with exponential phase cultures of the corresponding host *Campylobacter* isolate to a final

concentration of 10^6 CFU/ml. After stomaching, mixtures were incubated at 37 °C for 48 h under microaerobic conditions.

Method 4: Samples were processed and enriched as described for method 3 but incubated at 42 °C (instead of at 37 ºC).

Method 5: Samples were processed, enriched and incubated as described for method 4, but with gentle shaking during incubation.

Method 6: Samples were placed in filter sterile bags with Bolton broth supplemented with selective supplement of antibiotics and 5% of lysed horse blood (Oxoid). The preparation was enriched by inoculating exponential phase culture of the corresponding host *Campylobacter* isolate to a final concentration of 10^6 CFU/ml. Mixtures were stomached and incubated at 42 ºC for 48 h under microaerobic conditions.

Method 7: Samples were processed and incubated as described for method 6, but with no host bacterial enrichment.

After incubation under each condition, bacteriophages were tittered as explained in section 6. The limit of detection was considered the lowest inoculated phage titer at which phages were recovered in all replicates. Recovery efficiency data were calculated as the mean percentage of phage recovery ± standard deviation per chicken skin gram.

8. Bacteriophage characterization

8.1. Genome size determination

The genome size of all the bacteriophages was determined by PFGE. For agarose plugs, preparation, 600 µl of bacteriophage suspensions (approximately 10^8 PFU/ml) in SM

buffer were mixed with 200 µl of 2% molten Pulsed Field Certified agarose (Bio-Rad,) and dispensed into four plug molds each. Plugs were let solidify for 15 min at room temperature and introduced in sterile tubes with 2 ml TES lysis buffer (10 mM Tris, 100 mM EDTA, 1% sarkosyl and 0.1 mg/ml proteinase K). Tubes were incubated in a shaking water bath at 56 °C overnight for bacteriophage capsid digestion. Then, lysis buffer was removed, and plugs washed six times in washing buffer (20 mM Tris and 50 mM EDTA) at 56 °C for 20 min each wash. Plugs were then stored at 4 °C in TE buffer until used. PFGE was performed on 1 % Pulsed Field Certified agarose (Bio-Rad) gel in 0.5× TBE buffer and GelRed (Biotium, Fremont, CA, USA) using a CHEF-DR III PFGE system (Bio Rad). Three to five mm plug slices of each phage lysate were prepared and Lambda Ladder PFG Marker (New England Biolabs, Ipswich, MA, USA) was used as molecular size marker. The electrophoresis conditions were 18 h at 6 V/cm, included angle 120 and switch times of 6.8-35.4 s.

8.2. Host range analysis

Host range analyses were carried out by two different methods. Initially, 10 µl of each bacteriophage suspensions (approximately 10^6 PFU/ml) were spotted onto different *Campylobacter* bacterial lawns in NZCYM agar plates. After allowing the plates to dry for 20 min at room temperature, they were incubated at 37 °C for 24 h under microaerobic conditions. The appearance of 20 or more plaques was considered a positive result. These positive results were classified in four different lysis degrees: i) confluent clear lysis; ii) confluent clear lysis with a few discrete colonies; iii) confluent semi-clear or opaque lysis; and iv) more than 20 single plaques.

For the second method, bacteriophage suspensions of 10^6 PFU/ml were prepared and tenfold serial dilutions were performed in SM buffer (up to 10^{-5} PFU/ml). Both the undiluted suspension and 10 µl of each dilution were spotted on the bacterial lawns of the target *Campylobacter* strains. After incubation at 37 °C overnight under microaerobic conditions, sensitive host strains of *Campylobacter* were defined by the appearance of single plaques in the spotting zones.

8.3. Genetic diversity characterization

8.3.1. Bacteriophage DNA isolation

Bacteriophage DNA was obtained from 20 ml of 10^8-10^9 PFU/ml bacteriophage suspensions. These suspensions were centrifuged at 10,000 x g and filtered with 0,22 µm to remove all debris. Bacteriophage suspensions were concentrated using a method by polyethylene glycol (PEG) precipitation. Briefly, bacteriophage lysates were mixed (1:5 v/v) with a solution of PEG 8000 35% (w/v) in NaCl 2.5M and incubated at 4 °C overnight to enhance bacteriophage precipitation. After centrifugation at 10.000 x g and 4ºC for 60 min, the supernatants were carefully discarded and the pellet resuspended in 1 ml SM buffer. In order to degrade any remaining bacterial DNA or RNA, the resuspended solution was mixed with baseline zero reaction buffer (1:10 v/v) (10x buffer: 100 mM Tris pH 7.5; 15 mM $MgCl_2$; 5 mM $CaCl_2$) and 1 U baseline zero DNase and 10 U RNase. The mixture was incubated at 37 °C overnight. Bacteriophage DNA was finally obtained using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions. Isolated DNA concentration and purity was measured with a Nanodrop 1000 (Thermo Scientific, Vilnius, Lithuania) spectrophotometer.

8.3.2. Restriction analysis of group II campylophages

The restriction analysis of *Campylobacter* specific group II bacteriophages was performed with previously isolated DNA and RFLP analysis. The bacteriophage DNA was digested with *Smi*I restriction endonuclease (Thermo Scientific) according to manufacturer´s instructions and then, DNA digested fragments were separated by electrophoresis on GelRed stained agarose gel at 1% for 45 minutes at 90 V using Promega 1 kb ladder.

8.3.3. Restriction analysis of group III campylophages

Campylobacter specific group III bacteriophage genomes were subjected to digestion with *Hha*I restriction endonuclease (Thermo Scientific). For that purpose, 3-5 mm agarose plug slices from each bacteriophage were incubated with 20 U of the enzyme for 16-18 h at 37 °C. Restriction fragments were separated in 1% Pulsed Field Certified agarose as above mentioned, and Midrange PFG Marker (New England Biolabs) was used as molecular size marker. The electrophoresis was performed with CHEF-DR III system using the conditions of 14 h at 6 V/cm, included angle 120, and switch times 2-10 s.

8.3.4. RAPD-PCR

Four µl of each primer at a 100 µM concentration were used to amplify 50 ng bacteriophage DNA with 25 µl 2× Biomix, 3 µl $MgCl_2$ 25 mM and 2,5 µl 100% dimethyl sulfoxide (DMSO) in a final volume of 50 µl . The primers used were P1 (5´-CCGCAGCCAA-3´), P2 (5´-AACGGGCAGA-3´), OPL5 (5´-ACGCAGGCAC-3´) and RAPD5 (5´-AACGCGCAAC-3´) PCR was performed in a TProffesional thermocycler (Biometra GmbH, Göttingen, Germany). The thermal cycling program was: an initial

step of four cycles denaturation at 94 °C for 45 s, annealing at 30 °C for 120 s and extension at 72 °C for 60 s; followed by 26 cycles denaturation at 94 °C for 30 s, annealing at 36 °C for 30 s and extension at 72 °C for 60 s; and a final step of extension at 72 °C for 10 min. Amplification products were detected by electrophoresis gels on 1% agarose in 1× TAE buffer (w/v) with GelRed at 90 V for 45 min. Promega 1 kbp ladder was used as molecular size marker.

8.4. One step growth curve

Single-step growth experiments were performed in order to assess the latent period and burst size of the phages in a single round of replication. Host cells were grown to early exponential phase (OD_{600} nm = 0.4) in 8 ml BHI broth and incubated with shaking at 37 °C under microaerobic conditions. They were then infected with the particular phage at a multiplicity of infection (MOI) of 0.001. Samples were taken every 15 min for 4 h and the titer determined immediately by the spot test method in NZCYM agar. The burst size was determined by dividing the number of lysis plaques at the stationary phase by the number of plaques at the latent phase.

8.5. pH and thermal stability

The stability of *Campylobacter* bacteriophages was investigated at different pH values (2-9) and temperatures (4 °C - 70 °C) (Table 4). After propagation, phages were harvested in SM buffer previously adjusted to the appropriate pH value at 22 °C with HCl (to pH 2, 3.5 and 5.5) or NaOH (to pH 9). For thermal stability testing, bacteriophage solutions of the different pH values were maintained at the corresponding temperatures. Thereafter, aliquots of the buffered lysates were taken at different times

and were serially diluted to determine the phage titer by spotting dilutions onto NZCYM bacterial lawns of the corresponding host strain.

Table 4. Conditions tested in the stability assay of *Campylobacter* specific bacteriophages.

pH	Temperature (°C)			
	4	**22**	**42**	**70**
2.0	√		√	
3.5	√		√	
5.5	√		√	
7.5	√	√	√	√
9.0	√		√	

9. Fingerprinting data analysis

All PFGE, RFLP and RAPD-PCR gels were photographed with a ChemiDoc™ or a GelDoc™ EZ Imager imaging system (Bio-Rad) and phylogenetic relationships were determined using a temporary BioNumerics software version 7.6 evaluation license (Applied Maths, Sint-Martens-Latem, Belgium) (permission to publish received). Cluster analyses were performed using Dice similarity coefficient with the unweighted pair group method with arithmetic mean (UPGMA) and a tolerance of 1.5%. All band profiles were carefully checked by visual inspection to be correctly marked, and those profiles clustered in the dendrogram with more than 90% similarity were assigned to the same genotypic profile for both *Campylobacter* and bacteriophages analyses. Dendrograms combining different typing methods were created with BioNumerics software evaluation license using the Composite data sets.

The *Campylobacter* isolates diversity was calculated using the Simpson's Index of Diversity (SID), using $1-\Sigma pi2$, where pi is equal to the number of isolates of the same genotype divided by the total number of isolates.

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