Use of *Campylobacter* bacteriophages as biocontrol agents within the farm-to-fork process

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Campylobacteriosis is the most commonly reported foodborne disease in the European Union since 2005, with 246,158 confirmed cases in 2017 and an associated estimated annual cost of 2.4 billion €. Poultry is considered the natural reservoir of *Campylobacter* spp., and the consumption of raw and undercooked chicken meat the major source of human infection. In addition, the use of antimicrobials in primary production has caused the spread of antibiotic resistant *Campylobacter* strains, an additional threat to public health. Multiple mitigation strategies have been proposed for *Campylobacter* control in poultry. However, none of them have completely solve the problem. The application of specific bacteriophages appears as a promising alternative to antibiotics in order to reduce the burden of *Campylobacter* contamination within the farm-to-fork process. In this study, 304 phages were isolated from food and environmental samples. According to their genome size, new isolates were classified within campylophages of group II or *Cp220virus* (≈ 180 kb) and group III or *Cp8virus* (≈ 135 kb). The host range of all phages was also determined against a panel of 19 *Campylobacter* strains previously selected based on the typing results of a 100 strains collection. The characterization of their lytic capacities allowed the selection of the most promising 59 phages for their potential to be used as biocontrol agents. Their genetic diversity was then studied by restriction fragment length polymorphism (RFLP). *Hha*I RFLP classified 41 group III phages into five different patterns, while *Smi*I digestion did the same with 18 of group II. Further characterization of the selected phages is being carried out to determine their suitability for the future development of new effective campylophage-based products.

La campilobacteriosis es la enfermedad transmitida por alimentos más común en la Unión Europea desde 2005, con 246,158 casos confirmados en 2017 y un coste asociado de 2,4 billones de euros al año. Las aves de corral son el reservorio natural de este patógeno y el consumo de carne de ave cruda o poco cocinada la fuente más común de infección. Además, el uso generalizado de antibióticos en la producción primaria ha provocado la propagación de cepas resistentes, una amenaza adicional para la salud pública. Aunque se han propuesto diferentes estrategias de mitigación para el control de *Campylobacter*, ninguna de ellas ha podido solventar el problema. La aplicación de bacteriófagos específicos de *Campylobacter* se presenta como una prometedora alternativa para reducir la prevalencia de este patógeno desde la granja hasta la mesa. En este estudio, se aislaron 304 fagos a partir de muestras ambientales y alimentarias. Según su tamaño de genoma, se clasificaron como fagos del grupo II o *Cp220virus* (≈ 180 kb), o, del grupo III o *Cp8virus* (≈ 135 kb). Su rango de huésped se determinó frente a 19 cepas de *Campylobacter*, previamente seleccionadas en base al tipado de una colección de 100 cepas. La caracterización de su capacidad lítica permitió la selección de los 59 fagos con mayor potencial para su empleo como agentes de biocontrol. Posteriormente, se estudió su diversidad genética mediante la técnica de polimorfismos de longitud de fragmentos de restricción (RFLP). La RFLP mediante el enzima *Hha*I clasificó 41 fagos del grupo II en cinco perfiles distintos, mientras que el *Smi*I hizo lo mismo con 18 del grupo II. Se continúa caracterizando los fagos seleccionados para determinar su idoneidad para el futuro diseño de nuevos y efectivos productos a base de fagos.

Keywords: poultry, campilophages, food-safety, characterization, genetic diversity

Introduction

Campylobacter is the most commonly reported gastrointestinal bacterial pathogen in the European Union (EU) since 2005, with 246,158 confirmed cases of human campylobacteriosis in 2017, 18,860 of them in Spain (EFSA 2018). However, these figures only represent a small proportion of all clinical cases, as it has been estimated that there are approximately nine million cases of human campylobacteriosis per year in the EU, with an associated annual cost of 2.4 billion € (EFSA 2011). Campylobacteriosis 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 (Guillain-Barré and irritable bowel syndromes) and septicemia might occasionally follow infection (Hansson et al. 2018), its fatality rate is low (0.04%) (EFSA 2018).

Poultry is considered the natural reservoir of *Campylobacter* spp. and the consumption of raw and undercooked poultry products, especially chicken meat, 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 meat products at retail (Furuta et al. 2017). To face this problem, the EU has recently developed the Commission Regulation (EU) 2017/1495 amending Regulation (EC) No 2073/2005 as regards *Campylobacter* in broiler carcasses, which allows carcasses complied with a limit of 1,000 cfu/g, a number that should gradually decrease over the 7 years following the publication of this regulation.

Standard control measures at farm level rely upon the use of antibiotics, which promote the selection and spread of antibiotic resistant *Campylobacter* strains, an additional threat to public health (Kaakoush et al. 2015). In fact, the World Health Organization (WHO) included fluoroquinolone resistant *Campylobacter* in its global priority list of antibiotic resistant bacteria (WHO 2017). Therefore, it is critical to develop innovative non-antibiotic based strategies to reduce the burden of *Campylobacter* contamination within the farm-to-fork process.

Different mitigation strategies, such as strict biosecurity measures, reduction of slaughter age, vaccination or the use of pre-/pro-biotics or other antimicrobials (e.g. bacteriocins) as feed additives have facilitated the currently existing *Campylobacter* reduction (Hwang et al. 2009). However, their inability to solve the entire problem places them as complementary hurdles in a synergistic approach. Some decontamination processes have also been proposed as effective anti-*Campylobacter* treatments for carcasses. Nevertheless, no chemical decontamination treatments are currently authorized in the EU, and physical treatments, like freezing and heat treatment, have a negative effect on the product appearance and quality.

Other promising alternative to reduce the number of *Campylobacter* either at pre- or post-harvest level is the application of specific bacteriophages or phages (Goode et al. 2003;El-Shibiny et al. 2009; Carvalho et al. 2010b). Phages are viruses that specifically infect and kill bacteria, being 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; Tsuei et al. 2007; Connerton et al. 2011). The use of phages as a food safety strategy is desirable as they are specific towards the pathogen of concern, are harmless to humans, animals and plants, and do not affect the existing commensal microbiota or alter food organoleptic properties. However, not every phage is suitable for biocontrol applications. They must be virulent (i.e. strictly lytic), non-transducing and safe. Additionally, they should be effective against a broad host range of the pathogen of concern. To overcome the narrow host range phages often display due to their high specificity, and prevent the development of target bacteria resistances, the use of phage cocktails has been proposed as the best approach for phage-based biocontrol (Hansen et al. 2007).

The aim of this study was to isolate *Campylobacter* infecting phages from food and environmental samples, and, to characterize them in terms of their genome size, genetic diversity and lytic profile (host range) by using collection strains and contemporary typed *Campylobacter* isolates, in order to be able to select effective suitable phages as a promising alternative to antibiotics for future biocontrol applications in poultry.

Materials and methods

Campylobacter strains collection

A collection of one hundred *Campylobacter* strains from different origins was established by using 11 type collection strains, 26 human clinical isolates and 60 contemporary strains isolated from food and environmental samples (28 from broiler meat, 32 from chicken feces and 3 from pig feces). All *Campylobacter* species were identified following the multiplex PCR protocol described by Elbrissi et al. (2017). Bacterial typing was also carried out by *fla*A-RFLP typing (Llovo et al. 2003), PFGE (CDC's PulseNet protocol (CDC 2017)) and antimicrobial resistance profiling (EUCAST 2019).

Isolation of *Campylobacter* specific bacteriophages

More than 200 samples of chicken skin (purchased from different local supermarkets and butcher´s shops) and chicken and pig feces (collected from different local farms) were processed following the method described by Carvalho et al. (2010a) with minor modifications. The preparation was enriched by inoculating exponential phase cultures of ten *Campylobacter* strains, including 3 *C. jejuni*, 5 *C. coli* and 1 of each *C. lari* and *C. fetus,* to a final concentration of 10^6 CFU/ml. Mixtures were stomached and incubated at 42 ºC for 48 h under microaerobic conditions. After incubation, solutions were collected in sterile tubes, centrifuged at 5,000 x g at 20 °C for 10 minutes and chloroform was added (10% v/v). Phage presence was evaluated by spotting 10 µl of resulting solutions onto agar lawns of each of the ten strains. Plates were incubated overnight at 37 °C under microaerobic atmosphere and then examined for phage plaques presence. Detected campylophages were purified and amplified following the method described by Loc-Carrillo et al. (2007) with minor modification.

Bacteriophage genome size determination

Pulsed field gel electrophoresis (PFGE) plugs were prepared following the genome size determination protocol described by Sorensen et al. (2017). Three to five mm plug slices were loaded in a 100 ml 0.5× TBE buffer, 1% Pulsed Field Certified agarose gel (Bio Rad, Marmes la Coquete, France). Lambda Ladder PFG Marker (New England Biolabs, Ipswich, MA, USA) was used as molecular size marker. Gels were run for 18 h at 6 V/cm, included angle 120, and switch times 6.8-35.4 s in a CHEF-DR III system (Bio Rad).

Host range characterization

A total of 19 strains were chosen for host range characterization of new campylophage isolates, including 9 *C. jejuni*, 9 *C. coli* and 1 strain each of *C. fetus* and *C. upsaliensis*. Host range analysis was carried out by spotting 10 µl of 10^6 PFU/ml phage suspension onto prepared bacterial lawns (Janez et al. 2014). Plates were dried for 20 min at room temperature and incubated for 24 h at 37 °C under microaerobic conditions. The appearance of 20 or more plaques was considered a positive result.

Genetic diversity characterization

*Hha*I restriction analysis

Restriction analysis of group III phage genomes was carried out with *Hha*I restriction endonuclease (Thermo Scientific, Vilnius, Lithuania) as described by Sorensen et al. (2017). Digested slices were loaded in a Pulsed Field Certified agarose gel as explained before with Midrange PFG Marker (New England Biolabs) as molecular size marker. Gels were run in a CHEF-DR III system (Bio Rad) for 14 h at 6 V/cm, included angle 120, and switch times 2-10 s.

Phage DNA isolation

For DNA isolation, phages were concentrated by polyethylene glycol (PEG) precipitation. After concentration, 1 U DNase and 10 U RNase were added to degrade bacterial DNA and RNA. Phage DNA was finally isolated with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions. Isolated DNA concentration and purity was measured with a Nanodrop 1000 (Thermo Scientific) spectrophotometer.

*Smi*I restriction analysis

For restriction pattern analysis, phage DNAs were digested with *Smi*I restriction endonuclease (Thermo Scientific) according to manufacturer´s instructions. Fragments were separated by conventional gel electrophoresis at 70 V for 1 h with Promega 1 kpb ladder (Promega, Madison, WI, USA) as molecular size marker.

Results and discussion

Campylophages isolation

A total of 304 *Campylobacter* phages were isolated from 244 processed samples. The highest isolation rate (257%) was achieved for both chicken skin and pig feces samples, from which 280 and 18 phages were isolated from 109 and 7 samples, much higher than those reported by Tsuei et al. (2007) (0%) or Atterbury et al. (2003) (11%). Surprisingly, only 6 phages were isolated from a total of 128 chicken feces, which means and isolation rate of 5%. Chicken feces have been described as a common source of *Campylobacter* phages (Janež and Loc-Carrillo 2013) and several authors such as Connerton et al. (2004), Loc-Carrillo et al. (2007) and Aprea et al. (2018) achieved isolation rates of 42, 40 and 18% respectively, significantly higher than those obtained in this study.

Genome size determination

According to their genome size, campylophages are classified into three groups (Sails et al. 1998). Group I phages (320 kb) have been rarely isolated and have not been deeply described. Campylophages of group II (185 kb; *Cp220virus*) and group III (135 kb; *Cp8virus*) are most common and attach to different receptor-binding sites on the surface of the bacterial host during the first step in phage infection. Group II phages receptor binding protein binds the flagellum of *Campylobacter* strains while group III phages receptor is found in capsular polysaccharides (Sørensen et al. 2015).

The genome size of all the 304 phages was determined by PFGE allowing the classification of new phage isolates within the two major groups of campylophages. The 280 phages isolated from poultry meat and the 6 isolated from chicken feces displayed genome sizes of approximately 135 kb and, according to Sails et al. (1998), were classified as group III phages, nowadays included in *CP8virus* genus (Adams et al. 2016). These results are in concordance with the literature as group III *Campylobacter* phages are the most commonly isolated ones (O'Sullivan et al. 2018), specially from poultry sources (Connerton et al. 2011). The remaining 18 phages, all of them isolated from pig feces, possessed genomes of approximately 185 kb size and were, therefore, included in campylophages group II (Sails et al. 1998) and in *CP220virus* genus. Pig feces could be thereby considered a rich source in *Cp220viruses*. In contrast, it was not possible to isolate any group II phages from poultry sources, although other work used these sources for a number of campylophages isolation (Atterbury et al. 2003).

Host range analysis

The host range characterization of the 304 phages was determined against a 19 *Campylobacter* strains panel, previously selected based on the typing results obtained for the initially established collection of one hundred *Campylobacter* strains (data not shown). Four *Campylobacter* species were represented in the panel, including 9 *C. jejuni*, 8 *C. coli* and 1 strain each of *C. fetus* and *C. upsaliensis*.

All *C. jejuni*, *C. fetus* and *C. upsaliensis* strains were lysed by at least 1 phage, whereas only 5 out of 8 (62.5%) *C. coli* isolates showed sensitivity to phage infection. Two *C. jejuni* strains isolated from chicken feces (CJE078 and CJE079) displayed sensitivity to more than the 99% of *Cp8viruses* while resistance to all *CP220viruses*, suggesting their suitability to be used as reliable hosts for group III phages isolation. In the same way, 4 *C. jejuni*, 3 *C. coli* and the *C. upsaliensis* strain were only sensitive to group III phages. By contrast, 3 *C. jejuni* and 1 *C. coli* showed sensitivity against all group II phages, becoming good candidates for group II phages isolation.

Regarding the anti-*Campylobacter* effectiveness of group III phages, previous works described their ability to lyse *C. jejuni* strains, often more than those of group II (Hammerl et al. 2014; Jackel et al. 2019). Accordingly, in this study, group III phages showed lytic activity against all the 9 tested *C. jejuni* strains while those of group II against only 3. In particular, the phage Cph356 induced the lysis of 8 (89%) *C. jejuni* strains. Surprisingly, many group III phages were also able to lyse *C. coli*, *C. fetus* and

C. upsaliensis strains. Specifically, the 80% (228/286) of them infected *C. coli* CCO043 strain, and phages Cph109, Cph356 and Cph380 one to four *C. coli* strains more. Similarly, the 33% and 96% of group III phages infected the *C. fetus* and *C. upsaliensis* strains, respectively. Therefore, despite the small number of *Campylobacter* strains used in the host range analysis, this is the first work showing the ability of group III phages to infect not only *C. jejuni* but also other *Campylobacter* species.

Concerning phages of group II, they have been previously described to be able to lyse *C. jejuni* and *C. coli* species (Sørensen et al. 2015). Results of the present study showed that the 83% of new isolates were also able to lyse tested *C. fetus* strain.

Overall, the 304 phage isolates showed a variety of lytic spectra ranging from 16 to 74% infected strains among the used *Campylobacter* panel. The two phages (Cph356 and Cph380) showing the broadest host ranges (74 and 56%, respectively) as well as the 33 phages with the narrowest host range (16%) belonged to group III, while the group II phages´ lytic spectrum varied between 21 and 32%. Despite the narrow host range shown by some of the new phage isolates, the combination of the most promising bacteriophages in a cocktail could improve the lytic spectra of the phage-based solution as well as limit the potential of *Campylobacter* spp. to develop resistance to all phages included in the cocktail. As mentioned above, group II and group III phages diverge in terms of their host receptor and for that reason, phage cocktails should contain members of both groups. Moreover, phages within each group differ in their host range, so, their combination appears as the best strategy to design a successful phage cocktail. Therefore, based on their host range and the group they belonged to, 59 campylophages, including 41 from group III and the 18 from group II, were selected for further characterization.

Genetic diversity characterization

*Hha*I restriction profile

To analyze the genetic diversity among bacteriophages of group III, pulsed-field gels were produced to analyze phage genome fragments produced after restriction digest with *Hha*I endonuclease. All tested 41 phages yield clear restriction patterns, displaying between 6 and 9 bands after PFGE gel run. Five different restriction patterns were observed (Figure 1A): patterns I and II, which showed 6 bands, were observed in 9 and 3 phages respectively; patterns III and V, observed in 2 and 17 phages, showed 7 bands; and pattern IV, which was observed in 10 phages and showed the maximum bands. All phages shared five bands, the ones of smaller size, revealing some kind of similarity among them. As shown by other authors, *Hha*I restriction enzyme was useful to distinguish digestion patterns of *Campylobacter* group III phages genomic DNA.

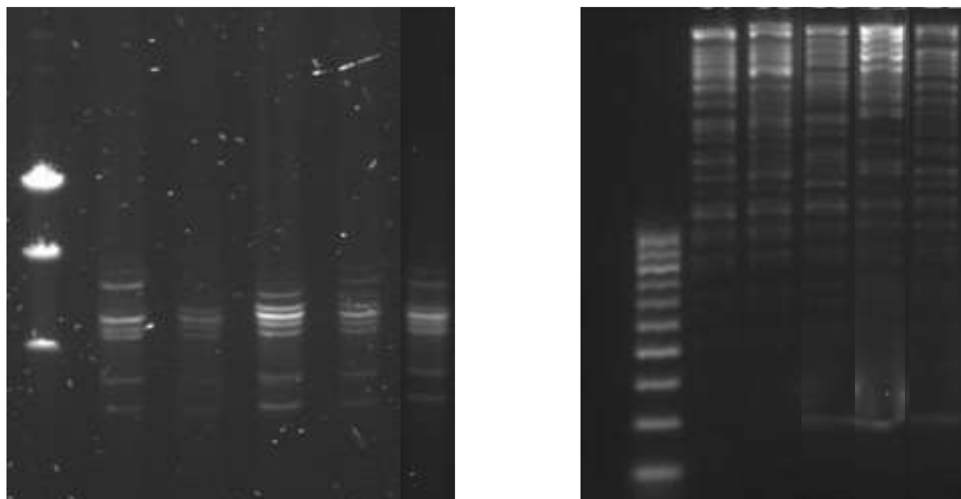

Figure 1. Restriction patterns of digested phage genomes representing different profiles. A. *Hha*I profiles of group III (≈135 kb) phage genomes. B. *Smi*I profiles of group II (≈185 kb) phage genomes.

*Smi*I restriction profile

Group II phages genome fragment analysis was carried out by cleaving purified DNA by *Smi*I endonuclease and posterior conventional gel electrophoresis. The 18 phages yield clear restriction

patterns, displaying lot of small size bands (<19 kb) which hindered the identification of the different profiles. Finally, five restriction patterns were observed (Figure 1B). Pattern I was the most commonly recorded one and was observed in 7 phages; pattern II was displayed by 1 phage, whereas patterns III, IV and V, in 5, 3 and 2 respectively. As suggested by Sørensen et al. (2015) and Jackel et al. (2019), *Smi*I restriction endonuclease successfully cleaved group II phages DNA.

Conclusion

In conclusion, poultry meat and pig feces, unlike chicken feces, were rich sources in *Campylobacter* bacteriophages of both group II and III. Host range analysis and genome size determination allowed the selection of the most promising 59 phages. Despite the narrow host range shown by some of them, the combination of different bacteriophages in a cocktail could improve the lytic spectra of the final phage-based solution and limit the potential of bacteria resistance to phages. Genetic diversity characterization identified five different phage types both within group II and group III phages. Further characterization is needed in order to define the most effective phage combination for its use as a biocontrol agent.

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