

RESEARCH REPORT

Control of *Campylobacter* spp. and *Yersinia enterocolitica* by virulent bacteriophages

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ABSTRACT

The efficacy of the *Campylobacter* (*C.*) phages NCTC12684 (group II) and CP81 (group III) and of the *Yersinia* (*Y.*) phage PY100 to reduce the numbers of *Campylobacter* and *Y. enterocolitica* in meat at 4°C applying different Multiplicities of Infection (MOIs) was analyzed. Initial experiments were carried out in broth at 4°C and 37°C to compare cell number reductions under chilling and optimized growth conditions, respectively. The results showed a 1 log₁₀ unit reduction of *Campylobacter* cell numbers at 37°C in broth. However, no reduction was observed in broth and meat at 4°C. In contrast, *Y. enterocolitica* cell numbers were reduced in broth at 4°C (up to 3 log₁₀ units after 24hr) and 37°C (5 log₁₀ units after 1.5hr) and also in meat at 4°C (2 log₁₀ units after 48hr). The highest cell number reductions were obtained at the highest MOIs.

KEYWORDS: *Campylobacter*, *Yersinia*, bacteriophage, food safety, biocontrol

INTRODUCTION

Campylobacter causes approximately 200,000 cases of campylobacteriosis in the European Union (EFSA, 2009) and thus is one of the most important bacterial foodborne pathogens. *Yersinia* infections are of secondary importance with about 7,500 cases yearly (EFSA, 2009). Complete elimination of both pathogens in the food chain is currently not feasible, but the quantitative load can be reduced by several pre- and post-harvest applications. Post-harvest approaches exploiting virulent phages have already been described and focus up to now on the control of *Listeria* (*L.*) *monocytogenes* and *Salmonella*. Due to the effectiveness and innocuousness of *Listeria* phages, two products were already approved by the FDA: ListShield, the LMP-102 phage preparation comprising six phages for the control of *L. monocytogenes* on ready-to-eat foods (Bren, 2007), and Listex P100 (phage P100) for the control of this species in

meat and cheese products (Carlton et al, 2005). P100 is able to eliminate or reduce *Listeria* up to 3.5 log₁₀ cfu/g under appropriate conditions (Carlton et al, 2005; Holck and Berg, 2009; Soni et al, 2009; Soni and Nannapaneni, 2010). With *Salmonella*, up to 4 log₁₀ unit reductions of cell number have been described in vegetables, chicken, chicken products, sausages and cheese (Modi et al, 2001; Goode et al, 2003; Leverentz et al, 2003; Whichard et al, 2003; Higgins et al, 2005). Some studies demonstrated the efficacy of phages on *C. jejuni*. Cell numbers were reduced by approximately 1 log₁₀ unit at 4-5°C on chicken skin, and on raw and cooked meat (Atterbury et al, 2003; Goode et al, 2003, Bigwood et al, 2008). No data are currently available on post-harvest application of *Y. enterocolitica* phages.

This work describes the potential of the *Campylobacter* phages NCTC12684 and CP81 and of the *Yersinia* phage PY100 to control their hosts in broth and meat.

MATERIALS AND METHODS

Bacterial strains and bacteriophages

Two *Campylobacter* strains and one *Yersinia* strain were used in this study: *C. jejuni* NCTC 11168 and *C. coli* NCTC 12668 were obtained from the National Collection of Type Cultures (NCTC), Health Protection Agency, United Kingdom. *Y. enterocolitica* 83/88/2 is a plasmid-cured derivative

of the serogroup O:5,27, biogroup 2 strain 83/88 (Hertwig et al, 2003). *Campylobacter* strains were grown on Mueller-Hinton-blood (MHB) agar (Oxoid, Wesel, Germany), modified Charcoal-Cefoperazon-Desoxycholat (mCCDA) agar (Oxoid) or in Sodium-NZamines-Casaminoacids-Yeast-Magnesiumsulfate (NZCYM) medium (Roth, Karlsruhe, Germany) at 37°C under microaerobic conditions. *Y. enterocolitica* 83/88/2 was grown on Luria Bertani (LB) agar

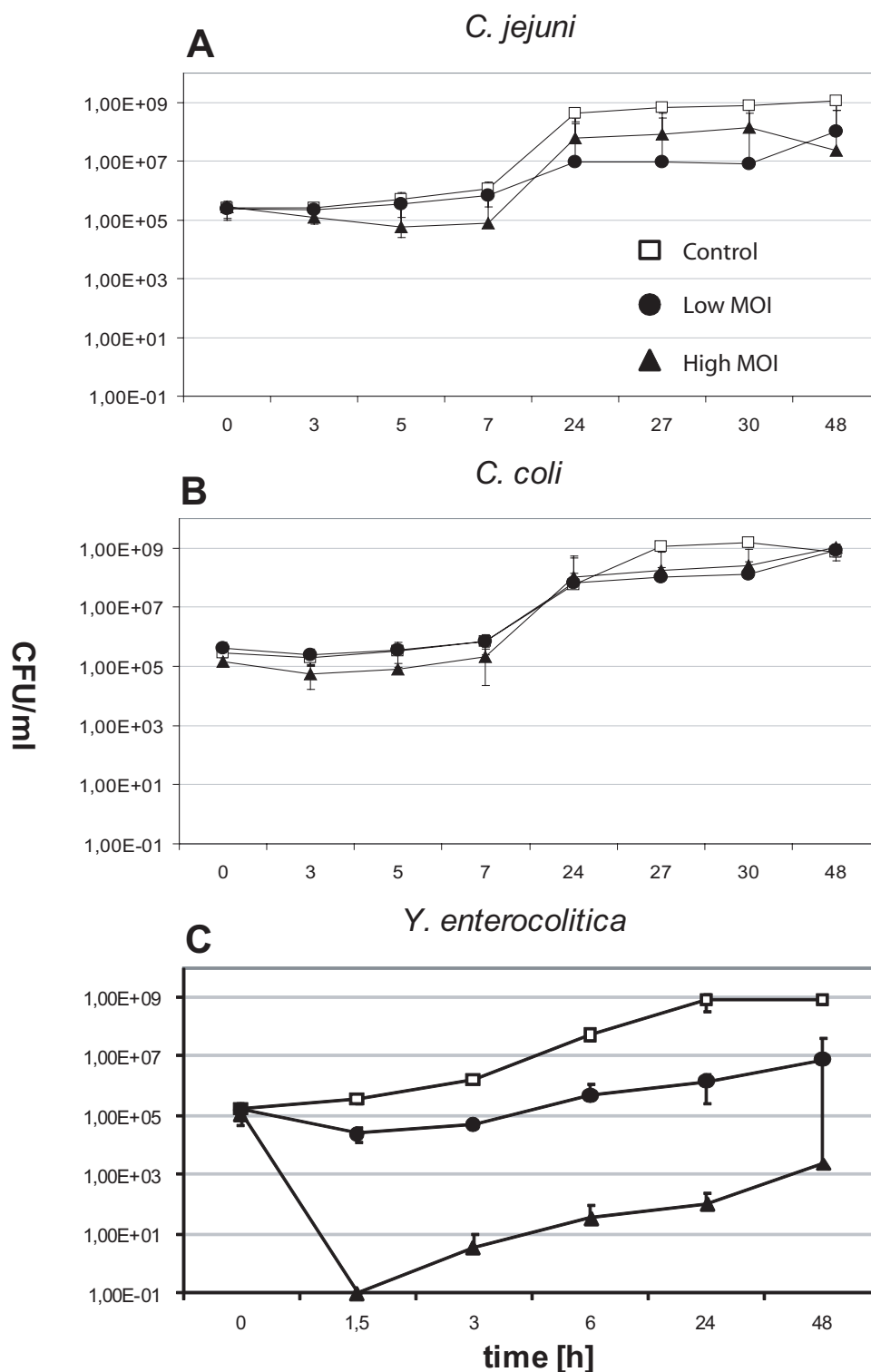


Figure 1. Phage-induced lysis of *Campylobacter* and *Yersinia* in broth at 37°C. (A) *C. jejuni* NCTC 11168 (Phage CP81). (B) *C. coli* NCTC 12668 (Phage NCTC12684). (C) *Y. enterocolitica* 83/88/2 (Phage PY100).

(Merck, Darmstadt, Germany), *Yersinia* selective (CIN) agar (Oxoid) or in NZCYM medium at 37°C under aerobic conditions. *C. jejuni* phage CP81 isolated from retail chicken meat has recently been characterized (Hammerl et al, 2011). The NCTC group II phage 12684 that infects *C. jejuni* and *C. coli* strains has also previously been described (Sails et al, 1998). PY100 is a broad host range *Yersinia*

phage lysing strains of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Schwudke et al, 2008).

Reduction of bacterial cell numbers in broth

Overnight cultures of bacterial strains were diluted to a final cell number of approximately 1×10^5 cfu/ml. 1ml phage lysate or SM buffer (negative control) was added to 1ml

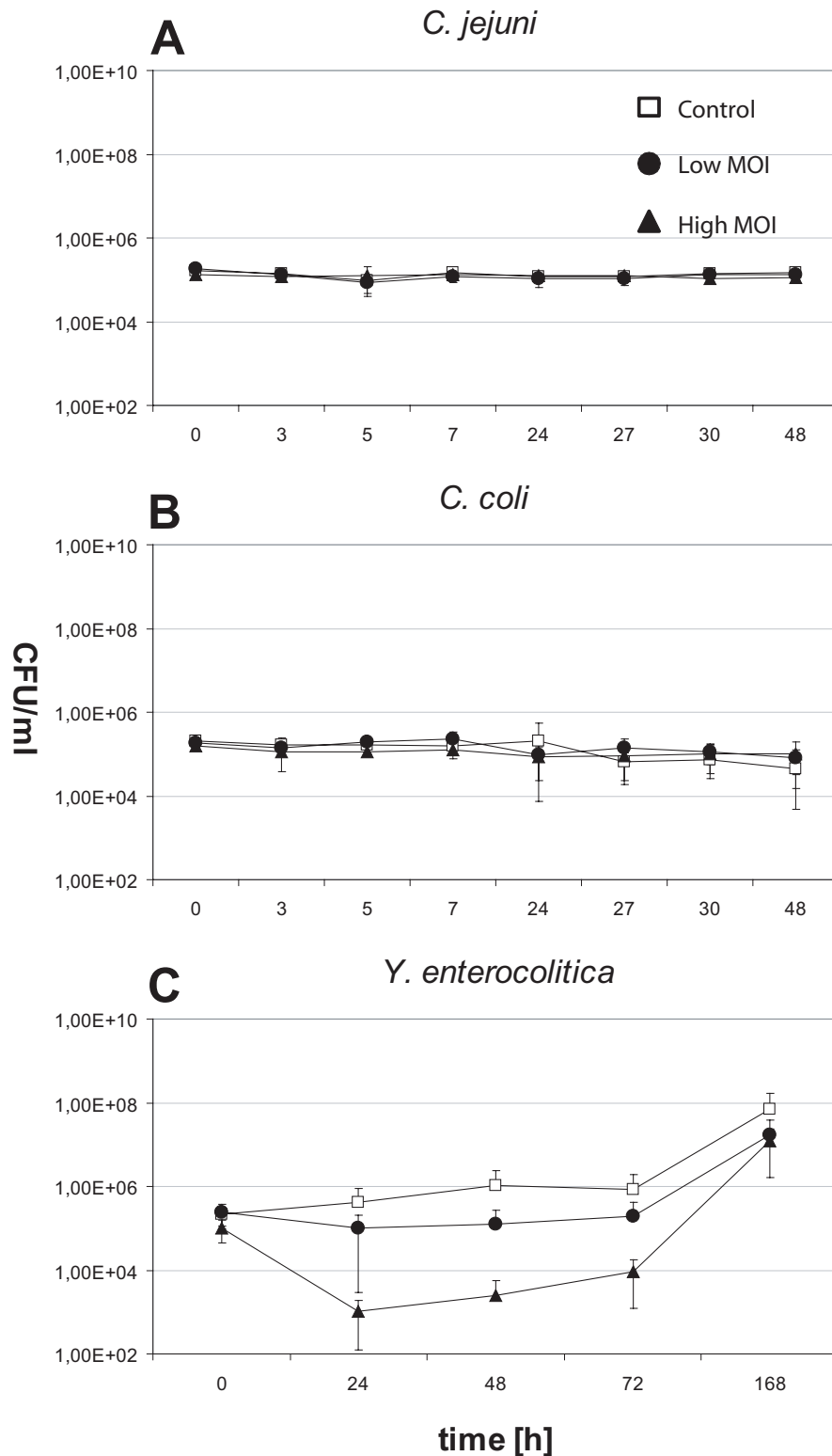


Figure 2. Phage-induced lysis of *Campylobacter* and *Yersinia* in broth at 4°C. (A) *C. jejuni* NCTC 11168 (Phage CP81). (B) *C. coli* NCTC 12668 (Phage NCTC12684). (C) *Y. enterocolitica* 83/88/2 (Phage PY100).

of the diluted culture. The mixture was incubated at 37°C (microaerobic/aerobic) or 4°C (aerobic). The initial bacterial host concentration was kept constant in all assays at approximately 1×10^5 cfu/ml. Bacterial counts were determined after 0, 6, 24, 27, 30, 48, 72 and 168 hours. The following MOIs were applied: high MOI for *C. jejuni* (10^2), and for *C. coli* and *Y. enterocolitica* (10^4); low MOI for *C. jejuni* and *C. coli* (10^1), and for *Y. enterocolitica* (10^2).

Reduction of bacterial cell numbers in meat

Raw chicken and pork meat was tested to be free of *Campylobacter* spp. and *Yersinia* spp. Meat was sliced aseptically into portions of 10gm, frozen at -20°C , and defrosted in a refrigerator 24hr prior to use. *Campylobacter* and *Yersinia* strains were grown overnight in NZCYM medium at 37°C under microaerobic (*Campylobacter*) resp. aerobic (*Yersinia*) conditions. Each meat portion was inoculated

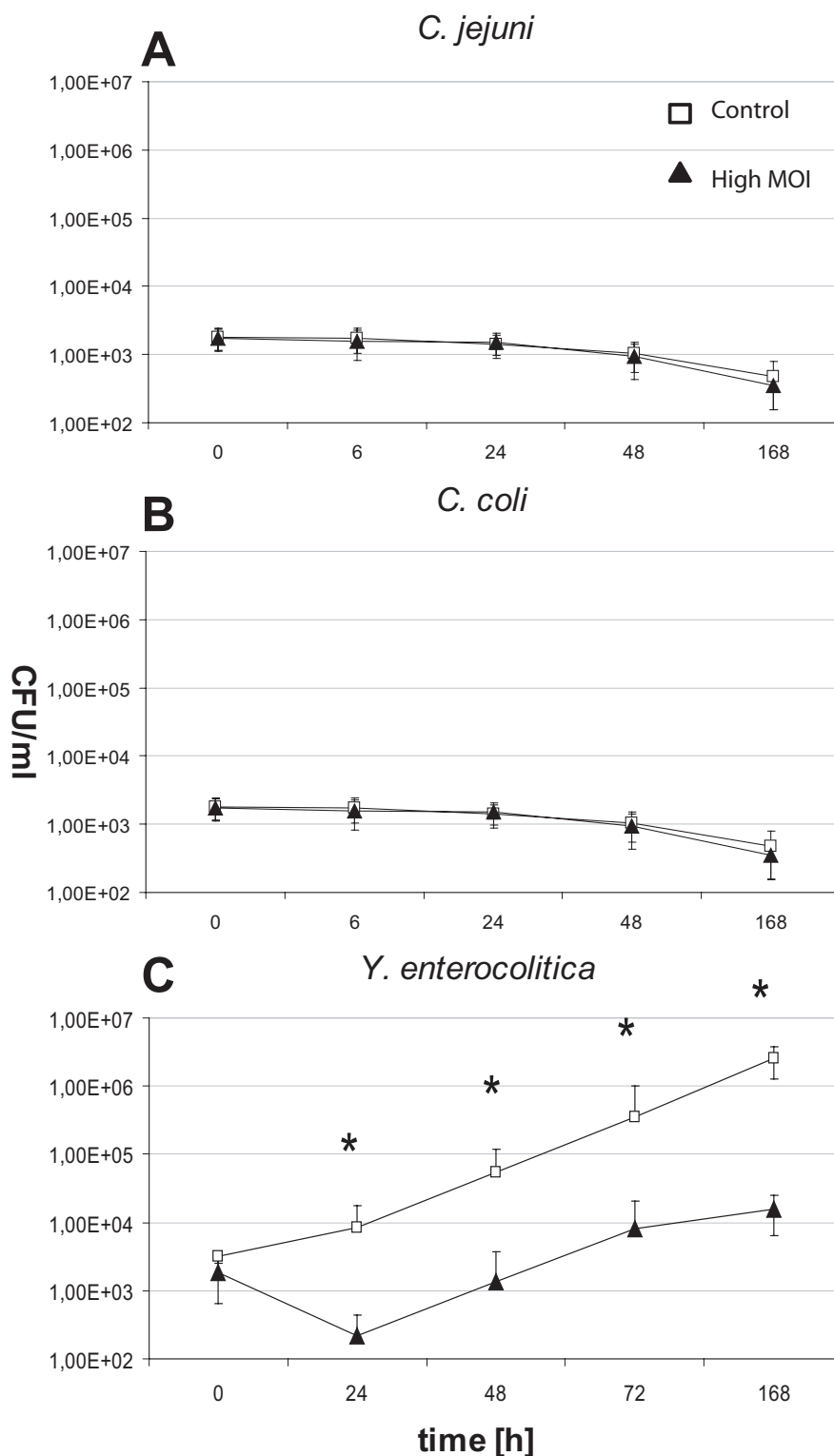


Figure 3. Phage-induced lysis of *Campylobacter* and *Yersinia* in meat at 4°C. (A) *C. jejuni* NCTC 11168 (Phage CP81). (B) *C. coli* NCTC 12668 (Phage NCTC12684). (C) *Y. enterocolitica* 83/88/2 (Phage PY100). (* $p < 0.05$)

with 100µl of the diluted bacterial suspensions (approximately 1×10^6 cfu/ml). Bacteria were allowed to attach to the matrix for 30min at room temperature. Thereafter, 1ml of the corresponding phage lysate or SM buffer was added. All samples were vacuum sealed and stored either at 37°C or at 4°C, depending on the experimental setup. At each sampling time, inoculated meat pieces were diluted 1:10 with NZCYM medium, blended for 2min, and serially diluted with NZCYM medium before being spread onto mCCDA or CIN agar plates. For the enumeration of the bacteria, plates were incubated for 48hr (*Campylobacter*) or 24hr (*Yersinia*). All experiments were performed in triplicates. Statistical differences between bacterial cell numbers in the samples were assessed by using the Mann-Whitney-U test.

RESULTS

All three phages reduced the numbers of their hosts in broth at 37°C. While the *Campylobacter* phages CP81 and NCTC12684 yielded reductions of only 1-2 log₁₀ units, respectively of the applied MOI, PY100 reduced *Y. enterocolitica* cell numbers at a MOI of 10² by up to 3 log₁₀ units (after 24hr) and at a MOI of 10⁴ by up to 5 log₁₀ units (after 1.5hr) (Figure 1). No growth inhibition of *C. jejuni* and *C. coli* was observed at 4°C in broth at any MOI (Figure 2 A and B). At this temperature, *Y. enterocolitica* cell numbers were reduced by up to 1 log₁₀ unit (low MOI) after 24hr and up to 3 log₁₀ units (high MOI) after 24hr (Figure 2C).

Due to restricted growth of *Campylobacter* at 4°C, experiments with chicken meat (*Campylobacter* assays) and pork meat (*Y. enterocolitica* assays) were exclusively carried out at high MOIs allowing lysis from without. Though, both *Campylobacter* phages did not lyse their host in chicken meat at 4°C (Figure 3A and B). By contrast, phage PY100 reduced the *Y. enterocolitica* cell numbers in pork meat significantly by approximately 2 log₁₀ units after 24hr (Figure 3C).

DISCUSSION

In this study the potential of the three phages to control foodborne pathogens at the post-harvest level was analysed. We ascertained that at 37°C the *Campylobacter* phages CP81 and NCTC12684 reduced the cell numbers of their respective host in broth, whereas no reduction was observed at 4°C, even at a high MOI (10² resp 10⁴). The phages probably did not cause lysis from without under these conditions. Other studies demonstrated phage-induced lysis of *Campylobacter* at 4°C, where an up to 1 log₁₀ unit reduction on chicken skin and in cooked and raw meat was achieved (Atterbury et al, 2003; Goode et al, 2003; Bigwood et al, 2008). As the lysis of *Campylobacter* at refrigeration temperatures is rather limited, post-harvest application of phages is apparently not a promising tool to reduce the *Campylobacter* load on carcasses or meat. On the other hand, high *Campylobacter* cell number reductions (2-5 log₁₀ units) by phage were obtained in chickens at the pre-harvest level (Loc Carrillo et al, 2005; Wagenaar et al, 2005; El-Shibiny et al, 2009).

Contrary to the *Campylobacter* phages, *Yersinia* phage PY100 significantly reduced cell numbers of its host at 4°C in broth and in pork meat. The most efficient reductions

occurred at the highest MOI. The data are consistent with previously published studies, in which a control of various pathogens was accomplished by application of high phage numbers (Goode et al, 2003; Leverentz et al, 2004; O'Flynn et al, 2004; Carlton et al, 2005; Guenther et al, 2009). To our knowledge, this is the first report on an application of phage to reduce *Yersinia* cell numbers in food.

CONCLUSION

An application of virulent bacteriophages for the control of *Y. enterocolitica* at the post-harvest level seems to be promising, whereas the potential of phages to control *Campylobacter* in food appears to be limited.

COMPETING INTERESTS

None declared.

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