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ORIGINAL ARTICLE

Impacts of treatment parameters on the inactivation of *Campylobacter jejuni* by high pressure: a statistical study of main effects and interactions

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Abstract

Aim: The influence of environmental (temperature and pH) and biological (strain) parameters on the inactivation of *Campylobacter jejuni* by high hydrostatic pressure (HHP) was investigated.
Methods and Results: Two clinical strains harvested in stationary phase were pressurized at 20°C and 37°C within a range of 50–400 MPa, in a phosphate (pH 7·0) or a citrate phosphate buffer (pH 5·6), for 10 min. Treatment efficiencies were determined by logarithmic comparisons of culturable cells on blood agar before and after treatment. Results were statistically compared using an ANOVA of culturable cells after treatment to evaluate the effect of all factors. At least a 7-log reduction in cell numbers was observed for both strains. The pH and the strains had no effect on HHP treatment at 20°C while at 37°C, both pH and strain influenced significantly the HHP treatment on *C. jejuni*.
Conclusions: The pressure efficacy on *C. jejuni* eradication was affected by both environmental and biological factors.
Significance and Impact of the Study: Depending on the treatment conditions, *C. jejuni* sensitivity to HHP can significantly vary. The determination of the inactivation treatment by HPP has to be normalized considering the interaction of environmental and biological factors.

Introduction

Campylobacteriosis has been recognized as the major bacterial foodborne infection worldwide. The incidence rates of this infection vary considerably according to countries: 12·9/100 000 confirmed cases were observed in the USA in 2004, while in northern European countries the incidence rate fluctuated between 60 and 90/100 000 (Murphy *et al.* 2006). Among the four pathogenic species, *Campylobacter jejuni* is responsible for 75–95% of campylobacterioses (Federighi 1999; Park 2002). In few cases, *C. jejuni* can cause complications as Guillain-Barré and Miller-Fisher syndromes (Young *et al.* 2007). Poultry meat products and raw milk are assumed to be the main food vectors for human campylobacteriosis (Federighi 1999; Solomon and Hoover 2004).

To inactivate foodborne pathogens in food products, preservation treatments have been recently developed. For the past few years, consumer demands oriented towards more 'fresh' and more 'natural' food have stimulated the development of alternative technologies which are used at ambient temperature, protecting heat-sensitive properties of food material like flavour, colour and nutrient value (Hayashi 1996). Among these nonthermal treatments, high hydrostatic pressure (HHP) is a promising preservative technology (Gould 2002). As traditional technologies used for food preservation, HHP treatment performance is evaluated by the ability to inactivate pathogen microorganisms in food.

The efficiency of HHP on inactivating vegetative bacteria depends on the conditions of application including temperature, procedures of pressurization and medium

properties and on the target micro-organisms considering its physiological state (Hoover *et al.* 1989; Mañas and Pagán 2005; Chapleau *et al.* 2006). Several studies have been carried out to compare sensitivity of pathogenic bacteria in different conditions (Alpas *et al.* 2000; Farkas and Hoover 2000; Patterson 2005; Chen 2007). Among the studied pathogens, *Campylobacter* spp have been rather neglected and only a few parameters have been evaluated, specifically intensity of the applied pressure, storage temperature after treatment, protective effect of milk and/or meat, growth phase and strain effect (Shigehisa *et al.* 1991; Solomon and Hoover 2004; Martinez-Rodriguez and Mackey 2005a,b). There is an apparent low resistance of *Campylobacter* spp. to high pressure (Shigehisa *et al.* 1991; Solomon and Hoover 2004). Furthermore, Martinez-Rodriguez and Mackey (2005b) observed that, conversely to other stresses, maximum resistance of *Campylobacter* spp. at high pressure occurred in early stationary phase. More recently, Lori *et al.* (2007) have developed a predictive model for inactivation of *C. jejuni* EB 1410/02 and *C. coli* A 19268/03 by heat and pressure in chicken meat for different treatment times. For *C. jejuni* EB 1410/02, temperature during pressurization can have a synergetic effect ($T > 45$°C), an antagonist effect ($T < 15$°C) or a protective effect on cell inactivation. The most protective temperature depends on the pressure treatment time: about 35°C for a 30-s holding time and 20°C for 8 min.

Besides these studies, there is little information on the variation in pressure resistance of *C. jejuni* according to experimental conditions. The purpose of this study was to investigate the effects of several combinations of factors (pressure intensity, temperature, pH and strain) on the inactivation of *C. jejuni*.

Materials and methods

Bacterial strains and growth conditions

Two clinical strains were studied. *C. jejuni* NCTC 11168 was isolated from a patient (Skirrow 1977) while *C. jejuni* 81–176, a particularly virulent strain (Hofreuter *et al.* 2006), was isolated in 1985 during an outbreak caused by the consumption of contaminated milk (Korlath *et al.* 1985).

Strains were stored at −70°C in brain heart infusion (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 15% glycerol (Nutri-Service, Villefranche-de-Rouergue, France). Prior to each experiment, cells were subcultured onto Columbia blood agar (Merck, Darmstadt, Germany) supplemented with 5% defibrinated horse blood (bioMerieux, Marcy l'Etoile, France) during 48 h, then one grown colony was suspended in 50 ml of Mueller-Hinton broth (MHB, Merck) and incubated for 24 h. Cultures were obtained by inoculating 50 ml of MHB with 0·5 ml of the last subculture and incubated for 16 h. All subcultures and cultures were grown at 42°C in microaerobic atmosphere: 5% O_2, 10% CO_2 and 85% N_2.

Pressure treatments

Two pH buffers were tested. Phosphate buffer at pH 7·0 [0·2 mol l^{-1} Na_2HPO_4 (Merck) and 0·2 mol l^{-1} NaH_2PO_4 (Merck)] and citrate phosphate buffer at pH 5·6 [0·2 mol l^{-1} Na_2HPO_4 and 0·1 mol l^{-1} citric acid (Merck)] were diluted using 1 ml of the final culture in 9 ml of the appropriate buffer in sterile polyethylene bags (AES, Combourg, France). Bags were then sterilely sealed and maintained at 4°C.

Pressurizations were carried out at 20 ± 2°C and 37 ± 3°C into the thermostated vessel of a 3·5-l reactor unit (ACB Pressure Systems, Nantes, France). Aluminium cylinders were used to reduce the volume of the pressure vessel (i.e. reduce time of pressurization) to 1·95 l and to reduce heating of sample during pressurization. Each sample was exposed to 50, 100, 150, 200, 250, 300, 350 and 400 MPa for 10 min. The come-up time to reach the target value lasted less than 1 min and the pressure release was almost immediate (4 s). Pressure holding time reported in this study does not include the pressure come-up or de-pressurization times. Three independent trials were performed.

Viable counts

The number of culturable cells was determined before and after treatment. Cell suspensions were serially diluted in buffered peptone water, pH 7·0 (Merck) and 0·1 ml volumes were twice plated on blood agar plates. For low numbers of survivors, 1 and 5 ml of treated suspensions were laid down on sterile plates and covered with blood agar. Plates were incubated 48 h at 42°C in microaerobic atmosphere.

Statistical analysis

In order to determine the individual effect of each variable and potential interacting effect during high pressure treatments, the first- and second-order interactions of the four studied variables (pressure, temperature, pH and strain) were calculated based on the values obtained from the three independent trials. Results were analyzed with Statgraphics Plus 5.1 software (StatPoint Inc., Herndon, Virginia, USA) using the General Linear Models designed to obtain ANOVA. The significance level was chosen at 95%; thereby an effect was significant if its P-value was lower than 0·05.

Results

Results of *C. jejuni* pressurizations were analyzed in terms of log (N/N_0), where N is the number of culturable cells of the experimental sample after treatment and N_0 the number of culturable cells without treatment. The control N_0 was achieved in the same buffer and exposed 10 min at 20°C or 37°C at atmospheric pressure. Figure 1 shows log (N/N_0) according to the applied pressure at 20°C (Fig. 1a) and 37°C (Fig. 1b), for both strains and both pHs. Inactivations performed at 20°C did not show any significant difference between the tested conditions (Fig. 1a). No *C. jejuni* colony was detected after 10 min pressurization at 400 MPa and 20°C, whatever the strain and the pH. However, when pressurizations were performed at 37°C, dramatic differences were observed for both strains (Fig. 1b). The decrease of pH from 7·0 to 5·6 emphasized *C. jejuni* sensitivity to HHP: the killing effect began at lower pressure and the >7-log inactivation was shifted by 50 MPa. A strain effect was also noticed; NCTC 11168 was more sensitive to pressure than 81–176.

The ANOVA was performed on log (N/N_0) values, with pressure, temperature, pH and strain as factors. As the P-value of the general linear statistical model fittings to results was less than 0·01, the correlation between inactivation and model parameters was statistically significant at a 99% confidence level (Table 1). P-values of all main effects were less than 0·01, and so all parameters were significant at a 99% confidence level (Table 2). All second-level interactions were also significant, except the interaction temperature × pH which P-value rated 0·068, so lightly higher than 0·05.

The weight of each significant factor and interaction is estimated based on F-ratios. As expected, the utmost factor was pressure intensity. The strain effect and the pH effect were, respectively, the second (58·74) and the third (47·12) most important effects after the pressure. This is illustrated by the means for all log (N/N_0) obtained after treatments were −2·75 for NCTC 11168, −1·58 for 81–176, −2·67 for pH 5·6 and −1·66 for pH 7·0. These differences meant that 81–176 was more resistant than NCTC 11168 and that decrease of pH from 7·0 to 5·6 enhanced the treatment efficiency. The most important interaction was temperature × strain with an F-ratio of 40·65. Interaction plot for these two factors clearly showed that the increase of temperature from 20°C to

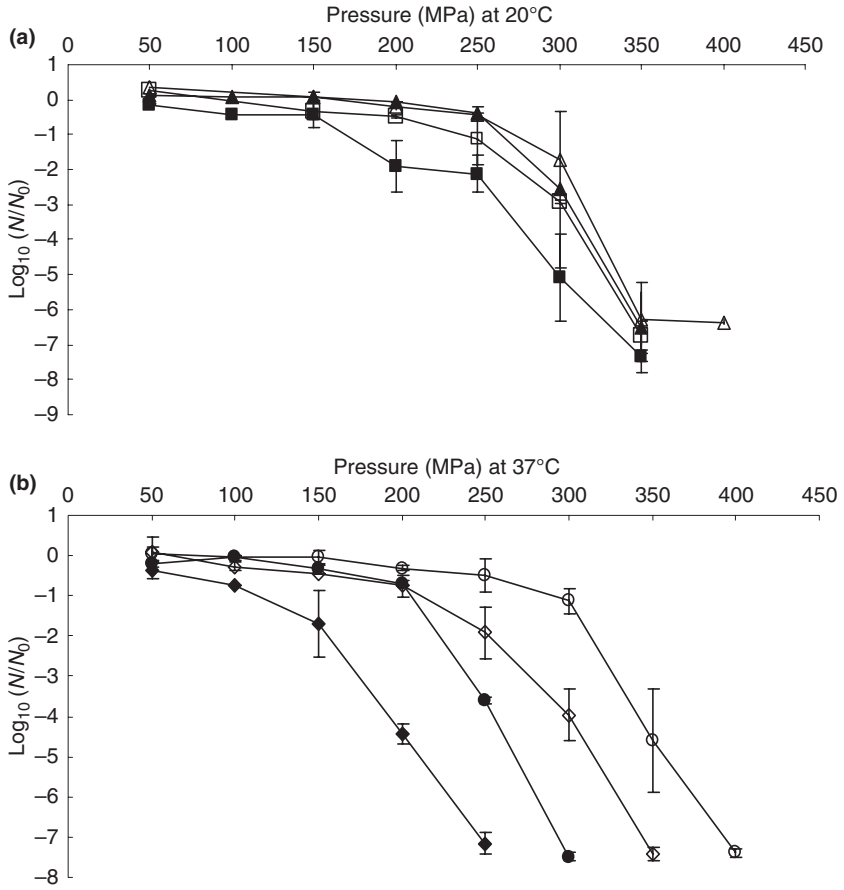

Figure 1 Inactivation of *Campylobacter jejuni* by high-pressure treatment during 10 min (a) at 20°C (NCTC 11168 at pH 7·0 (▲) and pH 5·6 (■), 81–176 at pH 7·0 (△) and pH 5·6 (□)) and (b) at 37°C (NCTC 11168 at pH 7·0 (●) and pH 5·6 (♦), 81–176 at pH 7·0 (○) and pH 5·6 (◇)). Error bars represent the standard deviation of three independent trials.

Table 1 Significance of linear model analysed by ANOVA

Source	Sum of squares	Df	Mean square	*F*-ratio	*P*-value
Model	816·719	30	27·224	45·28	<0·001
Residual	55·312	92	0·601		
Total (corrected)	872·031	122			

Table 2 Significance of factors and their interactions by ANOVA for log (N/N_0)

Source	Sum of squares	Df	Mean square	*F*-ratio	*P*-value
Main effects					
Pressure	578·505	6	96·418	160·37	<0·001
Temperature	12·123	1	12·123	20·16	<0·001
pH	28·331	1	28·331	47·12	<0·001
Strain	35·313	1	35·313	58·74	<0·001
Interactions					
Pressure × temperature	21·120	6	3·520	5·85	<0·001
Pressure × pH	10·692	6	1·782	2·96	0·011
Pressure × strain	38·942	6	6·490	10·80	<0·001
Temperature × pH	2·051	1	2·051	3·41	0·068
Temperature × strain	24·441	1	24·441	40·65	<0·001
pH × strain	4·074	1	4·074	6·78	0·011
Residual	55·312	92	0·601		
Total (corrected)	872·03	122			

R-squared (adjusted for Df) = 91·59%.

37°C induced inactivation for NCTC 11168 [log (N/N_0) shifted from −1·91 to −3·61], while it had no effect on 81–176 (data not shown).

Discussion

High-pressure processing has been found to be able to reduce Gram-negative food pathogens between 300 and 800 MPa (Hoover *et al.* 1989; Farkas and Hoover 2000). As expected, *C. jejuni* is dramatically reduced in this range of pressure, more than a 7-log reduction of culturable cells of NCTC 11168 and 81–176 occurred after 10 min treatment under 400 MPa in all test conditions. This confirms the particular sensitivity of this species to pressure (Shigehisa *et al.* 1991; Solomon and Hoover 2004).

In this study, four parameters were combined: pressure intensity, temperature of treatment, pH of medium and strain. To avoid any effects from the food matrix, the experiments were conducted using cell suspensions. The increase of temperature from 20°C to 37°C, or the decrease of pH from 7·0 to 5·6 did not have any inactivating effect on either test strain at atmospheric pressure. Conversely, after HHP treatment, the *C. jejuni* sensitivity increased with the temperature and the acidity of the medium.

The loss of resistance to HHP in acidic media has been already described for other foodborne pathogens like *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* (Hoover 1993; Alpas *et al.* 2000; Ritz *et al.* 2000; Lado and Yousef 2002). This has been explained by the fact that pressure favoured the nondissociated acid form, which enhanced the antimicrobial activity of organic acids (Smelt 1998). The synergetic effect of the temperature and high pressure was usually explained by a higher degree of damage on proteins (Mañas and Pagán 2005). In our work, a clear strain effect was detected in *C. jejuni*, with a higher resistance to HPP by *C. jejuni* 81–176 than by NCTC 11168. Similar results were obtained by Martinez-Rodriguez and Mackey (2005a) on six strains of *C. jejuni* at ambient temperature. However, no statistical analysis was performed. In our study, strain effect and second-order interactions between strains and the three other parameters were statistically proved.

Our results showed that significant differences between the two pHs appeared only at 37°C for both NCTC 11168 and 81–176. In addition, the strain effect, which was absent at 20°C, was revealed at 37°C. In spite of the statistical nonsignificance of the interaction temperature × pH ($P = 0{\cdot}068$), the change of temperature from 20°C to 37°C revealed a greater inactivation at pH 5·6 than at pH 7·0. This phenomenon could be explained by an influence of the temperature on the interaction pressure × pH, and the synergetic effect of the temperature and the acidity on the killing of *C. jejuni* by HPP could be attributable to the damage of proteins, including enzymes that could be involved in the response to acidic stress. The differences observed between NCTC 11168 and 81–176 behaviours at the two temperatures were not expected. Almost no difference was observed in 81–176 inactivation at pH 5·6 and 7·0 between 20°C and 37°C, while the increased sensitivity of NCTC 11168 respective to the increase of temperature was significant. While strain 81–176 has been reported to be clearly sensitive to treatment temperature during the oxidative stress (Garénaux *et al.* 2008), our results showed that 81–176 was much less sensible to temperature variation during HHP treatment. The absence of a temperature sensitizing effect, as we have found with 81–176, has never been described previously, to our knowledge, on bacterial foodborne pathogens after HPP treatment.

This study indicates that *C. jejuni* inactivation by HHP was strongly dependent on environmental conditions. Moreover, a single strain could behave similarly or differently to another strain according to the environmental factors. These aspects should be considered in the optimization

of HHP processing to reduce this pathogen, as an improvement of the inactivation of one strain may be ineffective for other strains.

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References

Alpas, H., Kalchayanand, N., Bozoglu, F. and Ray, B. (2000) Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *Int J Food Microbiol* **60**, 33–42.

Chapleau, N., Ritz, M., Delépine, S., Jugiau, F., Federighi, M. and de Lamballerie, M. (2006) Influence of kinetic parameters of high pressure processing on bacterial inactivation in a buffer system. *Int J Food Microbiol* **106**, 324–330.

Chen, H. (2007) Use of linear, Weibull and log-logistic functions to model pressure inactivation of seven foodborne pathogens in milk. *Food Microbiol* **24**, 197–204.

Farkas, D.F. and Hoover, D.G. (2000) High pressure processing. *J Food Sci Suppl*: Kinetics of Microbial Inactivation for Alternative Food Processing Technologies **65**, 47–64.

Federighi, M. (1999) *Campylobacter et hygiène des aliments*. Paris: Polytechnica.

Garénaux, A., Jugiau, F., Rama, F., de Jonge, R., Denis, M., Federighi, M and Ritz, M. (2008) Survival of *Campylobacter jejuni* strains from different origins under oxidative stress conditions: effect of temperature. *Curr Microbiol* **56**, 293–297.

Gould, G.W. (2002) The evolution of high pressure processing of foods. In *Ultra High Pressure Treatments of Foods* ed. Hendrickx, M.E.G. and Knorr, D. pp. 3–21 New York: Kluwer Academic/Plenum Publisher.

Hayashi, R. (1996) An overview of the use of high pressure in bioscience an biotechnology. In *High Pressure Bioscience and Biotechnology* ed. Hayashi, R. and Balny, C. pp. 1–6 Tokyo: Elsevier, Progress in Biotechnology.

Hofreuter, D., Tsai, J., Watson, R.O., Novik, V., Altman, B., Benitez, M., Clark, C., Perbost, C. *et al.* (2006) Unique feature of a highly pathogenic *Campylobacter jejuni* strain. *Infect Immun* **74**, 4694–4707.

Hoover, D.G. (1993) Pressure effects on biological systems. *Food Technology* **47**, 150–155.

Hoover, D.G., Metrick, C., Papineau, A.M., Farkas, D.F. and Knorr, D. (1989) Biological effects of high hydrostatic pressure on food microorganisms. *Food Technol* **43**, 99–107.

Korlath, J.A., Osterholm, M.T., Judy, L.A., Forfang, J.C. and Robinson, R.A. (1985) A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J Infect Dis* **152**, 592–596.

Lado, B.H. and Yousef, A.E. (2002) Alternative food-preservation technologies: efficacy and mechanisms. *Microbes Infect* **4**, 433–440.

Lori, S., Buckow, R., Knorr, D., Heinz, V. and Lehmacher, A. (2007) Predictive model for inactivation of *Campylobacter* spp. by heat and high hydrostatic pressure. *J Food Prot* **70**, 2023–2029.

Mañas, P. and Pagán, R. (2005) Microbial inactivation by new technologies of food preservation. *J Appl Microbiol* **98**, 1387–1399.

Martinez-Rodriguez, A. and Mackey, B.M. (2005a) Factors affecting the pressure resistance of some *Campylobacter* species. *Lett Appl Microbiol* **41**, 321–326.

Martinez-Rodriguez, A. and Mackey, B.M. (2005b) Physiological changes in *Campylobacter jejuni* on entry into stationary phase. *Int J Food Microbiol* **101**, 1–8.

Murphy, C., Carroll, C. and Jordan, K.N. (2006) Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J Appl Microbiol* **100**, 623–632.

Park, S.F. (2002) The physiology of Campylobacter species and its relevance to their role as foodborne pathogens. *Int J Food Microbiol* **74**, 177–188.

Patterson, M.F. (2005) Microbiology of pressure-treated food. *J Appl Microbiol* **98**, 1400–1409.

Ritz, M., Jugiau, F., Rama, F., Courcoux, P., Semenou, M. and Federighi, M. (2000) Inactivation of *Listeria monocytogenes* by high hydrostatic pressure: effects and interactions of treatments variables studied by analysis of variance. *Food Microbiol* **17**, 375–382.

Shigehisa, T., Ohmori, T., Saito, A., Taji, S. and Hayashi, R. (1991) Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. *Int J Food Microbiol* **12**, 207–216.

Skirrow, M.B. (1977) *Campylobacter enteritis* : a ''new'' disease. *Br Med J* **2**, 9–11.

Smelt, J.P.P.M. (1998) Recent advances in the microbiology of high pressure processing. *Trends Food Sci Technol* **9**, 152–158.

Solomon, E.B. and Hoover, D.G. (2004) Inactivation of *Campylobacter jejuni* by high hydrostatic pressure. *Lett Appl Microbiol* **38**, 505–509.

Young, K.T., Davis, L.M. and DiRita, V.J. (2007) *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol* **5**, 665–679.

