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

C. Bièche¹,², M. Ritz¹, O. Tresse¹, M. Federighi¹ and M. de Lamballerie²

¹ UMR-INRA 1014 SECALIM ENVN/ENITIAA, Ecole Nationale Vétérinaire de Nantes, Nantes, France
² UMR-CNRS 6144 GEPEA, ENITIAA, Nantes, France

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

Keywords

*Campylobacter jejuni*, high pressure, pH, temperature.

Correspondence

Marie de Lamballerie, UMR-CNRS 6144 GEPEA, ENITIAA, Rue de la Géraudière, BP 82225, F-44322 Nantes, Cedex 3, France.

E-mail: marie.de-lamballerie@enitiaa-nantes.fr

2008/0883: received 23 May 2008, revised and accepted 2 October 2008

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.
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; Martínez-Rodríguez 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, Martínez-Rodríguez 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 microaerobics atmosphere: 5% O2, 10% CO2 and 85% N2.

Pressure treatments

Two pH buffers were tested. Phosphate buffer at pH 7·0 [0·2 mol l−1 Na2HPO4 (Merck) and 0·2 mol l−1 NaH2PO4 (Merck)] and citrate phosphate buffer at pH 5·6 [0·2 mol l−1 Na2HPO4 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 microaerobics 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 \( \left( N/N_0 \right) \), 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 \( \left( N/N_0 \right) \) according to the applied pressure at 20°C (Fig. 1a) and 37°C (Fig. 1b), for both strains and both pHs. Inactuations 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 \( \left( N/N_0 \right) \) 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 \( \times \) 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 \( \left( N/N_0 \right) \) obtained after treatments were \(-2\cdot75\) for NCTC 11168, \(-1\cdot58\) for 81–176, \(-2\cdot67\) for pH 5–6 and \(-1\cdot66\) 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 \( \times \) 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.
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 = 0.068), 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.

Acknowledgements

The authors wish to thank Dr Philippe Courcoux for his assistance with the statistical analyses and Florence Jugiau and Florence Rama for their technical support.

References


