Effects of decontamination at varying contamination levels of Campylobacter jejuni on broiler meat

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ABSTRACT  When assessing effects of decontamination techniques on counts of Campylobacter spp. on broiler meat, it is essential that the results reflect the variations that may exist. Decontamination studies often use high inoculation levels (10^7 to 10^8 cfu) and one or few strains of Campylobacter jejuni, thereby restricting the results to reflect only a limited part of the true situation. This study presents results from physical and chemical decontamination of broiler meat medallions using different strains and different initial concentrations of C. jejuni. For 3 strains of C. jejuni, mean log reductions obtained by freezing at −20°C for 7 d was significantly higher for an initial concentration of 10^7 cfu/sample on the meat compared with an initial concentration of 10^3 cfu/sample. For freezing at −20°C for 24 h or application of 6% tartaric acid and subsequent storage for 24 h, no statistically significant difference in reductions was found for initial concentrations ranging from 10^3 to 10^7 cfu per sample. The mean log reductions obtained by all techniques were strongly dependent on the strain tested. The results reveal that reductions obtained with high inoculation levels of C. jejuni (10^7 cfu/sample) or single or few strains of the species (or both) should not be interpreted as a generic result for the species. If inoculation studies cannot be replaced by investigations of naturally contaminated meat, we advise using a mixture of strains found in the production environment at levels as close as possible to the natural contamination level.

Key words: Campylobacter, inoculation, contamination, decontamination, broiler meat

INTRODUCTION

Thermotolerant Campylobacter species, in particular Campylobacter jejuni, continue to be the most common cause of human gastrointestinal disease in Europe (European Food Safety Authority, 2011). In risk factor studies, poultry meat (Neimann et al., 2003; Friedman et al., 2004), especially fresh, unfrozen broiler meat, has been identified as the major risk factor for campylobacteriosis (Wingstrand et al., 2006). As a consequence, several countries have developed quantitative microbiological risk assessments for Campylobacter in the broiler production chain to support management decisions regarding the control of this pathogen (Hartnett et al., 2001; Rosenquist et al., 2003; Nauta et al., 2005, 2007; Lake et al., 2006). These risk assessments conclude, among other things, that reducing the Campylobacter concentration on the meat will have a considerable impact on the number of human cases associated with broiler meat. Consequently, decontamination of broiler meat during processing has gained increased interest as a tool to reduce the number of foodborne illnesses caused by Campylobacter.

One of the benefits of quantitative microbiological risk assessments is that the potential effects of control measures in the broiler production chain can be assessed. However, to obtain realistic risk estimates, the input data should be realistic. For example, decontamination studies should preferably be carried out in industrial scale using naturally contaminated meat (FAO/WHO, 2008; EFSA Panel on Biological Hazards (BIO-HAZ), 2010). However, this is not always feasible, and databases on artificially inoculated samples in pilot or laboratory scale are often generated, for instance when testing newly developed equipment for decontamination or new nonapproved chemical solutions. Multiple studies on the reduction of Campylobacter on broiler meat by physical and chemical decontamination at slaughter using artificial inoculation have been published (Park et al., 2002; Bhaduri and Cottrell, 2004; Özdemir et al., 2006; Corry et al., 2007; James et al., 2007; Riedel et al., 2009; Chun et al., 2010). However, the influence of different inoculation levels on reductions is rarely included in the investigations. This issue can be of great importance if the magnitude of reduc-
Preparation of Broiler Meat

In the vast majority of decontamination studies, which investigated decontamination of broiler meat, the initial inoculation levels have been high [i.e., around 10^7 to 10^8 cfu (Patterson, 1995; Lee et al., 1998; Zhao et al., 2003; Bhaduri and Cottrell, 2004; Kim et al., 2005; Zhao and Doyle, 2006; James et al., 2007; Ritz et al., 2007; El-Shibiny et al., 2009; Riedel et al., 2009; Chun et al., 2010)]. Hence, there is little information on whether or not a similar reduction can be expected when initial numbers are low.

Furthermore, a great proportion of decontamination studies only consider the effect on a single strain of *C. jejuni* (Lee et al., 1998; Park et al., 2002; Corry et al., 2007; James et al., 2007; Ritz et al., 2007; Riedel et al., 2009; Chun et al., 2010). In some cases, though, mixtures of more than one strain have been used (Zhao et al., 2003; Bhaduri and Cottrell, 2004; Kim et al., 2005; Zhao and Doyle, 2006; El-Shibiny et al., 2009), and in other cases different strains have been applied individually (Patterson, 1995; Özdemir et al., 2006), allowing for evaluation of strain variation. Studies of organic acid treatment and irradiation found statistical differences in reductions of varying significance between strains (Patterson, 1995; Birk et al., 2010), whereas no strain variation was found in a study evaluating acidified sodium chloride and trisodium phosphate (TSP; Özdemir et al., 2006). If effects of decontamination vary significantly for different strains of *C. jejuni*, strain variation will have to be considered when planning and evaluating decontamination studies.

The aim of the present study was to investigate if the magnitude of reductions obtained by physical (freezing for 24 h or 7 d) or chemical decontamination (application of 6% tartaric acid for 24 h or 10% TSP for 15 s) were influenced by the initial concentration of *Campylobacter* on the broiler meat and further if this reduction was strain specific.

**MATERIALS AND METHODS**

**Experimental Design**

The studies were carried out using a previously developed broiler meat model (Birk et al., 2010). Three different strains of *C. jejuni* were each inoculated onto medallions of broiler meat in the approximate levels: 10^3, 10^4, 10^5, and 10^7 cfu/sample, and challenged by physical decontamination (represented by freezing) and chemical decontamination (represented by application of tartaric acid or TSP). Experiments were carried out in triplicate.

**Preparation of Broiler Meat**

The study used frozen skinless breast fillets from *Campylobacter* negative broiler flocks, which were provided by a Danish slaughter plant. The *Campylobacter* status was determined by PCR analysis of cloacal swabs taken upon arrival at the abattoir. In every experiment, 2 pieces of meat were analyzed to verify the *Campylobacter*-negative status of the fillets. Frozen broiler breast fillets were thawed at ambient temperature for approximately 8 h and subsequently stored at 5°C until use (overnight). Meat medallions of 9.6 cm² were cut aseptically from the chicken breast fillets using a cork borer, and each piece of broiler meat was placed on gauze in a Petri dish.

For the freezing experiments, 26 medallions were prepared per replicate: 4 dilutions and 2 freezing treatments in duplicate, plus 8 unfrozen medallions, and 2 medallions to confirm the *Campylobacter*-negative status of the meat before inoculation. For each of the experiments with TSP or tartaric acid, 18 medallions were prepared per replicate: 4 dilutions in duplicate with and without treatment, plus 2 medallions to confirm the *Campylobacter*-negative status of the meat before inoculation.

**Preparation of Inoculum**

Three different *C. jejuni* strains were included in the study: NCTC 11168 (genome sequenced clinical human isolate from the National Collection of Type Cultures), 305 (genome sequenced poultry isolate considered robust to acid stress (Takamiya et al., 2011b), and 327 (genome sequenced poultry isolate considered sensitive to acid stress (Takamiya et al., 2011a). Inocula were prepared as previously described by Birk et al. (2010). Inoculum was adjusted to an optical density of 0.1 at 600 nm, corresponding to approximately 8 log_{10} cfu/mL and consecutively 10-fold diluted to a final concentration of 10^4 cfu/mL.

**Inoculation**

For each strain, 4 meat medallions were inoculated per decontamination technique: 2 for treatment and 2 for untreated controls. The medallions were stored in Petri dishes. Samples were inoculated by spreading 100 μL of inoculum of appropriate dilutions onto the top surface of the meat medallions with a pipette to obtain initial concentrations of the approximate levels: 10^3, 10^4, 10^5, and 10^7 cfu/sample. To allow for the cells to attach, the fillet pieces were left at ambient temperature for 30 min. The exact numbers of *Campylobacter* in the initial cultures were determined (cfu/mL), and numbers on medallions before treatment were verified by enumeration to confirm the approximate levels. After inoculation, samples were treated according to one of the following treatments.

**Treatment**

Freezing was chosen as a physical measure, commonly known to reduce *Campylobacter*. Samples were
placed in a freezer at \(-20^\circ C\) for 24 h or 7 d wrapped in a plastic bag to prevent desiccation.

Tartaric acid was chosen as an organic acid, which is allowed to be used as a food additive if labeled. The potential to reduce Campylobacter was therefore evaluated after storage for 24 h. Two hundred microliter volumes of 6% L(+) tartaric acid (Riedel-de Hae\~n) or (for controls) sterile water were spread over the top surface of the medallions with a pipette. The medallions were stored for 24 h before enumeration. Storage was aerobic at 4 to 5°C at high humidity to prevent desiccation.

Trisodium phosphate was chosen as an inorganic alkaline agent, as an example of a chemical decontamination aid, which according to regulation should be rinsed off carcasses using water. The potential to reduce Campylobacter was therefore evaluated immediately after treatment. Treatment with TSP (Sigma-Aldrich) was carried out by dipping medallions in a 10% (wt/vol, 25°C) solution for 15 s. Similarly, control samples were dipped in sterile water.

**Microbiological Analyses**

Counts of Campylobacter on medallions were determined by placing 2 meat medallions plus the underlying gauze in a stomacher bag with filter (Bie & Berntsen A/S, Herlev, Denmark) and adding 10 mL of maximum recovery diluent (CM733, Oxoid). The meat was homogenized in a stomacher 400 (Colworth) for 2 min. The homogenized samples were diluted 10-fold in maximum recovery diluent, and 20 μL of the dilution was spotted in 5 spots onto Campylobacter selective Abeyta-Hunt-Bark agar plates containing 1% triphe-nyl-tetrazolium chloride (Rosenquist et al., 2006; detection limit: 100 cfu/mL) and when required 20 spots to lower the detection limit to 25 cfu/mL. All plates were incubated under microaerobic conditions for 44 to 48 h at 41.5 ± 1°C. The Campylobacter colonies on the plates were counted, and cfu per sample were estimated. Colonies were confirmed as Campylobacter by microscopy of 5 colonies.

**Statistical Analysis**

Before analysis, Campylobacter counts (cfu/sample) were converted to log_{10} values to approximate the data to normal distributions. Data below the detection limit were set to one half of the detection limit. This was the case for only 2 samples, and in each case the duplicate was countable.

Models for ANOVA were carried out using the GLM procedure within the SAS Enterprise Guide statistical software, version 3.0 (SAS Institute Inc., Cary, NC). The significance of reduction was determined using a model including strain and initial inoculation level as classification variables and the interaction. The LSMEANS statement with the PDIFF option was used to estimate pairwise means comparisons between mean reductions for different inoculation levels. An α-value of 0.05 was used as the level of significance.

**RESULTS**

Mean log reductions obtained by freezing for 24 h and application of tartaric acid were not significantly dependent on the initial concentration of the 3 C. jejuni strains inoculated onto the meat medallions (P-values 0.256 to 0.488; Table 1). Reductions obtained by freezing for 7 d, however, were significantly dependent on the initial concentration (P = 0.036), i.e., a concentration of 10^7 C. jejuni on the meat medallions resulted in a statistically higher log reduction than a concentration of 10^3 cfu. The same was seen for TSP using the pairwise comparison, though only close to being statistically different in the GLM analysis (P = 0.064; Table 1). The difference in reductions was only evident for the lowest (10^3 cfu/sample) and the highest level of inoculation (10^7 cfu/sample); i.e., similar reductions were obtained for inoculation levels ranging from 10^3 to 10^7 cfu.

Mean log reductions obtained by all techniques were strongly dependent on strain (P-values <0.001 to 0.031; Table 1), i.e., significantly different reductions were obtained for the 3 C. jejuni strains included in the study. None of the strains was consistently more sensitive to all treatments than the others.

**DISCUSSION**

Several published decontamination studies on methods to reduce counts of Campylobacter on broiler meat, both chemical (Kim et al., 2005; Zhao and Doyle, 2006; Riedel et al., 2009) and physical (Patterson, 1995; Lee et al., 1998; Zhao et al., 2003; Bhaduri and Cottrell, 2004; James et al., 2007; Ritz et al., 2007; El-Shibiny et al., 2009; Chun et al., 2010), have used artificial inoculation. To our knowledge, only one other study has looked into different inoculation/contamination levels (Greer and Dilts, 1992). Therefore, the influence of inoculation level on the magnitude of reduction of Campylobacter on meat has not been well documented. When assessing the impact of a given control measure, for example in quantitative risk assessments, a realistic level of reduction is crucial to obtain credible risk estimates (Nauta et al., 2009).

In the present study, the differences in reductions were within a range of 0.5 logs. Statistical difference was not observed for freezing for 24 h or treatment with tartaric acid. Hence, for these latter treatments, it could be reasonable to conclude that equal log reductions are obtained for different initial contamination levels on the meat. Similar observations have been reported regarding C. jejuni on lean beef treated with organic acids where the magnitude of reduction varied inconsistently in relation to the initial inoculation level (Greer and Dilts, 1992). Nevertheless, this study also
revealed that reductions obtained with inoculation levels of $10^7$ to $10^8$ cfu should be interpreted with care because in some cases they may lead to a higher effect of decontamination than seen for lower levels of *C. jejuni*.

Significant strain variation in treatment of broiler meat with organic acids has also been reported in other studies (Birk et al., 2010). This means that reductions obtained with one or few strains of *C. jejuni* should not be interpreted as a general result for the species.

Our results support the statements in the EFSA guidance document on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods [EFSA Panel on Biological Hazards (BIOHAZ), 2010] and the criteria in the FAO/WHO report on benefits and risks of the use of chlorine-containing disinfectants in food production and food processing [FAO/WHO, 2008; EFSA Panel on Biological Hazards (BIOHAZ), 2010], which state that to provide the best data for use in efficacy evaluations and risk assessments, decontamination studies should preferably be carried out in industrial scale using naturally contaminated meat. Implicit in this are natural contamination levels and more strains of *Campylobacter*. If, for some reason, investigations of naturally contaminated meat cannot replace inoculation studies, we advise using a mixture of strains found in the production environment at levels as close as possible to the natural contamination level.

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**Table 1.** Mean reductions (log10) in cfu per medallion (3 replicates) for 3 strains of *Campylobacter jejuni*, SEM, and corresponding *P*-values from GLM including all inoculation levels1,2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc.3</th>
<th>NCTC11168</th>
<th>305</th>
<th>327</th>
<th>P-value</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
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</tr>
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<td>$10^7$</td>
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<td>−1.08</td>
<td>0.02</td>
<td>−1.19</td>
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</table>

1Pdiff indicates the pairwise means comparisons between mean reductions for different inoculation levels.

2Effect of interaction between strain and level was tested for all treatments but was not statistically significant.

3Approximate level of concentration before treatment.

4Different letters indicate a statistical difference ($P > 0.05$).


