Survival of *Campylobacter jejuni* in different gas mixtures

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**Abstract**

*Campylobacter jejuni* in fresh chilled chicken meat is known to be a major risk factor for human gastrointestinal disease. In the present study, the survival under chilled conditions of different *C. jejuni* strains exposed to different gas mixtures usually used for gas packaging of food was examined. Bolton broth and fresh, skinless chicken fillets were inoculated with six and four strains, respectively, and exposed to the gas mixtures 70/30% O2/CO2, 70/30% N2/CO2, and 100% N2 (the latter only investigated in broth) at refrigeration temperature (4–5 °C). In broth culture, the strains survived significantly longer when exposed to 100% N2 and 70/30% N2/CO2 than in the oxygen-containing gas mixture, 70/30% O2/CO2 (P < 0.0001). For the two anaerobic gas mixtures, the reductions only reached 0.3–0.8 log10 CFU mL−1 within the same period. In the presence of oxygen, the numbers of *C. jejuni* were reduced by a minimum of 4.6 log10 CFU mL−1 over 21 days. When inoculated onto chicken fillets, the *C. jejuni* strains also died significantly faster in the oxygen-containing gas mixture, 70/30% O2/CO2 (P < 0.0001), reaching reductions of 2.0–2.6 log10 CFU g−1 after 8 days. In the gas mixture without oxygen (70/30% N2/CO2), no reductions were observed.

**Introduction**

*Campylobacter jejuni* is one of the leading zoonotic causes of human gastrointestinal disease in Europe and the United States. About 95% of the campylobactersiosis cases are caused by the species *C. jejuni* (Anonymous, 2005; European Food Safety Authority, 2006). In rare cases, campylobactersiosis may be associated with chronic sequelae such as the demyelating disease Guillain–Barre syndrome and Reiter syndrome (Allos, 2001). The major risk factor for human *Campylobacter* infections is believed to be chicken meat (European Food Safety Authority, 2005; Anonymous, 2005). In Denmark, this has been specified to chilled chicken meat (European Food Safety Authority, 2005; Anonymous, 2006). Red colour of meat, and nitrogen is a metabolically inert soluble in water and fat tissues, which induces bacteriostatic effects in many bacteria (Davies, 1995). Oxygen maintains the red colour of meat, and nitrogen is a metabolically inert gas (Gill, 1988; Davies, 1995).

A quantitative risk assessment on *C. jejuni* in chicken products concluded that reducing the concentration of *C. jejuni* on contaminated chicken meat could lower the number of human cases. It was estimated that a reduction in the human cases by a factor of 25 could be obtained by reducing the concentration of *C. jejuni* by a factor of 100, i.e. 2 log10 CFU g−1 (Christensen et al., 2001). It is, therefore, of great importance to investigate and optimize new or existing methods for reducing counts of *C. jejuni*.

In laboratory studies, the growth of *Campylobacter* has been found to be sensitive to O2 in concentrations above 10% while CO2 was found to stimulate growth (Bolton & Coates, 1983). However, little is known about the influence of different gases on the survival of this organism under chilled conditions. Like red meat, the majority of the chicken fillets for retail sale produced in Denmark is packed in a modified atmosphere containing 70% O2 and 30% CO2. How this atmosphere affects the survival of *C. jejuni* has not been elucidated. The gas mixtures most frequently used in modified atmosphere packaging are CO2, oxygen, and nitrogen in various ratios. CO2 is an active gas, highly soluble in water and fat tissues, which induces bacteriostatic effects in many bacteria (Davies, 1995).
packaging of food. The survival of *C. jejuni* was examined in broth and on fresh, skinless chicken fillets.

**Materials and methods**

**Bacterial strains**

For survival experiments in broth, six different *C. jejuni* strains were included: NCTC 11168 (clinical human isolate from the National Collection of Type Cultures), 5-1, 503, and 305 (turkey isolates kindly provided by Thomas Alter, Leipzig), plus MS 16597, and MS 17113 (chicken isolates from the Danish surveillance of *Campylobacter* in food). On the basis of their survival in broth, two susceptible strains (5-1 and 503) and two less susceptible strains (NCTC 11168 and 305) were selected for survival experiments on chicken fillets.

**Gases**

In broth, three gas mixtures were studied: 100% N₂, 70/30% N₂/O₂, and 70/30% O₂/CO₂ (Air Liquide, DK). The aerobic and anaerobic CO₂-containing mixtures, 70/30% N₂/CO₂ and 70/30% O₂/CO₂, were used for the experiments on chicken fillets.

**Survival in broth**

**Experimental design**

One trial per gas mixture was performed. In each trial, two parallel series (= duplicates) of six 100 mL Blue Cap bottles containing 20 mL Bolton broth (Oxoid CM983, UK) were placed in a water bath at 4 °C. The bottles were connected with silicone tubes (internal diameter 3 mm) separated with sterile filters (0.45 μ, Sartorius Minisart®). The gas/medium ratio was 3.33.

The gas was humidified in sterile water before being led through the series of bottles at a pressure of 1.5–2 bar. To saturate the broth, this was exposed to the gas for 1 h before inoculation. Saturation with the CO₂-containing gases resulted in a lowering of the pH of the broth from 7.1 to 5.95 ± 0.05. To obtain comparable conditions within the three trials, the pH of the Bolton broth was lowered with HCl (1 mol L⁻¹) in the experiment with 100% N₂. After inoculation, the gas was continuously led through the two series of bottles for a period of 21 days at a constant pressure with gentle stirring of each bottle by magnetic stirrers.

**Culture preparation and inoculation**

Cells from frozen stock cultures [−80 °C in brain heart infusion broth with 15% (v/v) glycerol solution] were plated onto blood agar base no. 2 (Oxoid CM271, UK) with 5% horse blood. Plates were incubated at 41 ± 1 °C in sealed gas jars (2.5 L, Merck) for 21 ± 3 h under microaerobic conditions (8–10% CO₂, 5–7% O₂, and N₂) obtained by Anaerocult®C (Merck 3824 90 99). Cells were harvested with a sterile swab and transferred to Bolton broth (Oxoid CM983, UK). The cell concentration was adjusted using spectrophotometry at 600 nm to an initial cell density of c. 9 log₁₀ CFU mL⁻¹. This suspension was transferred to Bolton broth to give an initial concentration of 8.5 ± 0.3 log₁₀ CFU mL⁻¹ and a total volume of 30 mL.

**Quantification of *C. jejuni***

At each sampling point, the numbers of *C. jejuni* in the bottles were estimated. Hundred microliters of each sample was diluted in 900 μL 0.9% physiological saline solution with 0.1% peptone (NaCl, Merck 1.06404.1000; Peptone, BD 211677). This initial dilution was subsequently serial 10-fold diluted at least six times and five times 10 μL of each dilution were spotted onto Abeya–Hunt–Bark (AHB) agar (Heart infusion broth, Oxoid CM1032; Yeast extract, Difco 0127–17; Agar, BD 214010) with 1% triphenyltetrazoliumchloride (Rosenquist et al., 2006). Plates were incubated at 41 ± 1 °C in sealed gas jars (2.5 L, Merck) for 48 ± 4 h under microaerobic conditions obtained as mentioned above.

**Examination of cell morphology**

Cell morphology was investigated using an Olympus BH-2 microscope (Olympus, Japan) with ×100 magnification and pictures were taken using a microscope-adaptable camera mounted on the microscope (Olympus DP11). Cell sizes were analysed using the picture processing program IMAGEJ (National Institute of Health). A total of 100 cells of each combination of strain and gas were analysed using the ‘Analyze particles’ function. The threshold was adjusted to optimize the analysis. In addition, cell motility was visually evaluated.

**Survival on chicken fillets**

**Preparation of chicken fillets and inoculation**

Chicken fillets from the same batch were obtained from Danpo A/S. A total of 120 pieces of chicken fillets aseptically cut into 9 × 4.5 × 1 cm³ (L × W × H) were placed in oxygen-impermeable, polyethyleneephthalate containers with the dimensions 13.5 × 7 × 3 cm³ (L × W × H). The incula were prepared as previously described, except for the fact that the initial concentration was adjusted to c. 7 log₁₀ CFU mL⁻¹. For each strain, 24 pieces of chicken fillets were inoculated by spreading 100 μL inoculum onto the top
surface with a pipette. To allow for diffusion of the cells, the fillets were left at ambient room temperature for 15 min. The initial concentration on the fillets was $4.6 \pm 0.2 \log_{10} \text{CFU mL}^{-1}$. This relatively high concentration was chosen to be able to detect reductions of three to four log units. Another 24 pieces remained uninoculated (controls).

Packaging and storage

A Traysealer T200 (Multivac Sepp, Hagemüller GmbH & Co. KG, Germany) was used for sealing and gas filling. The sealing was an APET foil with low oxygen permeability ($< 5 \text{ cm}^3 \text{ m}^{-2} \text{ 24 h}^{-1}$). Half of the chicken fillets were packed with 70/30% $N_2/CO_2$ and the other half with 70/30% $O_2/CO_2$. The gas/product ratio was 7. The chicken fillets were stored at 5°C without exposure to light.

Microbiological examination

At regular intervals, the numbers of $C.\ jejuni$ on two independent chicken fillets (= replicates) were estimated using a Nordic standard procedure (NMKL no. 119, revised August 2004). Chicken fillets were washed in buffered peptone water (BPW) (Oxoid CM509) 1:1 (w/w) by stomaching the fillets for 120 s. Then, serial 10-fold dilutions of 100 μL sample in 900 μL BPW were made and five times 10 μL from appropriate dilutions were spotted onto dried AHB plates containing 1% triphenyltetrazoliumchloride. Initial experiments determined the recovery of inocula to be 98.6 ± 0.9%.

The numbers of naturally occurring mesophilic aerobic bacteria, lactic acid bacteria, and the spoilage bacteria Brochothrix thermosphacta on the chicken fillets were determined by surface plate spreading of appropriate dilutions onto plate count agar (Difco 247 940) (NMKL no. 86), de Man, Rogosa, Sharpe (Oxoid CM361) (NMKL no. 140), and streptomycin thallous acetate actidione agar (Oxoid CM881) (NMKL no. 141), respectively.

Analysis of meat colour

The meat colour was analysed using a Minolta Colorimeter (CR-300 series, Japan). Triplicate measurements were carried out for each fillet.

Statistical analysis

Statistical analyses were conducted to determine significant effects using SAS Enterprise guide 3.0 (SAS Institute Inc., Cary, NC). All positive bacteriological counts were transformed to $\log_{10}$ before conducting an ANOVA using General Linear Models (procglm). Analyses included the parameters strain and time with the reduction of $C.\ jejuni$ as the dependent variable. $P < 0.05$ was considered to be statistically significant.

Results

Survival in broth

$Campylobacter\ jejuni$ survived significantly longer ($P < 0.0001$) in the presence of 100% $N_2$ and $N_2/CO_2$ than in the presence of $O_2/CO_2$. Exposed to the oxygen-containing atmosphere, the various $C.\ jejuni$ strains were reduced by at least $4.6 \log_{10} \text{CFU mL}^{-1}$ over 21 days. Some were even non detectable, i.e. a reduction of more than $7 \log_{10} \text{CFU mL}^{-1}$ within 15 days. In 100% $N_2$ and $N_2/CO_2$, counts of $C.\ jejuni$ were only reduced by 0.3–0.8 $\log_{10} \text{CFU mL}^{-1}$ during the same period (Fig. 1).

In $O_2/CO_2$, the survival varied significantly between strains from the second day of the study ($P = 0.0093$). The

![Fig. 1. Relative reduction of Campylobacter jejuni in Bolton broth at 4°C exposed to (a) 100% $N_2$, (b) 70/30% $N_2/CO_2$, and (c) 70/30% $O_2/CO_2$. Initial cell counts were $8.5 \pm 0.3 \log_{10} \text{CFU mL}^{-1}$. Exposed to 100% $N_2$ and $N_2/CO_2$, no significant differences between strains were observed. In $O_2/CO_2$, the survival varied significantly between strains from the second day of the study. NCTC 11168 (■), 5-1 (□), 503 (♦), 305 (○), MS 16597 (▲), MS 17113 (△). The data presented are means ± SEM ($n=2$).](https://academic.oup.com/femsle/article-abstract/266/2/152/564732)
strain MS 17113 was the least sensitive, followed by NCTC 11168 and 305. The strains 5-1 and 503 were the most sensitive. No strain variability was observed when exposed to 100% N2 and N2/CO2.

Image analysis of the *C. jejuni* cells indicated that cells exposed to O2/CO2 were slightly longer and less coiled than cells exposed to 100% N2 and N2/CO2 (data not shown).

**Survival on chicken fillets**

On chicken fillets, *C. jejuni* also survived longer in the presence of N2/CO2 compared with O2/CO2 (*P* < 0.0001). In the oxygen-containing gas mixture, counts of *C. jejuni* decreased by 2.2–3.1 log10 CFU g−1 in 11 days, most notably within the first 8 days (2.0–2.6 log10 CFU g−1). No reduction was observed in N2/CO2 (Fig. 2). No significant differences between the four strains were observed (*P* > 0.05) regardless of the gas mixture. All control samples examined were negative for *Campylobacter*.

Contrary to *C. jejuni*, the total number of mesophilic aerobic bacteria as well as the number of lactic acid bacteria on the chicken fillets seemed independent of packaging gas as their numbers increased similarly in both gas mixtures within the storage period (Table 1). *Brochothrix thermosphacta*, however, developed more rapidly in the oxygen-containing gas mixture compared with the gas without oxygen (Table 1). In oxygen, numbers above 7 log10 CFU g−1 were reached at day 6 whereas without oxygen this level was not reached within the storage period (11 days). The numbers of mesophilic aerobic bacteria, lactic acid bacteria, and *B. thermosphacta* were not affected by the inoculated *C. jejuni* strains (Table 1).

Colour measurements and visual observations of the chicken fillets showed that the fillets packed in O2/CO2 remained more red throughout the shelf-life (7 days) compared with the fillets in N2/CO2 (data not shown).

**Discussion**

In Bolton broth, the numbers of the *C. jejuni* strains tested were significantly reduced when exposed to a high concentration of oxygen, the degree of reduction being strain specific. Marked strain variability among *C. jejuni* strains at 4 °C in broth has also been observed in other studies (Bolton & Coates, 1983; Chan et al., 2001). Chan et al. (2001) found that the human isolates tended to remain more viable at 4 °C than isolates from poultry. This was not observed in the present study. The reductions of *Campylobacter* were obtained in Bolton broth. As oxygen sensitivity has been shown to be dependent on the growth media (Hodge & Krieg, 1994), the reductions obtained do not necessarily apply to other substrates.

Exposed to oxygen, *C. jejuni* cells became slightly elongated and less coiled. In addition, they seemed to lose their

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**Table 1.** Total mesophilic count (TMC), lactic acid bacteria (LAB), and *Brochothrix thermosphacta* (*B. th.*.) on chicken fillets packed in different gas mixtures

<table>
<thead>
<tr>
<th>Days</th>
<th>Bacterial count (log10 CFU g−1)*</th>
<th>70/30% O2/CO2</th>
<th>70/30% N2/CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated samples</td>
<td>Controls†</td>
<td>Inoculated samples</td>
</tr>
<tr>
<td></td>
<td>TMC</td>
<td>LAB</td>
<td>B. th.</td>
</tr>
<tr>
<td>0</td>
<td>4.3</td>
<td>&lt; 2.0</td>
<td>&lt; 2.0</td>
</tr>
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<td>1</td>
<td>4.8</td>
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<td>3.9</td>
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<tr>
<td>11</td>
<td>8.6</td>
<td>8.3</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*The data are means of eight inoculated or two uninoculated samples.
†Uninoculated samples.
MO, missing observation.
motility. Pronounced elongation of cells has previously been reported for *Salmonella* and *Listeria monocytogenes* cells stressed by gas (100% CO₂) or low water activity (a_w = 0.93–0.98) within the temperature growth range (Jorgensen et al., 1995; Mattick et al., 2000; Nilsson et al., 2000; Jydegaard-Axelsen et al., 2005). Brief elongation of *C. jejuni* at 37 °C has also previously been observed in the transitional state from the exponential to stationary phase and again from the stationary to death phase (Thomas et al., 1999). In addition, loss of spiral morphology has been reported to be one of the stages before coccoid formation (Moran & Upton, 1987), which, among others, has been associated with oxidative stress and limited nutrients (Moran & Upton, 1987; Harvey & Leach, 1998). The cell elongation observed in the present study was therefore likely a response to the oxidative stress that the cells were exposed to and was probably due to unwinding of the typical coiled form. The reason for not observing coccoid cells could be due to sufficient amounts of nutrients. The tendency towards loss in motility was only based on visual examination of the cells and more studies are needed to verify this observation. A potential loss of motility due to oxygen and cold storage is very important in relation to the invasiveness of *C. jejuni*, which is conditioned by the motility of the cells (Snelling et al., 2005).

The survival of *C. jejuni* was the same when exposed to 100% N₂ and N₂/CO₂. This indicates that the survival of *C. jejuni* in Bolton broth was not affected by CO₂ per se. Other published results on the effect of CO₂ on the survival of *C. jejuni* have been carried out in chicken meat. In this medium, Wesley & Stadelman (1985) also found no effect of CO₂ compared with the ambient atmosphere, whereas Beuchat (1985) concluded that CO₂ afforded protective effects on some *C. jejuni* strains. This protective effect could, though, also be explained by a reducing effect of oxygen in the ambient atmosphere (~21%).

*Campylobacter jejuni* strains inoculated onto fresh chicken fillets also survived longer without exposure to oxygen. This finding is in accordance with the results of similar experiments carried out in the UK (J. Corry, pers. commun.). Exposure to 70% oxygen led to decreases in *C. jejuni* counts of more than 2 log units after 6–8 days of refrigerated storage. This reduction may contribute to food safety as a two-log reduction in numbers of *C. jejuni* has been estimated to cause a substantial reduction in human cases (Christensen et al., 2001). However, at the beginning of the shelf-life, the effect of oxygen was limited and cannot be relied on as the only reduction strategy to control *Campylobacter* in chicken meat. The packaging of chicken fillets in a high oxygen-containing atmosphere was also advantageous in relation to the appearance of the meat, because the oxygen maintained a red meat colour, as expected (Gill, 1988). Compared with for example bovine meat, the increased red colour of the chicken fillets is of less importance because of a much lower pigment content of this meat type.

The strain variation observed in broth using the oxygen-containing atmosphere was less pronounced in the fillet experiments. This was likely due to the inherent variations in experiments with solid food and independent samples.

The total mesophilic count was independent of the gas used, but the composition of the biota was different. The meat spoiler, *B. thermosphacta*, grew faster in the oxygen-containing atmosphere than in the gas mixture without oxygen. This was expected as oxygen stimulates the growth of *B. thermosphacta* (Kakouri & Nychas, 1994) and also the production of chemical components responsible for spoilage (Pin et al., 2002). High numbers of *B. thermosphacta* are therefore unfavourable in relation to the shelf-life of meat.

The level of lactic acid bacteria was similar in the two gas mixtures, although the species composition probably differed. The oxygen-containing gas mixture was expected to yield a greater proportion of heterofermentative lactic acid bacteria due to the stimulatory effect of oxygen on their growth (Kakouri & Nychas, 1994; Davies, 1995). The lactic acid bacteria grew faster than *B. thermosphacta*, as also stated by Dainty & Mackey (1992). The level of pseudomonads was not investigated, but these organisms were not expected to predominate the product, because of the inhibitory effect of CO₂ (Kakouri & Nychas, 1994; Davies, 1995).

In conclusion, the gas mixture containing 70% O₂ and 30% CO₂ significantly reduced the numbers of *C. jejuni* in broth as well as on fresh, skinless chicken fillets compared with an atmosphere with 70% N₂ and 30% CO₂. Hence, with regard to food safety, the high oxygen-containing gas was advantageous, but with regard to the shelf-life of the product this was not the case, as oxygen favoured the growth of the meat-spoiling bacterium, *B. thermosphacta*. This result leaves the common dilemma that food safety and food spoilage are different issues, which also have to be handled differently.

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Campylobacter jejuni in gas mixtures


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