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Review

Influence of measurement and control of microaerobic gaseous atmospheres in methods for *Campylobacter* growth studies

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ABSTRACT

Campylobacter is the leading cause of bacterial enteritis in the world. For this reason, this pathogen is widely studied. As a microaerophilic and capnophilic microorganism, this foodborne pathogen requires an atmosphere with reduced oxygen (O_2) and elevated carbon dioxide (CO_2) concentrations for its optimal growth *in vitro*. According to the procedure for *Campylobacter* spp. isolation and cultivation from food products and environmental samples, European and American standards recommend gas proportions of 5% O_2 and 10% CO_2, complemented with nitrogen (N_2). However, in the literature, the reported proportion of O_2 for microaerobic growth conditions of *Campylobacter* spp. can range from 2.5% to 15% and the reason for this variation is usually not explained. The use of different gas generating systems and media to detect and to grow *Campylobacter* from foodstuff and the lack of information about gas producing systems are the main sources of the loss of consistancy between data. In this review, the relevance, strengths and weaknesses of these methods and their impact on *Campylobacter* biology are discussed. In conclusion the minimum information concerning microaerobic gaseous atmospheres are suggested in order to better harmonize data obtained from research studies for a better understanding of *Campylobacter* features.

1. Introduction

The bacterial foodborne pathogen, *Campylobacter*, is the worldwide leading cause of campylobacteriosis in humans (Epps et al., 2013). This severe enteritis can lead to bacteremia and systemic infection, particularly in humans at the extreme ages or the immunocompromised. Campylobacteriosis is also associated with late-onset complications, such as Guillain-Barré and Miller Fisher syndromes (Moore et al., 2005). Reports from USA (CDC, 2013) and the EU (EFSA and ECDC, 2015), based on confirmed cases, clearly showed that the incidence of infections caused by *Campylobacter* has been significantly increasing. In addition, *Campylobacter* was the most common gastrointestinal zoonotic pathogen in 2013 in Europe, with 214,779 confirmed human cases (EFSA and ECDC, 2015). The last EU notification incidence of campylobacteriosis was more than twice that for salmonellosis. In particular, *Campylobacter jejuni* and *Campylobacter coli* are the most frequently isolated species from patients and food products (Wassenaar and Newell, 2006).

Campylobacter spp. are mainly hosted by birds and food-producing animals. Species living in the reproductive organs, intestinal tracts and oral cavities of mammals and birds are asymptomatic for them but pathogenic for humans (Vandamme et al., 2005; Wassenaar and Newell, 2006). The main source of infection is poultry, mostly fresh broiler meat; this constitutes the main risk factor for human infection as poultry flocks are colonized asymptomatically by *C. jejuni*. Its presence can be detectable in commercial chicken after the age of 1–2 weeks. Infection prevalence of up to 100% has been reported in some surveillance studies (Moore et al., 2005; Wassenaar and Newell, 2006). In 2008, throughout Europe, 71.2% of broiler batches were colonized by *Campylobacter* and 75.8% of the broiler carcasses were contaminated by this pathogen (EFSA and ECDC, 2010). For instance, 87.3% of the broiler carcasses in the UK were contaminated in 2008 with a *Campylobacter* load of over 1000 cfu.g^{-1} in 27.3% (Powell et al., 2012). In France, *Campylobacter* spp. were isolated from 77.2% of the poultry caeca and 87.5% of the broiler carcasses (Hue et al., 2010). As a consequence, in most of the developed and developing countries, at

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least 50% of poultry meats were positive for *Campylobacter* (Suzuki and Yamamoto, 2009). Evisceration at slaughterhouses is the food processing step which most contributes to the spread of contamination (Hue et al., 2010). Consequently, for food safety authorities, a reduction in *Campylobacter* in food products is the goal for the immediate future.

Campylobacter species are obligate microaerobic microorganisms (Kaakoush et al., 2007). This inability to survive in ambient levels of oxygen could be due to (i) a limited ability to resist ROS (reactive oxygen species), (ii) an inefficiency of protective enzymes against ROS, (iii) the presence of O_2-labile essential enzymes and (iv) a low respiratory rate (Krieg and Hoffman, 1986; Velayudhan et al., 2004). In oxygen-respiring organisms, the high oxidoreduction potential between O_2 and H_2O ($E_h = +820$ mV) favors ROS production as the O_2 final electron acceptor is not completely converted into water. ROS are mainly composed of superoxide anions (O_2^-), hydrogen peroxides (H_2O_2) or hydroxyl radicals (•OH). Adding a H_2O_2 quencher, such as pyruvate, can alleviate the aerobiotic constraints, confirming that *Campylobacter* is limited in ROS scavengers (Verhoeff-Bakkenes et al., 2008). Nonetheless, *C. jejuni* is not able to grow anaerobically, even though it can produce functional alternative electron acceptors to O_2, such as fumarate, nitrate, nitrite, trimethylamine-N-oxide or dimethylsulfoxide (Pittman et al., 2007; Sellars et al., 2002; Weingarten et al., 2008, 2009). This inability is attributed to the absence of an alternative to the O_2-dependent Class I type of ribonucleotide reductase, an enzyme essential for DNA synthesis (Sellars et al., 2002). In addition, *Campylobacter* species require high carbon dioxide concentration for growth. As a capnophilic bacterial species, *C. jejuni* is able to assimilate CO_2, a property that could be explained by a reverse reaction which produces pyruvate from CO_2 by a flavodoxin quinone reductase FqrB (Cjj_0584). This was demonstrated for the closely related species *Helicobacter pylori* (St Maurice et al., 2007). However, the pathway for this capnophilic feature remains elusive for these microorganisms.

Consequently, to fulfill the growth requirements of *Campylobacter* species, a combination of at least low O_2 and high CO_2 concentrations are required. In this review, the different methods used to create optimal atmospheres for *Campylobacter* growth, their relevance, strengths, weaknesses and impacts on *Campylobacter* biology, are discussed. Finally, the minimum information for microbial growth conditions in microaerobic conditions are recommended in order to better harmonize results from this emerging foodborne pathogen.

2. Gas composition and growth conditions from regulatory recommendations for *Campylobacter* species isolation

The International Organization for Standardization (ISO) provides a European standard, ISO 10272:2006, for research and numeration of thermotolerant *Campylobacter* spp. (ISO, 2006a, b). This method can be applied to food products for human or animal consumption and also to environmental samples related to food production. Technical recommendations of this standard are shown in Table 1. A constant microaerobic atmosphere is required with 5% ± 2% O_2, 10% ± 3% CO_2, ≤10% H_2 (facultative), complemented with N_2. Although the recommended gas proportion and composition is indicated, several different technologies, such as commercial gas producing sacs, are proposed to obtain a microaerobic atmosphere. Furthermore, a tightly closed bottle can be used without the addition of gas for the enrichment step in a selective liquid medium (Bolton broth) (Table 1). This variety of gas systems recommended by ISO results in microaerobic conditions which could not be as accurate as the specific gas ratio recommended in the regulation. This suggests a lack of continuity and accuracy

Table 1
Recommendation by the International Organisation for Standardisation ISO 10272:2006 (ISO, 2006a, b) for *Campylobacter* spp. selection and enumeration of microorganisms from food.

Atmosphere (v/v)	Gas system	Incubation			Media			
		Pre-enrichment	Enrichment	Isolation	Enumeration	Pre-enrichment/Enrichment	Isolation	Confirmation	Enumeration
O_2: 5 ± 2% CO_2: 10 ± 3% H_2: ≤10%[a] N_2: qsp	Chemical Gas Pack generators (according to manufacturer's procedures) or Gas blend with appropriate gas ratios or Uncontrolled microaerobic atmosphere[b] (only for enrichment)	37 °C (4–6 h)	41.5 °C (44 ± 4 h)	41.5 °C (40–48 h)	41.5 °C (40–48 h)	Bolton broth	mCCDA + or Skirrow agar or Karmali agar or Preston agar	Brucella broth + Blood Columbia agar	mCCDA

mCCDA: Modified Charcoal Cefoperazone Deoxycholate Agar.
[a] Facultative.
[b] Incubating the enrichment broth in bottle or flask tightly screwed (hermetic) with an empty space less than 2 cm.