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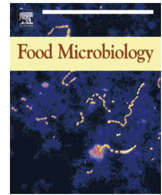

The influence of dissolved oxygen level and medium on biofilm formation by *Campylobacter jejuni*

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Campylobacter jejuni survival in aerobic environments has been suggested to be mediated by biofilm formation. Biofilm formation by eight *C. jejuni* strains under both aerobic and microaerobic conditions in different broths (Mueller-Hinton (MH), Bolton and Brucella) was quantified. The dissolved oxygen (DO) content of the broths under both incubation atmospheres was determined. Biofilm formation for all strains was highest in MH broth under both incubation atmospheres. Four strains had lower biofilm formation in MH under aerobic as compared to microaerobic incubation, while biofilm formation by the other four strains did not differ under the 2 atm. Two strains had higher biofilm formation under aerobic as compared to microaerobic atmospheres in Bolton broth. Biofilm formation by all other strains in Bolton, and all strains in Brucella broth, did not differ under the 2 atm. Under aerobic incubation DO levels in MH > Brucella > Bolton broth. Under microaerobic conditions levels in MH = Brucella > Bolton broth. Levels of DO in MH and Brucella broth were lower under microaerobic conditions but those of Bolton did not differ under the 2 atm. Experimental conditions and especially the DO of broth media confound previous conclusions drawn about aerobic biofilm formation by *C. jejuni*.

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1. Introduction

Campylobacter jejuni is one of the most frequent causes of bacterial gastrointestinal food-borne infection worldwide. The symptoms of *C. jejuni* infection include diarrhea, fever and abdominal pain and may also include neuropathies such as Guillain–Barre and Miller–Fisher syndromes (Joshua et al., 2006; Rees et al., 1995; Ropper, 1992). *C. jejuni* is commonly found in the gastrointestinal tract of a number of domestic animal species which serve as potential reservoirs for human *Campylobacter* infections. Of these domestic animals, poultry serves as the primary source of human *Campylobacter* infections (Aydin et al., 2001; Skirrow, 1994).

Campylobacter jejuni is fastidious in its growth requirements and is susceptible to various environmental and food processing induced stressors including oxygen stress (Martínez-Rodriguez et al., 2004; Murphy et al., 2006; Solomon and Hoover, 1999). These properties might make it difficult for *Campylobacter* to survive in natural aerobic environments or in the food chain (Nguyen et al., 2012; Park, 2002). In reality, however, *C. jejuni* is widely spread in the environment (Baylis et al., 2000; Martínez-Rodriguez et al., 2004). It has been suggested that biofilm formation may play a significant role in survival of *C. jejuni* in the environment (Buswell et al., 1998; Kalmokoff et al., 2006). Previous studies have shown that various environmental factors, including oxygen tension and nutrient availability, can influence the ability of *C. jejuni* to form biofilms (Reeser et al., 2007; Reuter et al., 2010).

Most studies of biofilm formation by *C. jejuni* have been carried out under microaerobic conditions which fail to simulate the ambient atmospheric conditions under which *C. jejuni* have been suggested to form biofilm (Teh et al., 2014). A clearer understanding of the ability of *C. jejuni* to form biofilms under ambient atmospheric conditions might contribute to the development of new strategies to prevent and eliminate biofilm formation by this pathogen. While work in this area has progressed, studies investigating biofilm formation by *C. jejuni* under aerobic conditions have used different media, for example, Brucella (Reuter et al., 2010) or Mueller-Hinton (Reeser et al., 2007) broth. Some of these studies have suggested that biofilm formation is enhanced under aerobic conditions (Reuter et al., 2010). The different media used in these studies might confound the conclusions drawn because some of

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these include oxygen scavenging compounds in their formulation which will influence the amount of oxygen available in the medium (Lee et al., 1988). A previous study showed that tolerance to oxygen concentration varied greatly in *C. jejuni* depending on the growth medium used, with some capable of supporting growth at higher levels of oxygen while others failed to do so (Hodge and Krieg, 1994). To address this issue we investigated biofilm formation by *C. jejuni* under aerobic and microaerobic incubation conditions in three different types of media. We also measured the level of dissolved oxygen in the different media under the different atmospheric incubation conditions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Seven *C. jejuni* strains (2862, 2863, 2865, 2866, 2868, 2869 and 2871) isolated from poultry obtained from retail outlets in Malaysia as reported in a study carried out by Wieczorek et al. (2013) were used. Molecular typing of these strains was carried out using PCR-Binary Typing scheme (P-BIT) by Wieczorek et al. (2013). *Campylobacter jejuni* ATCC 33291 from the American Type Culture Collection (Manassas, USA) was also used. All the strains were maintained at −80 °C in nutrient broth no. 2 (NB2, Oxoid, UK) supplemented with 15% glycerol. They were resuscitated on *Campylobacter* blood-free selective agar base (Oxoid, UK) with incubation at 37 °C for 48 h under microaerobic conditions. Microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) were established using CampyGen (Oxoid, UK) in an anaerobic jar (Oxoid, UK).

2.2. Biofilm quantification

The ability of the *C. jejuni* strains to form biofilm was determined in 96-well polystyrene microtiter plates (TPP®, Switzerland) using three different media, namely Mueller-Hinton broth (MHB) (Oxoid, UK), Bolton broth (Merck, Germany) and Brucella (BD, USA) broth by methods described by Reeser et al. (2007) with modifications. Briefly, the strains were grown as sessile cultures under microaerobic conditions for 48 h at 37 °C. After incubation, the colonies on the agar plates were harvested by suspending in 5 ml of phosphate buffer saline (PBS; 1st BASE, Singapore) and the optical density (at 600 nm) was adjusted in MHB, Bolton or Brucella broth to that equivalent to a cell concentration of 10^7 CFU/ml (as determined for each strain in a preliminary study). A 200 μl aliquot of the cell suspensions was transferred to a microtiter plate well. Six wells per microtiter plate were used for each strain for each media and a further six wells were filled with uninoculated medium which served as the negative control. Plates were then incubated statically under microaerobic (using CampyGen (Oxoid, UK) in an anaerobic jar) or aerobic conditions at 37 °C for 6 days. The assays were performed in triplicate. Biofilm formation was determined by examined the wells using the crystal violet staining method as described by Reeser et al. (2007) with slight modifications. Briefly, after incubation, medium, which contained unattached planktonic cells, was removed from the plate and the plate was air-dried for 15 min. The wells were then stained with 200 μl of 0.1% (w/v) crystal violet (CV) for 15 min. The colorant was discarded and followed by two subsequent washes with distilled water to remove excess stain, and the plate was air dried for 1 h. The bound CV was decolourised with 80% ethanol-20% acetone for 5 min. The absorbance of the solubilized dye of each well was then transferred to a new microtiter plate and the absorbance was measured at 550 nm using a microtiter plate reader (Tecan, Switzerland) to determine biofilm formation.

2.3. Dissolved oxygen (DO) determination

The level of dissolved oxygen in MHB, Bolton broth and Brucella

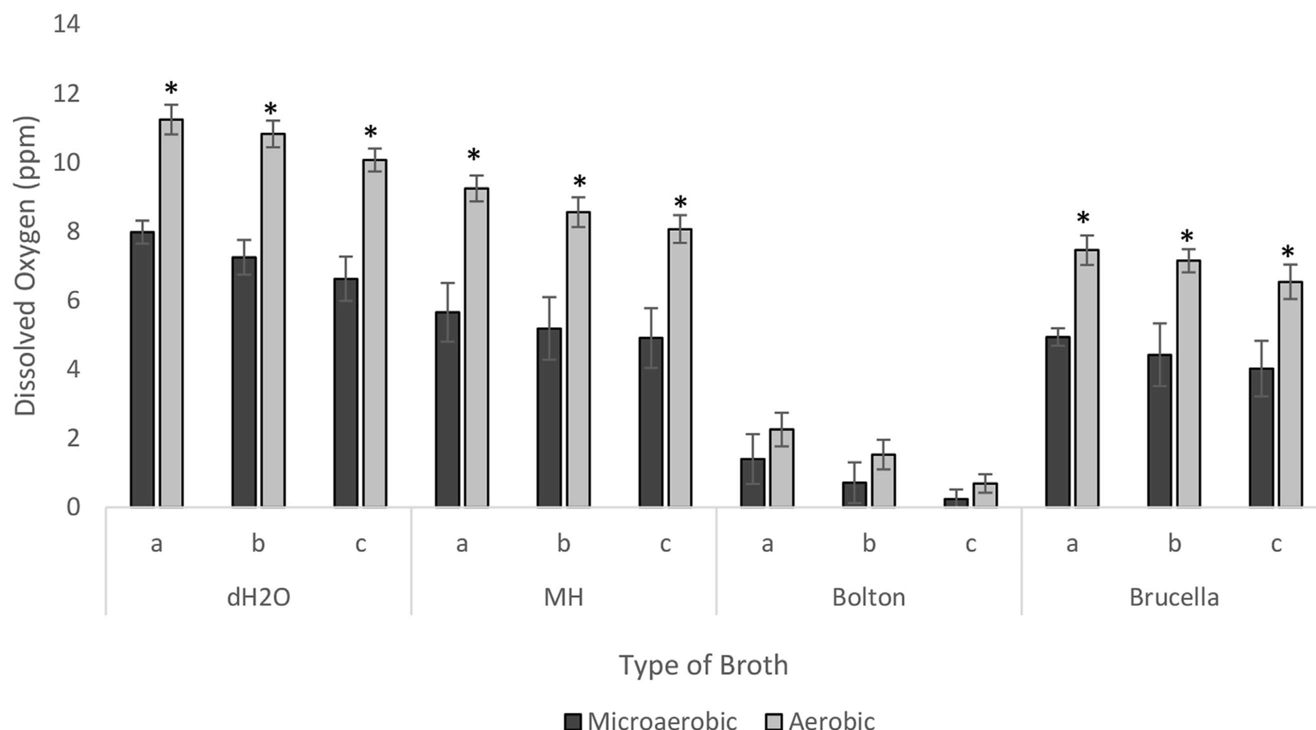

Fig. 1. Level of dissolved oxygen (ppm) in distilled water (dH_2O), Mueller-Hinton (MH), Bolton and Brucella broths incubated in aerobic and microaerobic atmospheres measured at three different depths from the surface (a = 1 cm; b = 3 cm; c = 5 cm). All results are presented as mean ± SD where n = 3; Symbol * indicates significant difference on dissolved oxygen level between microaerobic and aerobic conditions within the same medium measured at the same depth where $p < 0.05$.

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