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Biofilms of *Campylobacter concisus*: a potential survival mechanism in the oral cavity

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ABSTRACT

Campylobacter concisus, a member of the human's oral microflora, is a Gram-negative, fastidious, microaerophilic bacterium. However, it is debatable whether it should be recognised as a commensal of the human oral cavity, or an opportunistic pathogen as it has been linked to oral and gastrointestinal infections. But there is no doubt that its biofilm-forming capacity has enhanced its survival mechanism whether as a commensal or a pathogen. Hence, through our investigation to assess *C. concisus* biofilms, we believe that its survival strategy in the oral cavity is enhanced by being protected in the biofilm environment with other oral microbes. Our hypothesis is supported by the findings that oral isolates of this bacterium possess a significantly higher biofilm forming capability than those isolated from the gastrointestinal tract.

Keywords: biofilm formation, *Campylobacter concisus*, extracellular polymeric substances, hydrogen-requiring campylobacters, *luxS* gene.

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Association of *Campylobacter concisus* with the gastrointestinal tract

Campylobacter concisus is a Gram-negative, microaerophilic and hydrogen-requiring fastidious bacterium, which is a motile, spiral or curved-shaped rod.[1] *C. concisus* was first recognised and named as a member of the microflora of the human oral cavity in the early 1980s by Tanner *et al.*[2] Since then, it has been isolated from gingivitis, periodontitis, foot ulcers, gastritis, and from intestinal biopsies of patients with inflammatory bowel disease (IBD).[3] It is likely that the human gastrointestinal tract is the sole reservoir for *C. concisus*, as it has no known primary animal host. But it is debatable whether it should be recognised as commensal of the human oral cavity or an opportunistic pathogen. Macuch and Tanner[4] suggested that this bacterium colonises the oral cavity and is an opportunistic oral pathogen under certain medical conditions. The detection rate of *C. concisus* in permanent teeth was significantly higher than that of indeciduous teeth ($P < 0.001$).[5,6] The prevalence of *C. concisus* in the healthy human oral cavity was also confirmed by PCR[7,8] and by cultivation and culture-independent molecular methods such as *16S* rRNA sequencing.[9] The association of *C. concisus* with human periodontal diseases was well known since 1981,[2,6,10] as antibody levels against *C. concisus* were found to be higher in periodontally diseased subjects compared to the healthy controls.[11] Later on, it was reported to be associated with gingivitis and periodontal sites[12] and was isolated from enlarged lesions of gingivitis.[13] The association of *C. concisus* with periodontitis was also supported by significantly higher isolation rates when gingival crevicular fluid of patients was positive for aspartate aminotransferase (AST) compared to patients negative for AST in gingival crevicular fluid.[14]

Biofilm formation by *Campylobacter* spp.

Different *Campylobacter* spp., including *C. jejuni* and *C. concisus* are known to form biofilms,[15–17] which are microbial communities enveloped within an extracellular polymeric matrix that provides protection from physical, environmental and biological stresses, to persist in a diverse range of ecological niches.[18] In 2009, 14 *Campylobacter* species, both microaerophilic and hydrogen-requiring microaerophilic were tested on different surfaces such as glass, stainless steel, and polystyrene plastic for biofilm-forming ability. Of the eight microaerophilic *Campylobacter* species tested, *C. jejuni*, *C. coli*,

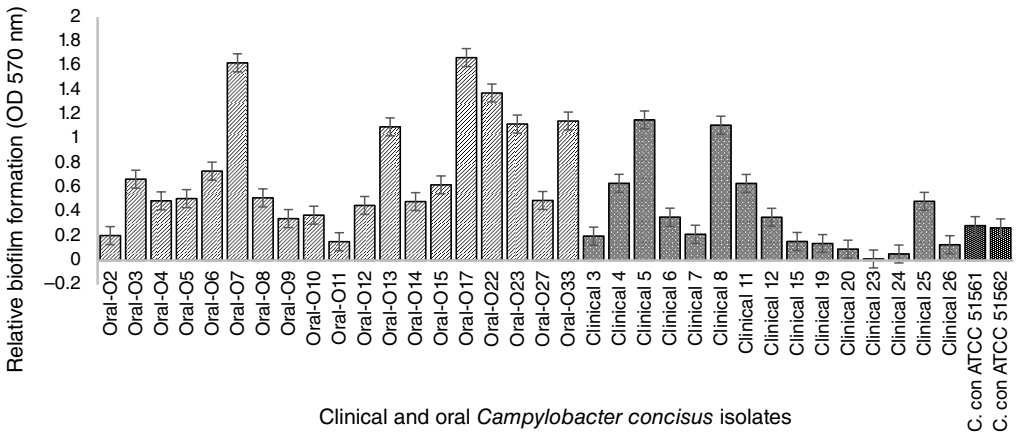

Fig. 1. Crystal violet, biofim quantitative assay of 34 *C. concisus* strains. The oral strains were isolated from saliva of healthy persons and the intestinal strains were isolated from patients with gastritis. The oral isolates exhibited significant biofilm forming potential than clinical isolates (by Mann–Whitney test; $P = 0.0354$). *C. concisus* ATCC 51561 and 51562 were used as control strains. The results represent the mean values and standard errors of three independent experiments. Each experiment was performed three times independently and in biological–technical triplicates.

C. lari, *C. upsaliensis*, *C. sputorum*, *C. hyointestinalis*, *C. helveticus* and *C. fetus*, only *C. jejuni* strain 81–176 reliably produced a visible biofilm on multiple surfaces. However, all six strains of the hydrogen-requiring microaerophilic *Campylobacter* species including *C. rectus*, *C. showae*, *C. mucosalis*, *C. concisus*, *C. curvus* and *C. gracilis* reliably produced visible biofilms on multiple surfaces.[17] Furthermore, Sampathkumar *et al.*[19] studied the transcriptional and translational expression profiles of *C. jejuni* 11168 biofilms. The proteomic analysis showed higher levels of expression of proteins involved in the motility complex, including the flagellins (FlaA, FlaB), the filament cap (FliD), the basal body (FlgG, FlgG2), and the chemotactic protein (CheA). Flagella and quorum-sensing (QS) are known to play important roles in biofilm formation. The inactivation of *flaAB* (flagella subunits) and *luxS* (responsible for QS) resulted in reduced biofilm formation. Hence, it was suggested that both *flab* and *luxS* are required for biofilm formation in *C. jejuni*.[16] Since then, many researchers investigated the role of *luxS* and demonstrated that this gene is involved in a variety of physiologic pathways, motility, autoagglutination, cytolethal distending toxin (CDT) expression, flagellar expression, oxidative stress, and animal colonisation in *C. jejuni*.[16,20–22]

Biofilm formation by hydrogen-requiring campylobacters

In following studies, *C. concisus* and *C. rectus* were detected in four of seven patients (57%) with Barrett's oesophagus (BO)[23] when micro-colonies were detected in the form of mucosal biofilm in biopsy samples taken from those patients. This study was supported by the research conducted by Blackett *et al.*[24], on oesophageal biofilms in patients with BO and gastro-oesophageal reflux disease (GORD), where *C. concisus* was the dominant species, therefore the authors suggested the emergence of *C. concisus* as the dominant species in the refluxed oesophagus. Moreover, *C. concisus*

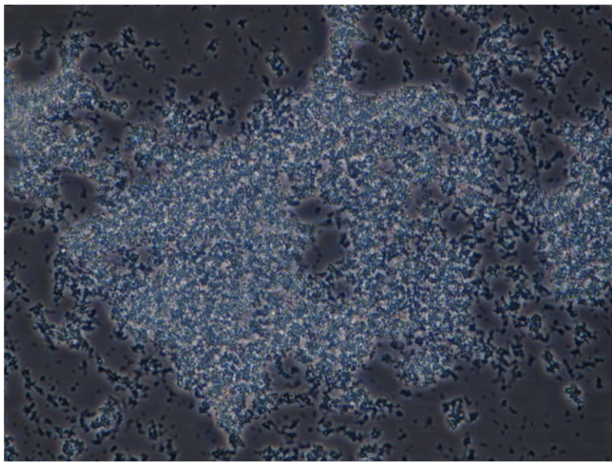

Fig. 2. Biofilm complex produced by a *C. concisus* oral isolate observed at 400× magnification by phase contrast microscopy showing the bacterial cells embedded within an extracellular material and cells evacuated interior portions of cell clusters, forming void spaces.

strains isolated from IBD patients, gastroenteritis and healthy individuals were reported to be capable of forming biofilms on glass coverslips.[25] *C. concisus* has recently been reported to have been isolated from subgingival microbiota of individuals with HIV[26] and from biofilms of the dental plaque.[27] Hence, biofilm formation by this bacterium being a fastidious oral cavity coloniser, is likely to be an important requirement for it to survive in this environment. However, to date, there has been no thorough investigation on *C. concisus* biofilm formation.

Investigation of *C. concisus* biofilms formed by oral and clinical strains

In the past few years, our research team focussed on the evaluation and characterisation of *C. concisus* biofilms. To assess the biofilm forming capability of strains isolated from different gastrointestinal tract sections, we tested 19 oral

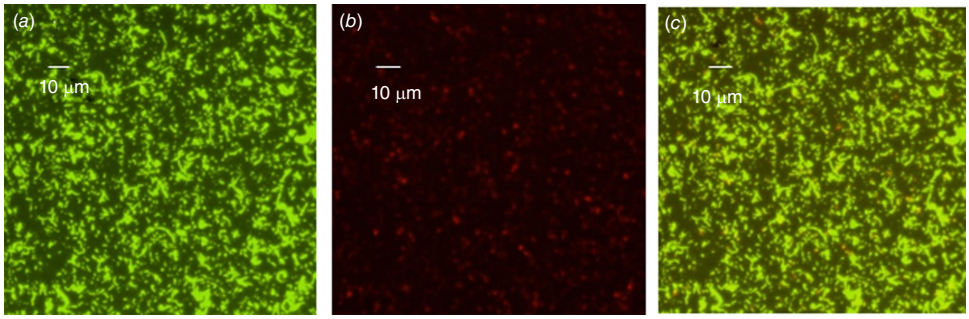

Fig. 3. Biofilms of a *C. concisus* oral isolate on glass coverslip observed by confocal laser scanning microscopy (CLSM) after 96 h of incubation showing a mixture of live and dead cells (*a*) stained with SYTO-9 (bright green for live cells), (*b*) stained with PI (red or orange, or loss of bright green for dead cells), (*c*) combined image of SYTO-9 and PI.

isolates collected from saliva samples of healthy volunteers at RMIT University, Melbourne, in addition to 14 intestinal clinical isolates previously collected using the Cape Town Protocol[28] from children suffering from mild to severe bloody diarrhoea at the Royal Children's Hospital, Melbourne.[29] Two *C. concisus* reference strains (ATCC 51561 and ATCC 51562) were also characterised in our research. All intestinal and oral *C. concisus* isolates were screened for biofilm formation by the modified quantitative crystal violet assay.[16] Among the 14 intestinal isolates, only 11 produced biofilms at low to moderate levels, whereas all tested oral isolates formed higher levels of biofilms (Fig. 1), indicating that the oral isolates are significantly more prolific biofilm producers than the clinical isolates ($P < 0.05$) including the two reference strains that also have a faecal origin. A possible explanation for forming more biofilm by the oral isolates could be an advantageous trait within the oral cavity to escape from toxic oxygen and other adverse conditions. In other studies, *C. jejuni* NCTC 11168 has been shown to develop biofilm more rapidly under environmental and food-chain-relevant aerobic conditions (20% O_2) than under microaerobic conditions (5% O_2, 10% CO_2).[30]

Microscopic observation of *C. concisus* biofilms

We also conducted phenotypic characterisation of biofilms formed by selected oral and intestinal *C. concisus* strains, which included phase contrast microscopy, and confocal laser scanning microscopy (CLSM). Different morphological stages of biofilm formation (attachment, maturation and dispersion) were observed by phase contrast microscopy. Cell clusters were observed to undergo alterations in their structure due to the dispersion of bacterial cells from their interior within 96 h (Fig. 2). These bacterial cells were observed to swim away from the inner portions of the cell cluster. The ability of bacteria to swim freely within the void spaces as observed by microscopy indicated the absence of dense polymers or other gel-like material in the void space. Completely developed biofilms were observed by CLSM after 96 h, with a mixture of dead and live bacteria within the biofilm's structure (Fig. 3).

In conclusion, we have shown that the ability of *C. concisus* to form biofilms is correlated with the source where it was collected from. Oral isolates comparatively produced higher biofilm levels *in vitro* than gut isolates, as only a few intestinal isolates could form good biofilms. This could be considered as a survival mechanism for the oral colonising bacterium in its normal habitat. Furthermore, *C. concisus* biofilms were observed to be complex matrices composed of live and dead cells embedded in an extracellular polymeric substance (EPS). Hence, expression of biofilm related genes in these oral and clinical isolates has currently been undertaken by our research team. Further studies to evaluate the environmental effects on biofilm formation and the role of chemotaxis related genes in this mechanism are also in progress.

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Data availability. Data related to this research topic are available from Dr Mohsina Huq's PhD thesis, 'Molecular characterization of biofilm production and whole genome sequencing of selected *Campylobacter concisus* oral and clinical strains', which is available through RMIT's Open Access Research Repository).[31]

Conflicts of interest. The authors declare that they have no conflicts of interest.

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Dr Mohsina Huq completed her MSc and PhD in Biotechnology at RMIT University, on research projects focussed on the detection and pathogenesis of the oral bacterium *Campylobacter concisus*, including biofilm formation and biofilm related genes. She has published several peer-reviewed research papers and a book chapter on the detection and virulence of this bacterium. She is currently an Assistant Professor at Qassim University, in Saudi Arabia.