

# Thermophilic and non-thermophilic Campylobacter species Emits Distinct Volatile Organic Compounds in Different Culture Media and Growth Phases

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# Abstract

Bacteria emits a multitude of volatile organic compounds (VOCs) into the headspace as a mean of interactions with the environments, as well as intra- and interkingdom communication for survival and persistence in the nature and within their hosts. *Campylobacter*, which is often found in poultry and ruminants, has shown great persistence in aquatic environments, making it one of the world's most dangerous foodborne pathogens, killing thousands of people annually. In this study, the VOCs emitted by both thermophilic (*C. jejuni*, *C. coli* and *C. lari*) and non-thermophilic *Campylobacter* (*C. fetus*) of clinical concerns, impacted by nutrients composition (media) and growth phase were identified. Most thermophilic *Campylobacter* were shown to release volatile alcohols and ketones (1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one and Isophorone) during early stationary and stationary phases using active sampling with active charcoal adsorbent and GC-MS analysis. *C. jejuni* cultured in the Brain Heart Infusion had 1-Heptadecanol in its headspace gas, but not in Bolton Broth. The non-thermophilic *C. fetus* did not produce alcohols or ketones, but rather a variety of unidentified chemicals that will require further investigation in the future. Overall, PCA analysis revealed that the five *Campylobacter* strains studied created distinct volatilomes, allowing for future *Campylobacter* identification based on VOCs.

## Introduction

The genus *Campylobacter* has been known for its challenging detection, isolation and identification in the laboratory, due to its physiological characteristics, such as non-glycolytic diet, low-oxygen requirement to grow (obligate microaerophile), and ability to enter in a coccoid form during non-favourable growth conditions, known as viable but non-culturable (VBNC) state<sup>1,2,3</sup>. However, despite the constraints of culturing this microorganism in vitro, *Campylobacter spp.* are ubiquitous in nature and colonise the intestinal tracts of many wild and domesticated animals<sup>4,5</sup>. These foodborne pathogens only require a small number of bacterial cells to cause human infection<sup>5</sup>, which is primarily transmitted through the consumption of poultry meat, particularly contaminated broiler chicken (*Gallus gallus*)<sup>2,6</sup>.

*Campylobacter jejuni subsp. jejuni* is the major species of the taxon and it is recognised as the leading-cause of human gastroenteritis worldwide<sup>7,8,9</sup>. The infection triggered by the pathogen is mainly characterised by mild and self-limiting symptoms, including watery to bloody diarrhoea, abdominal pain, fever, and vomiting<sup>2,10</sup>. However, severe sequelae may occur, such as Guillain–Barré syndrome, an autoimmune disease characterised by weakness of limbs and paralysis<sup>2,10,11</sup>. On the other hands, *C. coli* is the second most reported species of human campylobacteriosis. It is very similar to *C. jejuni*, and frequently isolated together with the *C. jejuni* from the contaminated poultry and faeces samples<sup>12,13</sup>. The third most clinically-significant *Campylobacter* species is *C. lari*. Although *C. lari* is infrequently associated with human infections, it has been isolated from patients with diarrhoea<sup>14,12</sup>. Another clinically important species is *Campylobacter fetus subsp fetus* that is an opportunistic human pathogen, that primarily affects patients with weakened immune system<sup>15,12</sup>.

The standard food safety testing is mainly based on culturing, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assays (ELISA) approach that is time-consuming, labour-intensive, and prone to false negative results<sup>3,16,17,18</sup>. To meet the demand for an increasing number of food testing for global food trade, new method for *Campylobacter* detection in foods that is simpler, rapid, and highly sensitive is required urgently<sup>10</sup>. In this regards, a number of studies have demonstrated that bacteria produce distinctive volatiles organic compounds (VOCs) during culturing, create a single VOC-emission pattern that could potentially be developed into a unique metabolic biomarker for bacterial identification with high specificity, simplicity and in real-time<sup>19,20,21,22</sup>.

The bacterial VOCs are detected by means of gas chromatographic techniques, particularly the gas chromatography-mass spectrometry (GC-MS), which is generally preceded by a sample preparation<sup>19,23</sup>. For instance, solid phase microextraction (SPME) has been largely used for sampling microbial VOCs<sup>19,24</sup>. However, owing to the lack of standardized methods, the nature of targeted sample matrix (i.e., the composition and the analytes' properties), the experimental requirements and constraints (i.e., costs and instrumental issues, etc.), select the most suitable sampling procedure to serve the purpose of VOC fingerprinting is challenging<sup>25,23,26</sup>. Nevertheless, sorption tube approach seems to be a suitable strategy that is widely adopted for gas sampling as it could retain a range of compatible VOCs at ambient temperature subjected to the interaction between the analytes and the sorbent material; the retained content could be desorbed for subsequent measurement<sup>25,26</sup>. This technique is simple, inexpensive, and more appropriate for thermally unstable compounds, when desorption via compatible solvent system is applied<sup>27,28,24</sup>.

Moreover, in contrast with the research attention that bacterial VOCs have drawn lately<sup>19,29,30</sup>, only few studies regarding to VOC-emissions of *Campylobacter* species have been published. The whole-cell fatty acids of *Campylobacter fetus subsp. venerealis* was investigated in a study that found hexadecanoic acid as the most significant volatile emitted by the bacterium<sup>31</sup>. Also, the profile of VOCs emitted by *C. jejuni* in contaminated human stools and by *C. jejuni* and *C. coli* in chicken faeces was previously explored<sup>32,33</sup>. Additionally, the prevalence of alcohols were observed in broth inoculated with *C. jejuni* after 20 hours of incubation<sup>34</sup>. However, when subjected to the type of media, the growth phase, bacterial strain, and sampling setting, the VOCs emitted by a particular bacterial species could vary considerably<sup>25,19,26,35</sup>. Hence, this study attempted to investigate the variation of VOCs emitted by the four clinically significant species of *Campylobacter* (*C. jejuni*, *C. coli*, *C. lari* and *C. fetus*) in different culture media and growth phase. This research would be a cornerstone for further development of VOC-based sensing technology for detection of foodborne *Campylobacter spp.* in food.

## Results

### Identification of putative bacterial volatiles produced by *Campylobacter*

Many peaks were detected during the GC-MS analysis of *Campylobacter* species. Figure 1 shows the similarity of the chromatograms of the bacterial samples and the blank media (negative control), demonstrating the complexity in identifying the putative biomarkers. Overall, the five *Campylobacter* strains included in this study were found to produce a total of 38 to 68 identified VOCs during growth in either Bolton or Brain Heart Infusion broth; of these VOCs identified, only 15.7 to 41.3% remained after removing the VOCs found present in the blank samples (i.e. VOCs produced by the culture media) and 3.1 to 10.8% of these VOCs were detected in at least two biological replicates (filtering criteria 2) (Table 1). The VOCs that passed both the filtering criteria 1 and 2 were then labelled as the putative VOC biomarkers.

Table 1

Number of VOCs detected in the bacterial samples by GC-MS and application of the filtering criteria.

Strain	Culture Media	Detected GC Peaks	Identified VOCs (MS+RI) <sup>a</sup>	VOCs Pass Criteria 1 <sup>b</sup>	VOCs Pass Criteria 2 <sup>c</sup>	Putative discriminant VOCs
<i>C. jejuni subsp jejuni</i> ATCC29428	Bolton broth	566	46	9 (19.5%)	5 (10.8%)	5
	BHI broth	531	40	14 (35%)	2 (5%)	2
<i>C. jejuni subsp jejuni</i> ATCC33291	Bolton broth	575	63	13 (20.6%)	2 (3.1%)	2
	BHI broth	553	46	19 (41.3%)	5 (10.8%)	5
<i>Campylobacter coli</i> ATCC43478	Bolton broth	545	38	6 (15.7%)	2 (5.2%)	2
<i>Campylobacter fetus subs. Fetus</i> ATCC24374	Bolton broth	601	43	9 (20.9%)	3 (7.0%)	3
<i>Campylobacter lari</i> ATCC35221	Bolton broth	432	68	16 (23.5%)	4 (5.8%)	4
<sup>a</sup> Identified VOC is compound that its identity has been confirmed by its Mass Spectrum (MS) and Retention Index (RI).						
<sup>b</sup> Criteria 1: VOC that is not found in the background (blank media).						
<sup>c</sup> Criteria 2: VOC that is detected in at least two biological replicates.						

A total of 16 VOC biomarkers were identified to be associated with *Campylobacter* species (Figure 2; Supplementary data A1). These VOCs were found to belong to alcohol (3), ketone (2), ester (1), phenyl alkene (1), phenol (1) and unidentified (8) (Figure 1). Some of the putative biomarkers were labelled as “not identified” due to a low matching with the mass spectrum and retention index. However, these

unidentified compounds were labelled according to their mass spectrum and calculated retention index for comparison between samples.

It is interesting to point out that there was no overlapping in the VOC biomarkers emitted by *Campylobacter* species cultured in Bolton and BHI broth (Figure 2). Both strains of *Campylobacter jejuni* (ATCC 29428 and ATCC 33291) emitted 1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one (ketone) when cultured in Bolton broth; while emitted 1-Heptadecanol (alcohol) when they were cultured in BHI broth (Figure 2).

While both *C. jejuni* strains shared only 1 similar VOC biomarkers growing in Bolton broth, *C. jejuni* ATCC 29428 shared more VOC biomarkers with *C. lari* ATCC 35221: 3-Dodecanol, 3,7,11-trimethyl- (alcohol), 1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one (ketone) and not identified 29 (Figure 2). The VOC not identified 29 was in fact found to be emitted also by *C. fetus* ATCC 27374. Another ketone, isophorone was emitted by *C. jejuni* ATCC 33291 and *C. coli* 43478. It is worthy to note that *C. coli* did not share similarity with *C. fetus* and *C. lari*, in term of VOC biomarkers (Figure 2).

## Bacterial Growth

The growth curve of *Campylobacter* species cultured in both Bolton and BHI broth were determined by MPN enumeration approach at 0 h, 12 h, 24 h, and 48 h of incubation. The growth curve was then plotted (Figure 3). All, but *C. jejuni* 33291 in BHI broth showed exponential growth in the first 12 hours of incubation, indicating of the exponential growth phase; which was then followed by a significant deceleration in the growth rate at 12h to 24h and 48h, indicating the late exponential phase entering into early phase of stationary growth phase (Figure 3). For *C. jejuni* 33291 cultivated in BHI broth, the exponential phase continued for another 12 hours before the growth rate started to slow down at 24 hours (Figure 2d).

### **Volatile organic compounds emitted by *Campylobacter* species during different growth phases**

Majority of the identified putative VOC-markers were detected after 12 hours of incubation, suggesting that the active production VOCs occur during the late exponential and early stationary phase of *Campylobacter* spp. under laboratory culturing conditions (Table 2). Generally, alcohol was detected in *Campylobacter jejuni* cultures at 24 hours and beyond; 1-Heptadecanol, which was only detected in *C. jejuni* ATCC 29428 BHI cultures was also detected after 12 hours of incubation (Table 2). Volatile ketones, 1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one and Isophorone, were mainly detected in the stationary phase at 48 hours (Table 2).

Table 2

Relative peak area (average) of bacterial VOCs detected at 12, 24 and 48 hours of incubation at 42°C.

Strain	Media	Class	VOC	Relative peak area		
				Exponential	Stationary	
				12h	24h	48h
<i>C. jejuni subsp jejuni</i> ATCC 29428	Bolton broth	Alcohol	1,8-Nonadien-3-ol	0	0.1075	0
		Alcohol	3-Dodecanol, 3,7,11-trimethyl-	0	0.102	0.0458*
		Ketone	1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one	0.0478	0	0.0592
		Unknown	Not Identified – 29	0	0	0.122
		Unknown	Not Identified – 16	1.324	0	0
	BHI broth	Alcohol	1-Heptadecanol	0	0.0416	0.3796
		Ketone	Isophorone	0	0	0.2128*
<i>C. jejuni subsp jejuni</i> ATCC 33291	Bolton broth	Ketone	Isophorone	0	0	0.2319
		Ketone	1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one	0	0.0345	0
<i>C. jejuni subsp jejuni</i> ATCC 33291	BHI broth	Alcohol	1-Heptadecanol	0	0.0226	0.0314*
		Ketone	Isophorone	0	0	0.2991*
		Ester	Diisobutyl phthalate	0.5166*	0.0647	0.0671
		Phenyl Alkene	Benzene, (1-propyloctyl)-	0	0.03625	0
		Unknown	Not Identified – 73	0.1580	0	0
		Unknown	Not Identified – 84	0.1994	0	0
<i>Campylobacter coli</i>	Bolton broth	Ketone	Isophorone	0	0	0.2770
		Phenol	Phenol, 3,5-bis(1,1-dimethylethyl)-	0	0	5.80

\* VOC detected in only one bacterial sample.

<i>Campylobacter fetus subs. Fetus</i>	Bolton broth	Unknown	Not Identified – 29	25.79	15.28	0
		Unknown	Not Identified – 40	0	16.55	0
		Unknown	Not Identified – 58	0	2.26	0
		Unknown	Not Identified – 42	0	5.07	0
<i>Campylobacter lari</i>	Bolton broth	Alcohol	3-Dodecanol, 3,7,11-trimethyl-	0	0	6.25
		Ketone	1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one	0	0	6.64
		Unknown	Not Identified – 25	0	10.40	0
		Unknown	Not Identified – 29	0	0	13.3
* VOC detected in only one bacterial sample.						

## Discussion

The measurement results of bacterial VOCs depend greatly on the analytical methods adopted for fingerprinting, especially the gas sampling strategy. Although the charcoal-based matrix is a sufficiently suitable adsorbent for a wide range of compounds<sup>27</sup>, one of the major drawbacks of using the charcoal adsorbent used in this study was the loss of volatiles, which could be caused by the solvent dilution factor, lesser affinity with polar compounds, and overload of the sorbent matrix<sup>27,28,24</sup>. These might explain the detection of less putative VOC-markers in this study as compared to other reported study that used different adsorbents<sup>34</sup> and technique such as thermal desorption<sup>28,35</sup>. However, the charcoal adsorbent has been proven to perform sufficiently well to detect the most predominant bacterial volatiles that were commonly reported by other researchers, such as alcohols and ketones.

In this study, alcohols were the most commonly detected VOCs in *Campylobacter spp.*, produced mainly in the early stationary and stationary growth phase (Table 2). The detected alcohol-based VOCs included the fatty alcohols 3,7,11-trimethyl-3-dodecanol and 1-heptadecanol, as well as the non-cyclic alkene alcohol 1,8-nonadien-3-ol (Figure 2). The probable pathways for bacterial synthesis of fatty alcohol and long-chain alcohols are through hydrogenation of methyl esters of fatty acids<sup>36</sup>, as well as by the  $\beta$ - or  $\alpha$ -oxidation of fatty acid by-products<sup>37</sup>. For instance, the production of fatty alcohols in *E. coli* begins with a thioesterase-mediated conversion of a fatty acyl-ACP (acyl carrier protein) to a free fatty acid. The free fatty acid is then converted to a fatty acyl-CoA by a fatty acyl-CoA synthase. The resulting fatty acyl-CoA can subsequently be metabolised via the  $\beta$ -oxidation route or reduced to its corresponding fatty alcohol by the generation of its corresponding fatty aldehyde in a NADPH-dependent fatty acyl-CoA reductase-catalysed process<sup>38</sup>. Additionally, 1,8-nonadien-3-ol has been reported to be emitted by *Pseudomonas putida* as an antimicrobial volatile against plant pathogens<sup>39</sup>, and commercial fermentation starters

during food fermentation<sup>40</sup>. On the other hand, 1-heptadecanol was also found to be one of the antifungal volatiles emitted by *Pseudomonas spp.* isolated from canola and soybean<sup>41</sup>.

The two most predominantly isolated volatile ketones emitted by *Campylobacter spp.* in the current study were isophorone and 1s,4R,7R,11R-1,3,4,7-tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one (Figure 2 and Table 2). The ketones were found to be emitted by the thermophilic *Campylobacter* (*C. jejuni*, *C. coli* and *C. lari*) in both Bolton broth and Brain Heart Infusion culture (Table 2). However, it is noteworthy to point out that ketones were detected generally after 12 hours of incubation at 24 and 48 hours, corresponding to the early phase of stationary and stationary growth phase of *Campylobacter spp.* in vitro (Table 2). Similarly, Reese et al.<sup>35</sup> also reported on the observation of detection of ketones with long carbon chains in the stationary phase and beyond. Isophorone is an unsaturated cyclic ketone that has a peppermint-like smell, and it evaporates faster than water<sup>42</sup>. It can be naturally found in cranberries<sup>42</sup>, honey<sup>43</sup> and as a beetle pheromone<sup>44</sup>. Also, it has been already reported by Schulz and Dickschat<sup>45</sup> as a volatile emitted by the cyanobacteria *Oscillatoria perornata*. Whereas 1s,4R,7R,11R-1,3,4,7-tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one is sesquiterpene ketones occurs naturally in fungi and plants<sup>46</sup> and it has an important antibiotic role as a bacterial secondary metabolite<sup>47</sup>. 1s,4R,7R,11R-1,3,4,7-tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one has been also reported as one the volatiles of lignin isolated from oil palm empty fruit<sup>48</sup>.

Generally, the biosynthesis pathway of ketones, particularly methyl ketones, involve the decarboxylation of fatty acids<sup>49</sup>. However, isophorone and 1s,4R,7R,11R-1,3,4,7-tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one are terpenoids with a molecule of oxygen as the functional group of a ketone. Hence, the bacterial metabolic routes of terpenoids, unlike methyl ketones, are synthesised from the five-carbon building blocks, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). These molecules are likely to be originated from pyruvate and glyceraldehyde-3-phosphate in the methylerythritol-4-phosphate (MEP) and the Mevalonate (MVA) pathways<sup>50</sup>.

The presence of alcohols and ketones in the VOC profile of *C. jejuni* is in accordance with other studies that observed the prevalence of these compounds in the cultures of *C. jejuni* and in matrices contaminated by the bacterium. Probert et al.<sup>32</sup> also noticed the presence of these chemicals in stool of patients contaminated with *C. jejuni*. Alcohols and ketones were detected as discriminant VOCs of chicken faeces contaminated with *C. jejuni* and *C. coli*<sup>33</sup>. Alcohols and ketones were also abundant in the bacterial samples of *C. jejuni* (ATCC 33560) cultivated in BHI broth after 20 hours of incubation<sup>34</sup>.

The VOC profiling of other clinically important *Campylobacter* species, such as *C. coli*, *C. fetus subsp. fetus* and *C. lari*, was a pioneering step towards the characterization of the volatilome of these pathogens. Isophorone and 3,5-bis(1,1-dimethylethyl)-phenol were discriminant volatiles emitted by *C. coli*, 3,7,11-trimethyl-3-dodecanol and 1s,4R,7R,11R-1,3,4,7-tetramethyltricyclo [5.3.1.0(4,11)] undec-2-en-8-one were identified as biomarkers of *C. lari*. However, the profile of *C. fetus* consisted of only not-identified compounds, that despite of their unconfirmed identity, these compounds were important to



demonstrate the distinction of *C. fetus* among the other species. Nonetheless, future research should be able to widen the identification of the volatiles of these foodborne bacteria by employing more sensitive VOC-analytical methods.

The PCA analysis demonstrated clear distinguishable markers based on the VOCs profile emitted by the various strains of *Campylobacter spp.* included in this work; as well as VOCs profiles that is growth phase dependent (Figure 4 and Figure 5). PCA has been used by numerous researchers to identify similarity and variation in the multivariable dataset, such as Milanowski and co-workers<sup>51</sup> that has demonstrated discrimination of salivary bacteria (*Hafnia alvei*, *Pseudomonas luteola* and *Staphylococcus warneri*) based on their volatiles' profiles; and discrimination of *F. tularensis subsp. novicida*, *Bacillus anthracis Sterne* and *Bacillus anthracis Ames* in the study by Reese and co-workers<sup>35</sup>.

The study of the variation in the VOCs emitted by the various clinically important *Campylobacter spp.* including *Campylobacter jejuni subsp. jejuni* in vitro via active sampling with an activated charcoal sorbent, revealed the culture- and growth phase-associated volatilome profiles of thermophilic and non-thermophilic *Campylobacter spp.* The findings will provide an insight into the potential roles of bacterial VOCs in their growth and survival in various niches in environment and its host. The findings may also provide a baseline to development of VOC-based detection method of the fastidious *Campylobacter spp.* Nevertheless, more in-depth studies are required to identify the unknown VOCs detected in this study. Also, it will be meaningful to investigate other influencing factors, such as coculturing of *Campylobacter spp.* with other bacteria population to fully understand the mechanisms and role of bacterial VOCs.

## Methods

### Strains, Culture Media, and Laboratorial Maintenance

In this study, five bacterial strains of *Campylobacter* genus were targeted: *Campylobacter jejuni subsp. jejuni* (ATCC 33291 and ATCC 29428), *Campylobacter coli* (ATCC 43478), *Campylobacter fetus subsp. fetus* (ATCC 27374), and *Campylobacter lari* (ATCC 35221). These strains were acquired from Microbiologics (Minnesota, USA). The strains of *Campylobacter spp.* were revived by inoculating the swab into sterile Bolton broth (BB; Oxoid, UK) supplemented with 5% of lysed horse blood. The lysed horse blood was prepared from fresh horse blood, which was purchased from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The fresh horse blood was subjected to multiple rounds of freezing and thawing until no separation into two layers was observed<sup>53</sup>. The inoculated media were incubated at 37 °C for 48 h in a sealed universal bottle with 20% of headspace, creating the microaerophilic condition.

After 48 hours of incubation, the turbid broth was aliquoted into sterilized Eppendorf tubes (1.5 ml of volume) and spun down by centrifugation at 6,500 RPM (Rotation Per Minute) for 2 minutes. The supernatant was discarded and 0.5 ml of double strength Brain Heart Infusion broth (BHI; Himedia, India) and 0.5 ml glycerol were added into the vials, then it was thoroughly mixed by pipetting. The glycerol stocks were then divided into two parts to be maintained in the freezers at -20 °C and -80 °C.

The strains of *Campylobacter* were revived from glycerol stocks for the headspace-air sampling as follows: one hundred microliters were transferred from the glycerol vial to a sterile Bolton broth tube supplemented with 5% of lysed horse blood and incubated for 48 h at 37 °C in a sealed universal bottle with 20% of headspace. After the growth, the inoculum was streaked onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA; Oxoid, UK) in triplicate and incubated under microaerophilic atmosphere generated by Campygen 3.5 L (Oxoid, Basingstoke Hampshire, UK) at 37 °C for 48 h. Five to ten colonies were then transferred to a test tube containing BB and gently mixed. After that, 1 ml of the bacterial suspension was pipetted into a 100 ml screw cap bottle (Duran, Mainz, Germany) containing 80 ml of culture media and leaving 20 ml of headspace volume for the headspace gas sampling and VOC detection.

## Headspace-air Sampling And Experimental Conditions

Briefly the VOCs were collected by forcing the headspace gas in the bacteria culture bottle to pass through an ORBO activated coconut charcoal (100/50 mg) adsorbent tube (6 × 70 mm) (Merck, Darmstadt, Germany) at a fixed rate by a pump. The adsorbent tube was then removed, sealed, and pending for solvent extraction and subsequent GC-MS analysis. However, preliminary tests were performed to adjust the volume of headspace-air that needed to pass through the adsorbent matrix to ascertain detectable signals for subsequent investigations. The apparatus used for the sampling was assembled inside a anaerobic jar with a lit candle to generate the microaerophilic condition. The sample bottle was equipped with a GL 45 screw cap with two ports (Duran, Mainz, Germany). Both inlet and outlet ports were connected by Tygon tubes (4.0 mm of internal diameter). The inlet tube was connected to a portable battery air pump DC-900 (Xilong, China) operated at a flow rate of approximately 2 L/min and the sorbent tube was inserted in the outlet tube on the other hand. The duration of the headspace-air sampling was according to the length of each incubation period.

The *C. jejuni* strains (ATCC 29428 and 33291) were inoculated individually in two different high-protein media: BB supplemented with 5% of lysed horse blood and double strength BHI; with initial concentration of approximately  $10^4$  MPN/ml. All the samples were incubated at 37 °C and the headspace air was sampled during three time-points: 12 h, 24 h, and 48 h. The others *Campylobacter* species: *C. coli*, *C. fetus subs. fetus* and *C. lari* were inoculated only in BB supplemented with 5% of lysed horse blood and with initial bacterial concentration of approximately  $10^5$ ,  $10^3$ , and  $10^3$  MPN/ml, respectively. They were also incubated at 37 °C with continuous active headspace-air sampling for 12 h, 24 h, and 48 h.

## Solvent Desorption And Gc-ms Analysis

After the headspace air sampling, the sorbent tube was removed, and the sorbent content was transferred into a 1.5 ml vial. Then, 1 ml of carbon disulfide (Merck, Darmstadt, Germany) was added into the vial for

desorption of the VOCs. The vial was thoroughly shaken using a vortex mixer, and the extract was further transferred into another clean GC vial before sending for immediate GC-MS analysis<sup>27,28</sup>.

The carbon disulfide extract was analysed under splitless mode using a GC-MS (Brand: Shimadzu; Model: QP2010 ULTRA 7890A GC/MS Agilent 5975) based on the following settings: a capillary column RTX-5 MS (length: 30 m, internal diameter: 0.25 mm, and film thickness: 0.25  $\mu\text{m}$ ) was used; the temperature of the injection port was at 230°C; the oven was held at 40°C for 1 min, then the temperature was raised to 280°C at a rate of 6°C min<sup>-1</sup>, and the final temperature was held for 1 min; a solvent cut of 3 min was performed and the length of the analysis was 25 min per sample.

For the samples containing BHI broth, the temperature program was further refined as the preliminary results indicated the need to improve the data collection. In this case, the temperature of the injection port was increase to 250°C; similarly, the oven was held at 40°C for 1 min, then the temperature was raised to 250°C at a rate of 6°C min<sup>-1</sup>, and the final temperature was held for 11 minutes; leading to a 46 min run time. For both settings, a full scan mode was used for the mass spectrometry analysis.

For referencing purposes, a series of n-alkanes standard (Merck, Darmstadt, Germany) was run under the exact same GC-MS operating conditions; the retention index calculated from the alkane runs is rather system independent and thus reproducible<sup>54</sup>.

## Enumeration Of Bacterial Count

Before the incubation periods (12, 24, and 48 hours) and after the headspace air sampling 1 ml of the enrichment broth was collected from the sample for three-tube Most Probable Number (MPN) analysis. The aliquot was serially diluted using fresh BB with 5% of lysed horse blood in a 10-fold dilution until maximum 10<sup>-9</sup>, or depending on the expected growth phase, and incubated in triplicate at 37 °C for 48 h under microaerophilic condition<sup>53</sup>.

The tubes were then checked for turbidity. The tubes that became turbid were plated on mCCDA and incubated for 48 h at 37 °C under microaerophilic condition to confirm the presence of *Campylobacter* in the tubes. The concentration of bacteria cells in the tube (MPN/ml) was then calculated with a MPN calculator developed by the US governmental environmental protection agency (EPA)<sup>55</sup>. Also, the growth curve of *Campylobacter* species (MPN/ml) for all replicates throughout the experiment was then plotted.

## Data Analysis

The VOCs were identified by taking both mass spectra and retention index into account. The mass spectrum of each compound was matched against the reference spectrum available in NIST mass spectral library, only those with high degree of similarity (>70%) were further considered<sup>56</sup>. In addition to library mapping, GC retention index (RI) provide complementary information to aid the identification of VOCs. The normalized retention time (non-isothermal Kovats retention index) was calculated according to

the formula of Van Den Dool & Kratz<sup>57</sup>. For further identification, the calculated RIs were compared with the reference RIs of same kind of stationary phase; those with 5% or more of relative standard deviation were rejected<sup>35</sup>.

Moreover, two criteria were adopted here to decide the putative volatiles, that are most likely produced by the microorganism, with relative consistency (see Results, Table 1). First criterion, the compound is not present in the background (blank media) at the same timepoint (incubation period) of the bacterial samples, and second criterion, the compound was detected in at least two samples throughout the repetitions. Only filtered VOCs were considered for statistical comparison of bacterial strains and growth phases. The relative peak area of these selected compounds was subjected to PCA to explore the similarities/dissimilarities in VOCs patterns that might associated with the bacterial strains, the production of VOC during different growth phases and culture media. The calculation was performed using SAS JMP 15 software.

## Declarations

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### Author contributions

FMSDS is the MSc student conducted bench work, collected data, analysed data and drafted the article; LKH is a chemist and co-PI of the project, experimental design, advised on methods for detection and identification of VOCs, analysed data, wrote and corrected the article; CLC is a microbiologist, the PI of the project, developed the idea, experimental design, analysed data, wrote and corrected the article.

### Competing Interests Statement

The authors declare no competing interest.

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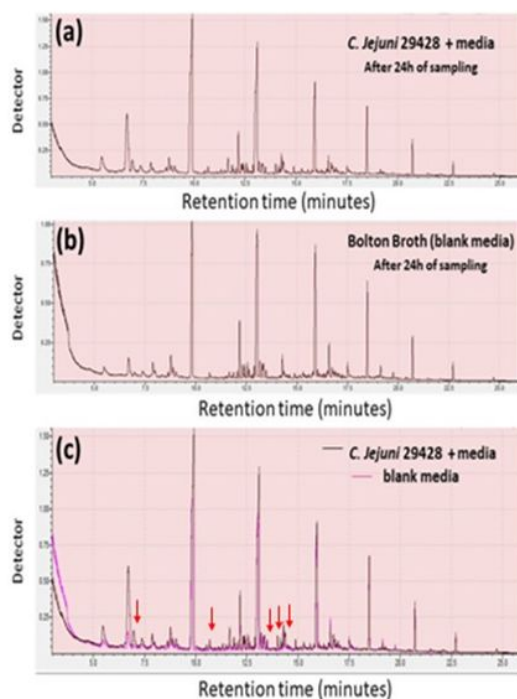


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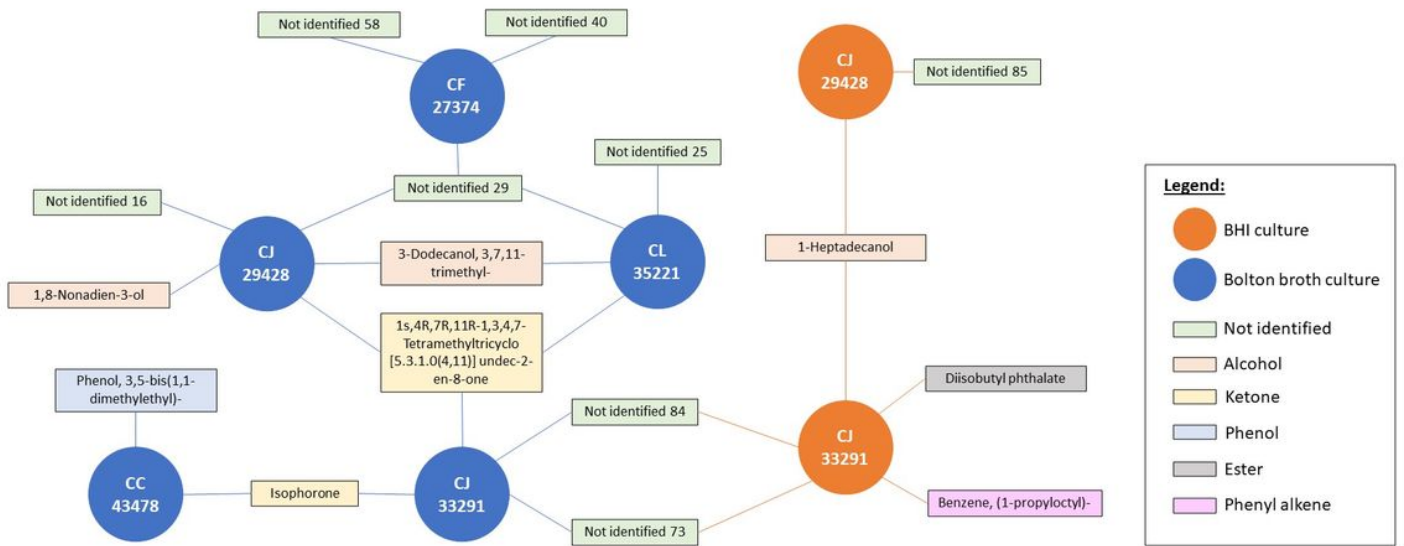
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## Figures



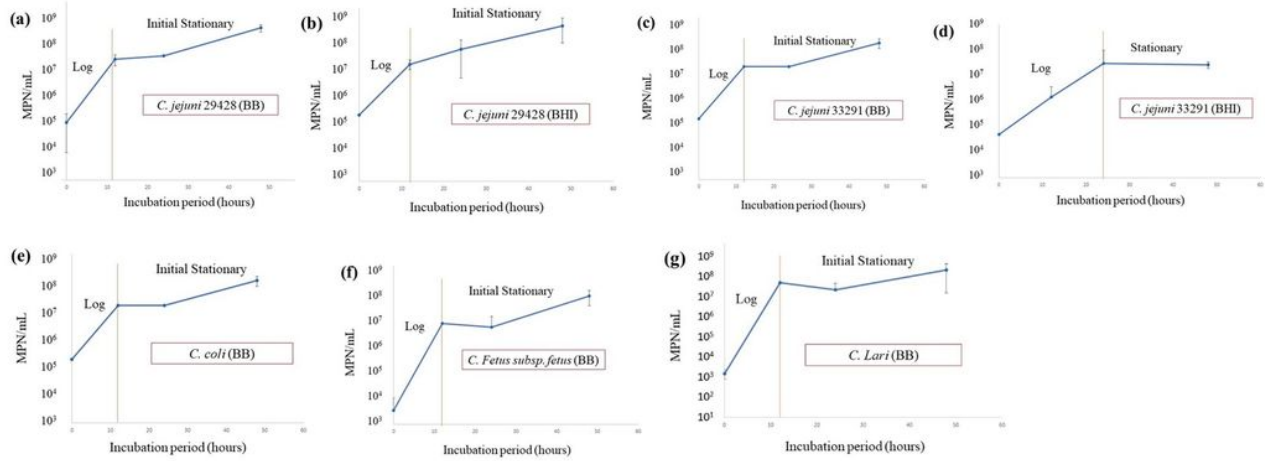
**Figure 1**

Comparison between chromatograms. Note: (a) *C. jejuni* 29428 after 24 hours of incubation and sampling, (b) negative control (blank Bolton Broth), and (c) overlap of chromatograms with the negative control peaks coloured in pink; the red arrows in part (c) represent the peaks that were not detected in the blank media.



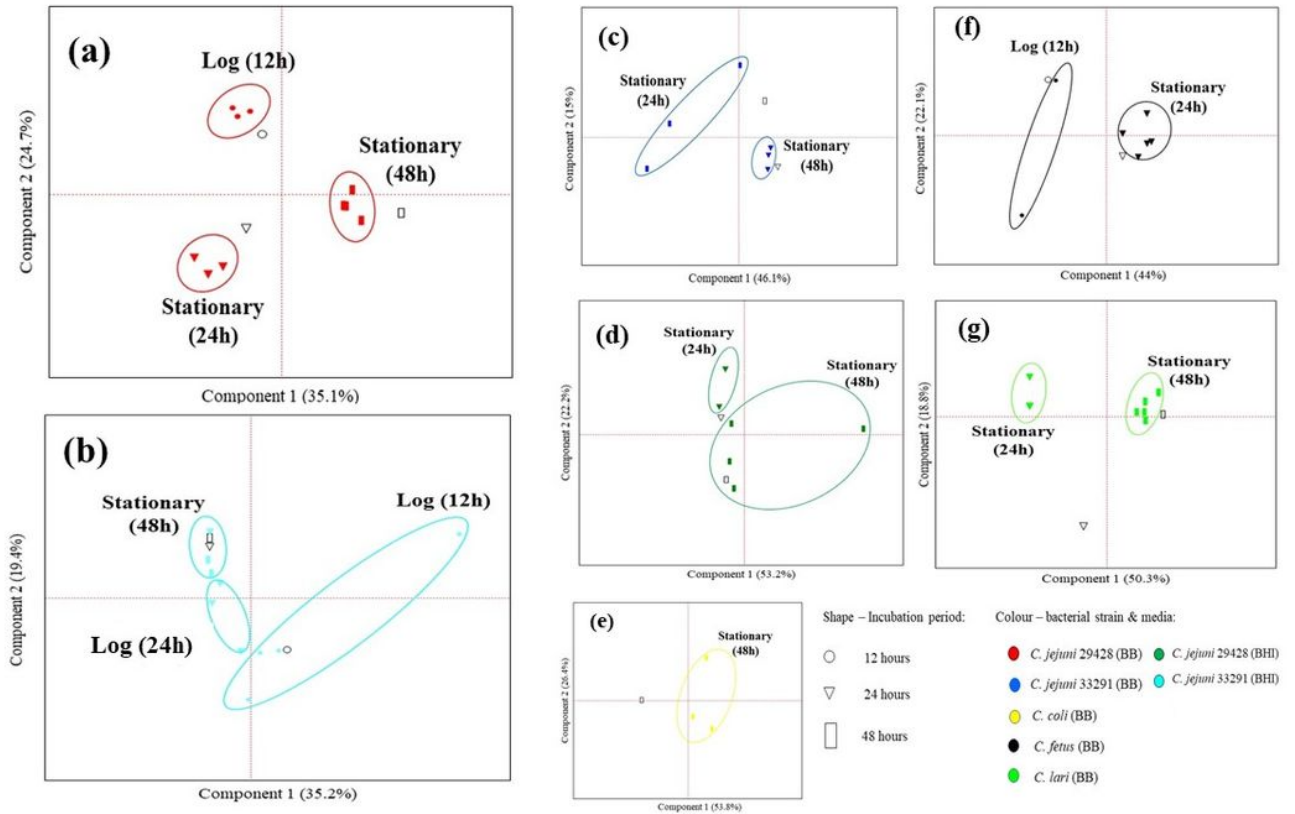
**Figure 2**

VOC biomarkers produced by *C. jejuni* (CJ) ATCC 29428, *C. jejuni* ATCC 33291, *C. coli* (CC) ATCC 43478, *C. lari* (CL) ATCC 35221, *C. fetus* (CF) ATCC 27374 cultured in Bolton broth and BHI broth for 48 hours.



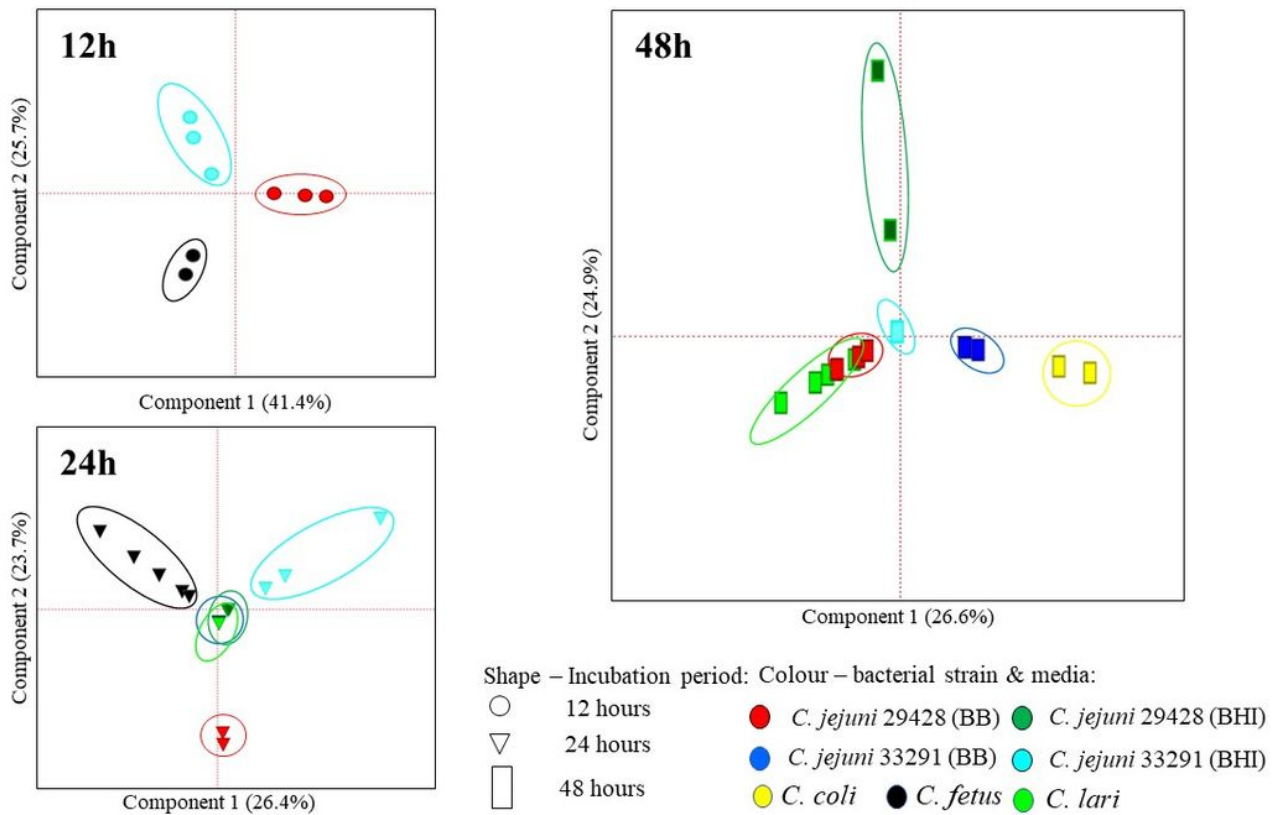
**Figure 3**

Growth curves in MPN (Most Probable Number) per mL of *Campylobacter* spp. (a) *C. jejuni* ATCC 29428 in Bolton broth (BB), (b) *C. jejuni* ATCC 29428 in BHI, (c) *C. jejuni* ATCC 33291 in BB, (d) *C. jejuni* ATCC 33291 in BHI, (e) *C. coli* ATCC 43478, (f) *C. fetus* subsp. *fetus* ATCC 27374, and (g) *C. lari* ATCC 35221, over incubation periods of 0, 12, 24, and 48 hours throughout all repetitions. The error bars represent the standard deviation calculated within the MPN/mL of all the 5 repetitions at each timepoint.



**Figure 4**

PCA score plots of each strain of *Campylobacter* at all incubation intervals with detected putative VOCs. Note: (a) *C. jejuni* 29428 (BB); (b) *C. jejuni* 33291 (BHI); (c) *C. jejuni* 33291 (BB); (d) *C. jejuni* 29428 (BHI); (e) *C. coli*; (f) *C. fetus*; (g) *C. lari*; each point represents a bacterial sample with detected VOC-markers; non-coloured shape represents the blank media (negative control).



**Figure 5**

PCA score plots of all bacterial samples divided by each incubation period (12h, 24h, and 48h). Note: each point represents a bacterial sample with its detected VOC-markers; the strains absent in one of intervals means that no putative VOC-markers were identified in these strains in that interval.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryA1.pdf](#)