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Article

Innovative Sensor Approach to Follow *Campylobacter jejuni* Development

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Abstract: Campylobacter infection affects more than 200,000 people every year in Europe and in the last four years trend shows an increase of campylobacteriosis. Main vehicle for transmission of the bacterium is contaminated food like meat, milk, fruit and vegetables. In this study, the aim was to find characteristic VOCs of *C. jejuni* in order to detect its presence with an array of MOX gas sensors. Using a starting concentration of 10^3 CFU/mL, VOCs were analyzed using GC-MS with SPME technique at the initial time (T0) and after 20 hours (T20). It has been found that Campylobacter sample at T20 is characterized by a higher number of alcohol compounds that the one at T0 and this is due to sugar fermentation. Sensors results showed the ability of the system to follow bacteria curve growth from T0 to T20 using PCA. In particular, this results in a decrease of $\Delta R/R_0$ value over time. For this reason, MOX sensors are a promising technology for the development of a rapid and sensitive system for *C. jejuni*.

Keywords: Campylobacter jejuni; VOCs; GC-MS SPME; nanowire sensors; PCA.

1. Introduction

Nowadays we live in the safest environment regarding the food industry. Organizations as EFSA (European Food Safety Agency), WHO (World Health Organization), and FAO (Food and Agriculture Organization), determine, organize and regulates all the normatives that controls every single aspect in the food safety, security and trading. Even if the risk perception regarding the food industry is not one of the first concern of the population in non-developing countries [1,2], food poisoning it's still the first cause of hospitalization in the world. CDC (Center of Disease Control) estimates that each year 48 million people get sick from a foodborne illness, 128,000 are hospitalized, and 3,000 die just in the USA [3].

FoodNet (Foodborne Diseases Active Surveillance Network) division of the CDC affirms on its 2017 report summarizes preliminary surveillance data from 2016 to 2017 that the incidence of infections per 100,000 people was highest for Campylobacter and Salmonella, which is similar to previous years. The situation in Europe for Campylobacter infections are illustrated by the Campylobacteriosis-Annual epidemiological report [4] published the 30th January of 2017 by the ECDC (European Center of Disease Control) from data collected in 2014. The report affirms that 240,379 confirmed cases were reported in 2014 with a rate of campylobacteriosis of 59.8 cases per 100,000 population in the EU/EEA, representing a 13% increase compared with the previous year. Human campylobacteriosis was more common in children below five years of age and in general was slightly higher for males than females across all age groups. Campylobacteriosis shows a clear seasonality, with a sharp peak of cases in July, trend that it is confirmed by CDC as well. At the

beginning of the summer of 2018 in Pescara the center region of Italy 180 cases of intoxicated children were reported and identified as campylobacteriosis infection.

The most representative etiologic agent for campylobacteriosis is *Campylobacter jejuni.* It is a slender, spirally curved rod that possesses a single polar flagellum at one or both ends of the cell. It is oxidase and catalase positive, is microaerophilic, requiring small amounts of oxygen (3–6%) for growth, its optimum growth temperatures on solid media are 37◦C, and grow well at pH 5.5–8.0. *C. jejuni* is associated with warm-blooded animals, actually a large percentage of all major meat animals have been shown to contain these organisms in their feces. Some strains of *C. jejuni* produce a termolabile enterotoxin (CJT). That have been reported to have some similar properties with the enterotoxins of *Vibrio cholerae* (CT) and *Escherichia coli* (LT) [5, 6]. Many meat warm blood animals can carry in their intestines, liver and giblets cells of *C. jejuni* that can be transferred to other edible parts of an animal when it's slaughtered. In the USA, National Antimicrobial Resistance Monitoring System (NARMS) testing found *C. jejuni* on 33% of raw chicken bought from retailers.

Campylobacter infection can also be transmitted through unpasteurized milk ingestion when a cow has a Campylobacter cells in her udder or when milk is contaminated with manure [7]. Moreover, most problematic foods regarding Campylobacter infection are foods that mostly are eaten raw such as fruits and vegetables that can be can become contaminated through contact with soil containing feces from cows, birds, or other animals. Animal feces can also contaminate water sources as lakes and streams.

Today there are many techniques that can be used in the identification of this type of contamination, many of which have limits related to the collection time of responses, the high complexity or the possibility of being re-used several times.

In the last years, different kinds of sensors have been developed for Campylobacter detection. They are essentially DNA-biosensors, whose specificity is due to oligonucleotide probes covalently immobilized on the sensing surface. Several techniques such as optical [8], acoustic [9] and electrochemical [10] have been proposed for traducing the hybridization with the specific target nucleic acid to the pathogen detection [11]. As an example, quartz crystal microbalance (QCM) immunosensors, using monoclonal and polyclonal antibody systems coupled with the use of gold nanoparticles (AuNPs) to increase the sensitivity, were used [12]. In this way, a limit of detection (LOD) of 10^4 CFU/mL has been reached; however, this kind of sensors is limited for a single use and consequently not low usage costs. Same LOD was reached using a colorimetric aptasensor, that can be used for on-line applications and gives its response in 30 minutes [13].

As an example, to overcome time consumption and single-use limitations, approaches based on nanowire gas sensors technology could be employed. Nanowire gas sensors based their action principle on the analysis and recognition of the volatile fingerprint of a determinate sample. This kind of approach have already successfully been applied in many different fields as human microbiota monitoring [14], and environmental monitoring [15, 16]. In particular regarding food microbial contamination nanowire tech was able of recognize the presence of a determinate microorganism throughout the set of Volatile Organic Compounds (VOCs) emitted when growing in a determinate matrix [17, 18, 19]. In comparison with the aforementioned sensor technologies nanowire gas sensor exhibit the advantages of the nanostructured materials as long term stability for sustained operations, high rate surface/area, drastically reduced time of response and the possibility to be reused as well. In this study, an array of these sensors has been used inside a portable device called Small Sensor System (S3), described in detail in *Section 2.3*.

The aim of this work was to find and identify the VOCs set that characterize *C. jejuni* through Gas-Chromatography Mass-Spectrometry (GC-MS) and to assess the capability of S3 to distinguish between samples inoculated with this microorganism and control specimens in order to use it as an efficient tool for prevention of illnesses and food quality control.

2. Materials and Methods

2.1. Samples preparation

The sample were prepared using *Campylobacter jejuni* subsp. *jejuni* type strain purchased from DMSZ, DSM number 4688, (ATCC 33560, CCUG 11284, CIP 702, NCTC 11351) and Brain Heart Infusion Broth (BHI) media purchased from Sigma Aldrich (Merck). Tubes containing 9 mL of sterile BHI were inoculated with *C. jejuni* cells and where incubated for 24 h at 35° C in order to produce enough biomass to proceed with the next step of analysis. After the incubation the culture was used to inoculate tubes of sterile BHI media in order to real the same optical density (OD) of the number 3 standard of McFarland that correspond to a concentration of $9x10^8$ CFU (Colony Forming Unit) by mL. Subsequently serial dilutions using sterile BHI media were performed until the concentration was decreased of 4 orders of magnitude to $9x10^4$ CFU/mL, that was used for the inoculation of the analyzed vials.

Sterile chromatography 20 mL vials containing 4 mL of BHI were inoculated with 100 µL of the $9x10^4$ CFU/mL solution reaching a final concentration of $2.20x10^3$ CFU/mL. Control vials were performed as well keeping the vials containing 4 ml of BHI with no innoculus. Furthermore, in order to control the effective number of cells at the beginning and the end of the analysis, plate count technique was applied using four plates for each time (T0 and T20).

2.2. GC-MS Analysis

The Gas Chromatograph (GC) used during the analyses was a Shimadzu GC2010 PLUS (Kyoto, KYT, Japan), equipped with a Shimadzu single quadrupole Mass Spectrometer (MS) MS-QP2010 Ultra (Kyoto, KYT, Japan) and an autosampler HT280T (HTA s.r.l., Brescia, Italy). The GC-MS analysis was coupled with the Solid-Phase Micro Extraction (SPME) method in order to find the most characteristic VOCs for each sample.

The fiber used for the adsorption of volatiles was a DVB/CAR/PDMS-50/30 µm (Supelco Co. Bellefonte, PA, USA). The fiber was exposed to the headspace of the vials after heating and shaking the samples in the HT280T oven, thermostatically regulated at 40 °C for 15 min, with the aim of creating the headspace equilibrium. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the GC-MS for 6 min at 240 °C.

The gas chromatograph was operated in the direct mode throughout the run, with the mass spectrometer in electron ionization (EI) mode (70 eV). GC separation was performed on a MEGA-WAX capillary column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, Santa Clara, CA, USA). Ultrapure helium (99.99%) was used as the carrier gas, at the constant flow rate of 1.5 mL/min. The following GC oven temperature programming was applied. At the beginning, the column was held at 40 °C for 3.5 min, and then raised from 40 to 90 °C at 5 °C/min. Next, the temperature was raised from 90 °C to 220 °C, with a rate of 12 °C/min; finally, 220 °C was maintained for 7 min.

The GC-MS interface was kept at 200 °C. The mass spectra were collected over the range of 40 to 500 *m/z* in the Total Ion Current (TIC) mode, with scan intervals at 0.3 s. The identification of the volatile compounds was carried out using the NIST11 and the FFNSC2 libraries of mass spectra.

Four samples were analyzed: control and Campylobacter at times T0 and T20.

2.3. S3 Analysis

S3 device used in the present work has been completely designed and constructed at SENSOR Laboratory (University of Brescia, Italy) in collaboration with NASYS S.r.l., a spin-off of the University of Brescia. It has been described in other works [20-23]. Briefly, the tool comprises three parts: pneumatic components, that transfer VOCs from the headspace of samples to the sensing

chamber; electronic boards, that manage the acquisition and transmission of the data from the device to the dedicated Web-App and allow the synchronization between S3 and the auto-sampler; sensing chamber, that can host from five to ten different MOX gas sensors and is thermostated and isolated in order to avoid any influence of the surrounding environment. To function properly, the sensors need a reference value, which has been obtained by filtering the ambient air with a small metal cylinder (21.5 cm in length, 5 cm in diameter) filled with activated carbons.

Eight MOX gas sensors were used. Three of them are MOX nanowire [24,25]. Two of them are tin oxides nanowires sensors, both grown by means of the Vapor Liquid Solid technique [26], using a gold catalyst on the alumina substrate and functionalizing one of them with gold clusters; the third sensor has an active layer of copper oxide nanowires. The working temperature is 350 °C, 350 °C and 400 °C, respectively. The other three sensors are prepared with RGTO thin film technology [27]; one is tin oxide functionalized with gold clusters (working at 400 °C), while the other two are pure tin oxide (working at 300 °C and at 400 °C, respectively). The last two are commercial MOX sensors produced by Figaro Engineering Inc. (Osaka, Japan). In particular, they are the TGS2611 and TGS2602, which are sensitive to natural gases and odorous gases like ammonia, respectively, according to the datasheet of the company. Commercial sensors have been mounted on our e-nose in order to evaluate the performances of nanowire sensors. Details of S3 sensors made at SENSOR Laboratory are summarized in Table 1. Response to 5 ppm of ethanol, selectivity (response ethanol/response carbon monoxide) and limit of detection (LOD) of ethanol are also included.

Table 1. Type, composition, morphology, operating temperature, response (ΔR/R), selectivity (response ethanol/response carbon monoxide) and limit of detection (LOD) of ethanol for S3 sensors made at the SENSOR Laboratory.

Materials (Type)	Composition	Morphology	Operating Temperature (°C)	Response to 5 ppm of Ethanol	Selectivity	Limit of Detection (LOD) of Ethanol (ppm)
SnO_2Au (n)	SnO_2 functionalized with Au clusters	RGTO	400 °C	6.5	3	0.5
SnO_2 (n)	SnO_2	RGTO	300 °C	3.5	2.5	1
SnO_2 (n)	SnO_2	RGTO	400 °C	4	2	0.8
SnO_2Au+Au (n)	SnO_2 grown with Au and functionalized with gold clusters	Nanowire	350 °C	7	2.5	0.5
SnO_2Au (n)	SnO_2 grown with Au	Nanowire	350 °C	5	2.1	1
CuO (p)	CuO	Nanowire	400 °C	1.5	1.5	1

The autosampler headspace system HT2010H (HTA s.r.l., Brescia, Italy) was coupled with S3. It supports a 42-loading-sites carousel and a shaking oven to equilibrate the sample headspace. 40 vials were placed in a randomized mode into the carousel. Among these vials, 5 were control samples analyzed at times 2.5 h, 5.5 h, 8.5 h, 11.5 h and 14.5 h. Each vial was incubated at 40 °C for

10 min in the autosampler oven. The sample headspace was then extracted from the vial in the dynamic headspace path and released into the carried flow (100 sccm). The analysis timeline can be divided into three different steps for a duration of 30 min per sample divided as follows: 5 min to analyze samples, 5 min to clean the tube that carries VOCs from sample headspace to sensing chamber and 25 min to restore the base line. Thanks to the processor integrated in the S3 instrument, the frequency at which the equipment works is equal to 1 Hz.

2.4. S3 Data Analysis

Data analysis was performed using MATLAB® R2015a software (MathWorks, USA). First of all, sensors responses in terms of resistance (Ω) were normalized when compared to the first value of the acquisition (R_0). For all the sensors, the difference between the first value and the minimum value during the analysis time was calculated; hence, $\Delta R/R_0$ has been extracted as feature.

Principal Component Analysis (PCA) was applied to this data matrix in order to evaluate the ability of the system to follow variation over time of the number of bacteria and therefore also the quantity of VOCs emitted.

3. Results and Discussion

3.1. GC-MS Results

Chromatograms of analyzed samples were compared to highlight differences between control samples and Campylobacter ones and to see the variation of emitted VOCs in the headspace between T0 and T20.

The comparison between control and Campylobacter specimens at time T0 underlines no significant differences in terms of number and amount of VOCs. In Table 2, the list of compounds with their retention time (RT) and abundance in arbitrary unit is reported. Correlation coefficient has been calculated to get how similar the two samples were; a value equal to 0.9965 has been obtained. This proves that during the conditioning period of 15 min before fiber exposure in GC injector, Campylobacter VOCs production was too small to change headspace composition.

Table 2. List of VOCs for *C. jejuni* and control samples with their retention times (RT) and abundance in arbitrary units at time T0.

RT	VOC	Abundance	
		Campylobacter	*Control*
1.552	3-Butynol	5488041	5289186
2.674	Isovaleraldehyde	28336125	25535856
5.386	Dimethyl Disulfide	5401037	6048406
8.666	3-O-Methyl-D-fructose	912105	904062
9.281	1-Hydroperoxyheptane	533178	418838
12.332	2,5-Dimethylpyrazine	623139	560597
14.432	Nonanal	512867	227918
14.624	6-Methyloctadecane	549617	794739
15.419	4-Methyl-2-oxovaleric acid	457664	412957
15.805	2-Acetylamino-3-hydroxy-propionic acid	27861	51993
16.017	1-(2-Methoxy-1-methylethoxy)-2-propanol	519216	240149
16.372	Ethylhexanol	404819	389367
16.866	Benzaldehyde	12509648	13515034
17.430	3-Trifluoroacetoxydodecane	92701	168299
18.455	3-Hydroxycyclohexanone	145875	206891

18.695	Acetophenone	2201980	1987623
19.545	[(2-Ethylhexyl)methyl]oxirane	93105	281168
19.950	Methoxy-phenyl-oxime	1545714	1301333
20.871	Heptanoic acid	268343	304130
21.205	Benzyl alcohol	280516	246860
21.555	2-[2-(Benzyloxy)-1-(1-methoxy-1-methylethoxy)ethyl]oxirane	226967	202114
22.021	1-Dodecanol	222887	482480
22.465	Phenyl carbamate	51364	93104
23.580	Octanal	121544	130790
24.480	Octadecanal	131841	76150
25.078	2,6-Bis(tert-butyl)phenol	691529	597086
27.565	N,N-Dimethylformamide ethylene acetal	53073	24830

On the contrary, samples analysis after 20 h has indicated changes in vial headspace due to microorganisms metabolism activity and to slow release of VOCs contained in BHI broth. In Table 3, the list of VOCs is shown. Main differences between the specimens reside in the presence of alcohol compounds, such as 1-pentanol, acetoin, 2,7-dimethyl-4,5-octanediol, 2-propyl-1-pentanol, bicyclo[3.2.1]octan-6-ol, 1-nonanol, γ-methylmercaptopropyl alcohol and (9E)-9-hexadecen-1-ol greater in Campylobacter samples than control one. This result points out how sugar fermentation went on during 20 h incubation period at 37°C. Furthermore, this heating phase could be also responsible of the formation of new compounds derived from pyrazine, like trimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine. In this case, correlation coefficient is equal to 0.2666, indicating the samples were strongly diverse.

Table 3. List of VOCs for *C. jejuni* and control samples with their retention times (RT) and abundance in arbitrary units at time T20.

RT	**VOC**	**Abundance**	
		Campylobacter	*Control*
1.540	3-Butynol	8834835	2135579
2.266	Isovaleraldehyde	221889809	3852176
4.142	Dimethyl Disulfide	42167678	0
8.377	1-Pentanol	236112172	0
9.229	Isoamyl Alcohol	0	737186
10.673	Acetoin	1974910	0
11.125	2-Methylbutyl isovalerate	1684776	0
12.299	2,5-Dimethylpyrazine	0	693770
14.400	Nonanal	0	123487
14.483	Trimethylpyrazine	0	258896
14.600	6-Methyloctadecane	0	60501
15.275	2-Ethyl-3,6-dimethylpyrazine	243733	0
15.392	4-Methyl-2-oxovaleric acid	454870	273645
15.702	Ammonium acetate	812172	572057
15.903	2,7-Dimethyl-4,5-octanediol	945696	0
16.186	1-(2-Methoxy-1-methylethoxy)-2-propanol	0	79930
16.295	2-Propyl-1-pentanol	308880	0
16.340	Ethylhexanol	267747	301354
16.845	Benzaldehyde	0	14760479
17.366	1-Octanol	972272	256675

17.550	Bicyclo[3.2.1]octan-6-ol	164638	0
17.946	2-Undecanone	103647	0
18.419	3-Hydroxycyclohexanone	0	79109
18.581	Benzeneacetaldehyde	5087169	0
18.668	Acetophenone	0	669190
18.740	1-Nonanol	1331499	0
18.914	Methyl 4-hydroxybutanoate	0	204876
19.446	γ-Methylmercaptopropyl alcohol	690884	0
19.923	E-11,13-Tetradecadien-1-ol	2393731	532815
20.575	β-Phenethyl acetate	131503	0
20.844	Heptanoic acid	488771	215935
21.187	Benzyl alcohol	342619	146035
21.540	Phenylethyl Alcohol	26577900	1429647
21.750	m-Tolunitrile	0	60783
21.996	1-Dodecanol	492208	270053
22.316	Tropone	165612	56068
22.443	4-Hydroxybenzenephosphonic acid	0	77574
22.684	Nerolidyl acetate	0	116357
22.876	Octanoic acid	178530	73913
23.555	1,3,2-Dioxaborolane, 2-ethyl-4-(3-oxiranylpropyl)-	0	48973
23.822	(9E)-9-Hexadecen-1-ol	188001	0
25.047	2,4-Di-tert-butylphenol	204650	200860
26.632	Pyrindan	103007890	14885449
27.555	N,N-Dimethylformamide ethylene acetal	40116	40546

Growth of *C. jejuni* is confirmed by microbiological analysis, too. There were $(8.57\pm1.18)\times10^4$ CFU/mL at time T0 in terms of mean ± standard deviation calculated on four plates and $(1.38\pm0.40)\times10^7$ CFU/mL at time T20.

3.2. S3 Results

First step of S3 data analysis consisted of checking which of the eight sensors were more performing. Sensor responses were normalized in order to highlight the variation of the resistance once sensing materials were exposed to VOCs. Five sensors showed the best performances: two RGTO (SnO_2Au and SnO_2 heated at 300°C), SnO_2Au nanowire, copper oxide and TGS2611. Resistance variation over time for all measures is shown in Figure 1 for three kinds of sensors, *i.e.* RGTO, nanowire and commercial MOX.

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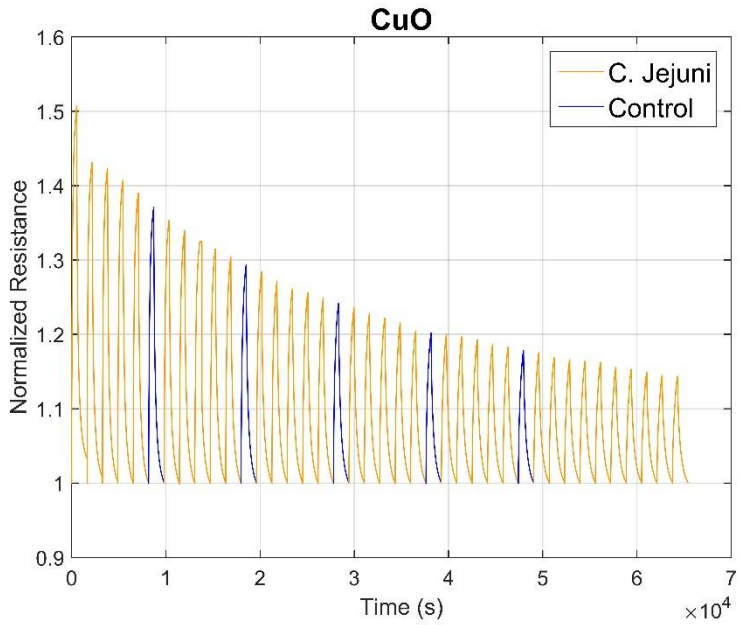

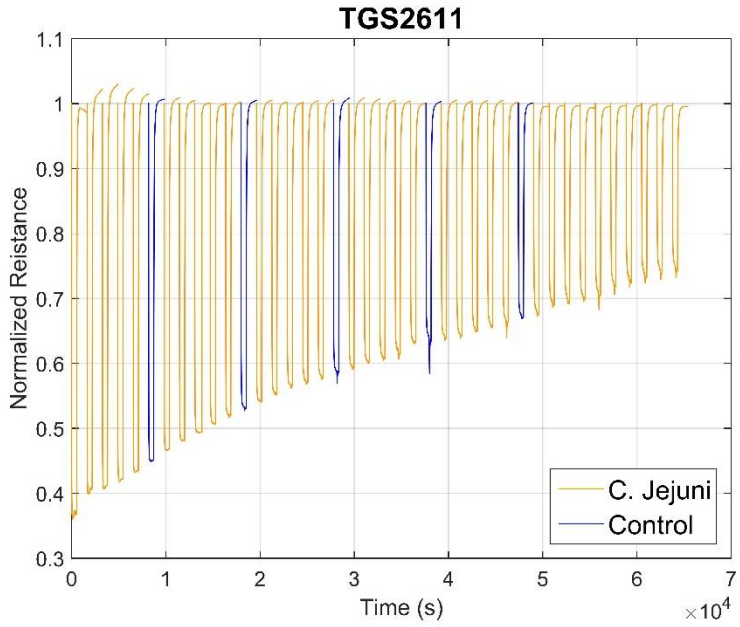

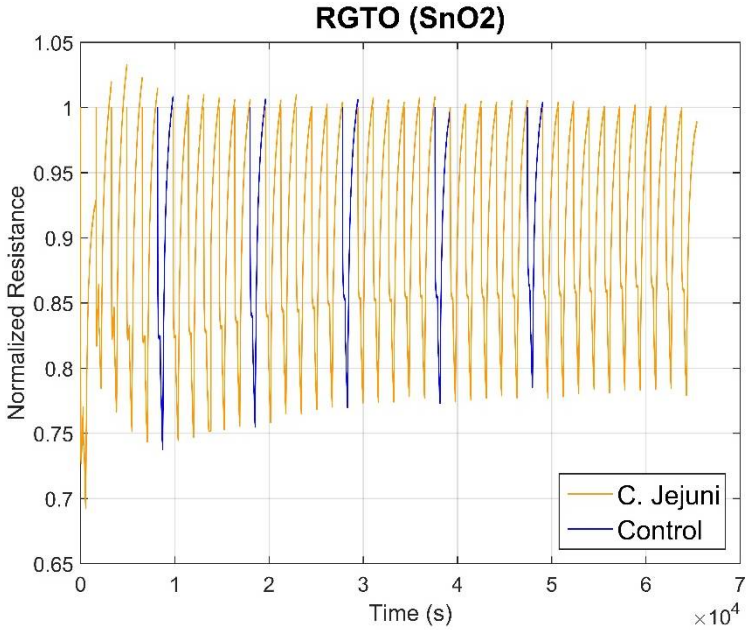

Figure 1. Resistance variations of three sensors once exposed to VOCs. From top to bottom: copper oxide nanowire, TGS2611 and tin oxide RGTO. On the x-axis there is time in seconds, on the y-axis normalized resistance.

CuO sensing material exhibits an increase in resistance respect to R_0 value, while TGS2611 and SnO_2 RGTO have an opposite behavior due to their n-type semiconductor characteristic. However, all of them are characterized by the decreasing of ΔR with the growth of time. This trend is more evident for CuO and TGS2611 sensors. Conversely, RGTO has an increase of ΔR for the first 5 samples, while from the seventh specimen it has the same kind of response of the other two even if it is less accentuated. This tendency could be explained considering that the number of microorganisms grows over time very quickly; it has been shown that they double their number in BHI broth in 75 minutes (average value) [28]. Hence, many VOCs could be used by *C. jejuni* to feed, thus subtracting them from the headspace. At the same time, new compounds are emitted from bacteria and pass in the gaseous phase, as shown in Table 2 and 3 of the previous section. It is important to underline that for 20 hours not only alcohols have increased in number and amount, but also other compounds. Among them, the one present in greater quantity is pyrindan, a bicyclic compound containing a pyridine ring. The reduction of $\Delta R/R_0$ value could be due to action of this compound. Furthermore, it can be noticed that the first sample of the analysis produced a ΔR significantly different respect to the others; it is higher for all sensors, especially for RGTO and nanowire sensors. Since this could be the result of a different conditioning, it has been discarded for the following analysis.

Figure 2 refers to PCA that has been performed using the five aforementioned sensors. First two Principal Components (PC) were used for a total explained variance equal to 99.08% (91.77% in PC1 and 7.31 in PC2). It is possible to identify two different trends. For *C. jejuni* samples, first four samples (0.5-2.5 h) are characterized by descending scores along PC2 axis, while the other specimens (3-20 h) are distributed essentially along PC1 ascending scores. Furthermore, the distance between points decrease as time goes on and it can be explained by the typical growth curve of microorganism. Indeed, it is characterized by four phases: A) lag phase (bacteria adapt themselves to growth conditions and are yet not able to divide), B) log phase (cell doubling), C) stationary phase (growth rate and stationary rate are equal due to a growth-limiting factor such as the depletion of an essential nutrient) and D) death phase (bacteria die). In PCA, lag phase corresponds to the first four points that move along PC2 axis, log phase to the following eleven samples (3-8 h) and stationary phase to the remaining ones (8.5-20 h). Instead, control samples assume higher scores along both PCs axes with increasing time. It is due to the slow release in the headspace of some VOCs contained in the BHI broth. The only exception is the fourth control sample that does not follow the parabolic trend of the others, but it is closer to *C. jejuni* points.

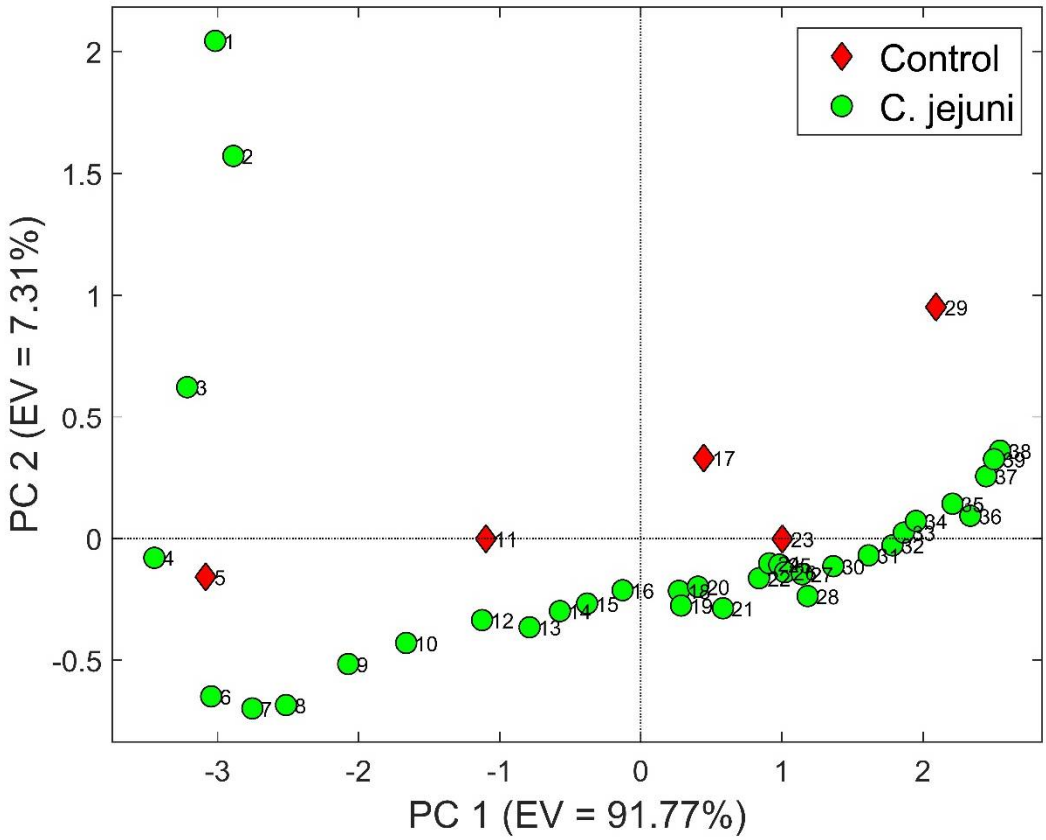

Figure 2. PCA done with first two components (total variance equal to 99.08%). Green circles are *C. jejuni* samples, red diamond control ones.

4. Conclusions

This work demonstrates the potential of this technology for the development of a rapid and sensitive detection method for *C. jejuni*. The sensors used for this study have proven to be very good at identifying and characterizing microbiological contamination. In particular, PCA done with four sensors shows the capability of the system to follow bacteria growth along a period of 20 h. During which the sensors used were able to associate the response faithfully following the growth curve of the contaminated microorganisms. We will plan to continue this study by focusing on reducing detection threshold in order to use this tool to individuate the presence of *C. jejuni* at low concentration and to avoid human infections. We will evaluate in future works how food matrix where *C. jejuni* develops and grows will influence sensor responses and their LOD.

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