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FULL ARTICLE

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Nanoplasmonic sensor for foodborne pathogens detection. Towards development of ISO-SERS methodology for taxonomic affiliation of *Campylobacter* spp.

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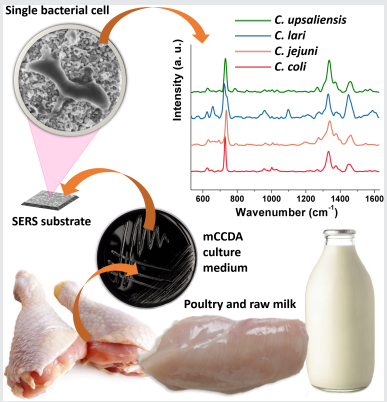
Abstract



According to EU summary report on zoonoses, zoonotic agents and food-borne outbreaks in 2017, *Campylobacter* was the most commonly reported gastrointestinal bacterial pathogen in humans in the EU. Unfortunately, the standard methods for the detection of thermotolerant *Campylobacter* spp. in foods are time-consuming. Additionally, the qualified staff is obligatory. For this reason, new methods of pathogens detection are needed. The present work demonstrates that surface-enhanced Raman scattering (SERS) is a reliable and fast method for detection of *Campylobacter* spp. in food samples. The proposed method combines the SERS measurements performed on an Ag/Si substrate with two initial steps of the ISO standard procedure. Finally, the principal component analysis (PCA) allows for statistical classification of the studied bacteria. By applying the proposed ISO-SERS-PCA method in the case of *Campylobacter* bacteria the total detection time may be reduced from 7 to 8 days required by ISO method to 3 to 4 days in the case of SERS-based approach.

KEYWORDS

Campylobacter spp., foodborne bacteria, ISO, PCA, SERS, surface-enhanced Raman spectroscopy

1 | INTRODUCTION

Food-borne illnesses represent a significant and serious health hazard. Besides the health problems, they are responsible for economic burden worth billions of dollars worldwide [1]. The allowed limit of bacterial pathogens, namely *Salmonella* spp., *Listeria monocytogenes*, *Cronobacter sakazakii* in food samples equals zero and is regulated by the Commission Regulation (EU) 2073/2005 [2], while the detection procedure, based mostly on conventional microbiology, is standardized by International Organization for Standardization (ISO) [3–5].

In 2017, a new regulation (Commission Regulation [EU] 2017/1495) [6], amending the previous one, appeared.

The new document concerns the presence of *Campylobacter* spp. in broiler carcasses, as according to the EFSA Scientific Opinion handling, preparation and consumption of broiler meat accounts for 20% to 30% of human cases of campylobacteriosis (illness caused by *Campylobacter* spp.), while 50% to 80% can be attributed to the chicken reservoir as a whole [7]. The document also indicates that food manufactures and business operators are responsible for the production and delivery of safe food, but it does not impose an obligation to check the food safety on poultry sellers. For this reason, campylobacters can be easily found in chicken meat available in the grocery stores.

The topic concerning campylobacteriosis is of imperative significance, as the mentioned illness is the most common among foodborne diseases in the European Union. The number of reported confirmed cases was 246 158 with an EU notification rate of 64.8 per 100 000 population [7]. This number is around 2.7 times higher than in the case of illnesses caused by *Salmonella*. As the campylobacteriosis is the third most common cause of mortality among the foodborne pathogens, monitoring of *Campylobacter* in food, mainly in fresh meat from broilers or turkeys, milk, and milk products should be harmonized and respected by the countries of the European Union.

Currently, there exist several quantitative and qualitative methods for the detection and identification of bacteria species based on biochemical [8], immunological (e.g., enzyme-linked immunosorbent assays, ELISA [9, 10]), and nucleic acid approaches (e.g., polymerase chain reaction, PCR [11]), as well as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [12]. However, the majority of them are cost and time-consuming, complex, labor exhaustive, and commonly require several steps of cultivation of bacterial cells and assistance of trained microbiologists. The most popular methods, ELISA and PCR, permit multiplex detection of several bacterial cells, but require huge libraries of specific antibodies and primers, respectively. Moreover, in the PCR and ELISA techniques, the false-positive and false-negative results may appear. Vibrational and fluorescence spectroscopy have also been employed for bacteria identification [13–15]. Nevertheless, these methods have some limitations, for example, the fluorescence spectroscopic technique lacks specificity of the chemical information of analyzed samples, and IR spectroscopy cannot be applied in measurements in aqueous solutions. Additionally, all these techniques are not suitable for routine in situ microbial analysis. Therefore, the development of a rapid, sensitive, simple, portable, high-throughput and reliable system for label-free pathogens detection and identification in complex samples is important from scientific and industrial points of view. Ideally, the designed technique should ensure also the deeper insight into biochemical and structural information.

Within the last decade, the popularity of surface-enhanced Raman scattering (SERS) has extended significantly, as it may serve as "whole-microorganism fingerprint" tool in medical and biological studies [16–22]. This vibrational method is relatively simple, non-destructive and it provides direct information about chemical and biochemical composition at the molecular level with high sensitivity and specificity.

Signal enhancement in this technique is mainly ascribed to the combination of two main mechanisms. The first and most important is known as the electromagnetic mechanism (EM) [23, 24]. Interaction between electromagnetic radiation and metallic nanostructure causes the excitation of collective oscillation of free electrons. This effect, called localized surface plasmon resonance (LSPR) leads to the presence of strong electromagnetic fields at the metal surface, which influences the Raman signal enhancement of molecules adsorbed to this surface [25–28]. The excitation of localized surface plasmons strongly depends on the type, shape and size of metal nanostructures obtained during the production of a SERS substrate. Appropriate metals for this purpose are noble metals, for example, gold, silver or copper, due to their special properties associated with the ability to excite plasmon resonance with UV, visible and near-infrared light [29].

The second mechanism, known as chemical mechanism (CT) is related to situation when the analyte chemically bonds to the surface. As a result, the charge (electrons or holes) is transferred from the metal surface into the analyte and vice versa. More precisely, this charge transfer can occur from the metal state near the Fermi level to the lowest unoccupied molecular orbital (LUMO) of the adsorbed molecules or, less frequently, from the highest occupied molecular orbital (HOMO) of adsorbed molecules to the Fermi level of the metal. This in turn causes changes in polarizability of the analyzed molecule and activates new paths of metal-molecule and molecule-metal charge transfer resonances [30, 31].

This mechanism has been proposed due to the dissimilarities between normal Raman and SERS spectra, for example, band shifting, differences in relative intensities and spectral shape [32].

Due to these two mechanisms, Raman spectroscopy became a powerful approach for analytical studies and biomedical sensing. Nowadays, Raman spectroscopy is known for practical applications in the studies of proteins [33], nucleic acids [34], eukaryotic cells [35, 36] (including cancer cells [37–39]), drugs [40, 41] and trace materials [42]. In order to calculate and visualize important characteristics of SERS spectra for a specific analyte, the principal component analysis (PCA) can be applied. The main features of this mathematical approach are: (a) extracting the biochemical information from spectra,

(b) arrangement of the statistical classification of measured substances and (c) identifying the spectrum of an unknown sample by comparison to the database of SERS spectra.

In this paper, we show a new approach applying silver/photovoltaic-based (Ag/PV) SERS substrates [43] in the detection of foodborne bacteria, namely thermotolerant *Campylobacter* spp., in different poultry samples. The topic of *Campylobacter* spp. detection by non-biological methods has been already described in scientific literature, however, the proposed methods need labels in the form of specific antibodies or do not show the procedure of direct detection of campylobacters from food samples [44, 45]. Additionally, we show for the first time that there is a substantial match between SERS spectra of the *Campylobacter jejuni* reference strain obtained from National Collection of Type Cultures (NCTC) collection and the SERS spectra of the *C. jejuni* strain detected in poultry sample (they are located in close proximity to each other in PCA). This result proves that the method presented in this work may serve as a rapid, label-free and ultrasensitive way of *Campylobacter* spp. detection, which additionally allows for strain identification and is in accordance with EU standards. The combination of Raman technique and ISO methods has been presented so far for *Salmonella* spp., *Listeria monocytogenes* and *Cronobacter sakazakii* [46, 47]. The method presented here of *Campylobacter* spp. identification is based on the SERS technique challenges the standard biochemical methods in terms of simplicity, specificity and rapidity, and thus may significantly influence the future of microbiology and medical diagnosis.

2 | MATERIALS AND METHODS

2.1 | Bacteria strains and growth media

Four different *Campylobacter* species, namely *C. jejuni* NCTC 11351, *C. coli* ATCC 33559, *C. upsaliensis* ATCC 43954, and *C. lari* ATCC BAA-1060, as well as *P. aeruginosa* ATCC 27853, were obtained from the Department of Applied Microbiology, Faculty of Biology, University of Warsaw (Poland). Cultures were maintained at −80°C in Brucella Broth supplemented with 20% glycerol.

Bolton broth, as well as modified charcoal-cefoperazone-deoxycholate agar (mCCDA), Skirrow agar, and Columbia blood agar were purchased from Oxoid (Basingstoke, United Kingdom). Sodium chloride, acetone, isopropyl alcohol, sucrose, hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), and lysozyme were purchased from Linegal Chemicals (Warsaw, Poland), mutanolysin—from A&A Biotechnology (Gdynia, Poland), GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit—from EUR_X (Gdansk, Poland), *Pfu* polymerase and GeneJET Gel Extraction and DNA Cleanup Micro Kit—from Thermo Fisher Scientific (Waltham, Massachusetts).

2.2 | SERS database of thermotolerant *Campylobacters* spp. control strains

C. jejuni NCTC 11351, *C. coli* ATCC 33559, *C. upsaliensis* ATCC 43954 and *C. lari* ATCC BAA-1060 were cultured on mCCDA, Skirrow agar and Columbia blood agar in a microaerobic atmosphere at 41.5°C for 44 ± 4 h. Next, the bacteria were subjected to SERS experiments according to the procedure described in Section 2.5. The cultivation process for each *Campylobacter* sp. and on each type of culture medium was performed in triplicate and the obtained SERS results were averaged and placed in the bacterial SERS database.

2.3 | Food samples

The standard ISO-based experiment (ISO 10272-1:2017) may be conducted in two variants to detect *Campylobacter* spp. by enrichment in products with:

1. low numbers of campylobacters and low level of background microflora and/or with stressed campylobacters (procedure A); for example, cooked and frozen products;
2. low numbers of campylobacters and high level of background microflora (procedure B); for example, raw milk and meats.

Due to ISO standards, food samples should be homogenized in enrichment medium—Bolton (procedure A) or Preston broth (procedure B), depending on the type of food sample. Subsequently, the obtained mixtures are incubated in a microaerobic atmosphere, at 37°C for 5 hours and then at 41.5°C for 44 hours (procedure A) or at 41.5°C for 24 hours (procedure B). In the next steps, the surfaces of mCCDA and a second medium, for example, Skirrow agar (procedure A) or only mCCDA agar (procedure B) are inoculated with appropriate mixtures via 10 μL loop. The samples are then incubated in a microaerobic atmosphere at 41.5°C for 44 ± 4 hours.

At least one typical or suspect colony of *Campylobacter* spp. is next selected and streaked onto non-selective blood agar plate, for example, Columbia blood agar. After cultivation in microaerobic atmosphere at 41.5°C for 24 to 48 hours, well-isolated freshly grown colony/

colonies is/are used in further confirmation and/or identification.

Ten samples of fresh, raw chicken (from 10 different retail stores) dedicated for the detection of thermotolerant *Campylobacter* sp. were transported to the laboratory inside portable insulated cold boxes. The samples were transported to the laboratories in cooler boxes and analyzed immediately. These transport conditions guarantee the chemical and biological stability of samples over time. The samples (in the amount of 10 g each) were taken in an aseptic manner and homogenized in enrichment broth (90 mL) and then subjected to microbiological analysis. The enrichment and selective isolation steps were performed according to ISO 10272-1:2017 (see Figure 1, procedure B), under microaerobic conditions generated by Anaxomat Mark II (KendroLab, Poland). All types of bacterial colonies that have grown on mCCDA were subjected to SERS experiments. Moreover, typical or suspect *Campylobacter* sp. isolate, selected from mCCDA, was streaked on Columbia blood agar and after incubation in microaerobic atmosphere at 41.5°C for 44 ± 4 h was subjected to SERS experiments. All obtained SERS spectra wee then processed with PCA.

All typical and non-typical bacterial colonies, selected from mCCDA, were additionally identified due to molecular analysis based on *16S rRNA* gene sequence (see Section 2.4).

2.4 | Molecular identification of bacterial strains

All types of strains isolated from poultry meat and selected from mCCDA were streaked on Columbia blood agar. After incubation in microaerobic atmosphere at 41.5°C for 44 ± 4 h they were identified by amplification of the *16S rRNA* gene. For this purpose, chromosomal DNA was isolated from 4 to 5 colonies, which were suspended in TES

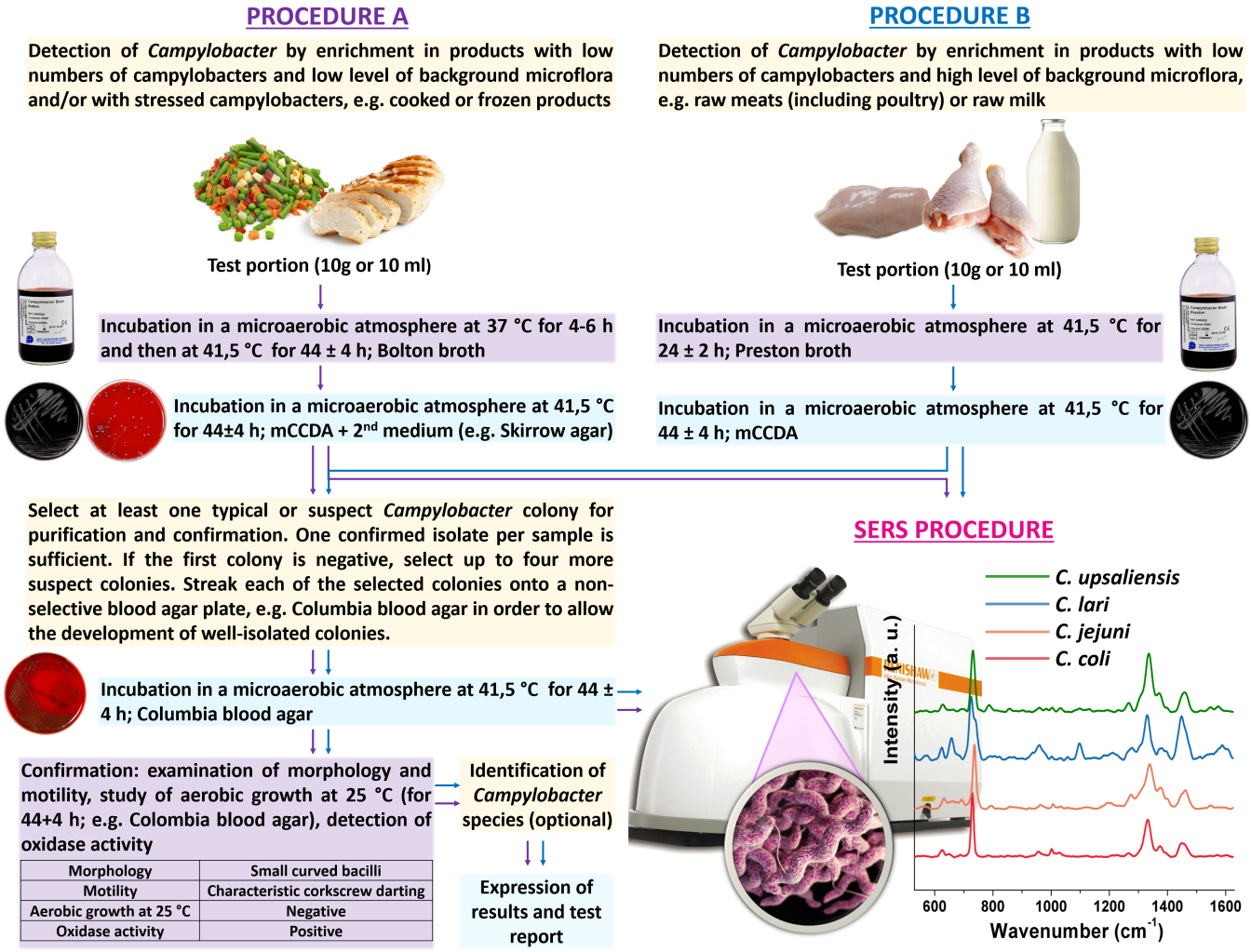


FIGURE 1 The scheme representing different paths for *Campylobacter* spp. detection in food samples, including both ISO standards and surface-enhanced Raman scattering-based approach

buffer (25 mm sucrose, 50 mm Tris HCl pH 8.0, 10 mm EDTA) containing lysozyme (20 mg/mL) and mutanolysin (500 U/mL). After incubation for 1 hour at 37°C under constant shaking, chromosomal DNA from the bacterial strains was isolated using GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit. For amplification of the *16S rRNA* gene, universal primers F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 (5′-GGTTACCTTGTTACGACTT-3′) were used with an expected PCR product size of 1.5 kb [48]. The PCR was carried out in 50 µL of reaction mixture containing: DNA (50 ng), dNTPs (0.2 mM), of each primers (0.2 mM), *Pfu* polymerase (1 U), and PCR buffer. The PCR conditions were (a) 5 minutes at 95°C, (b) 20 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 1.30 minutes at 72°C, and (c) 15 cycles of 30 seconds at 95°C, 30 seconds at 46°C, and 1.30 minutes at 72°C, and (d) 10 minutes at 72°C. The PCR products were purified with GeneJET Gel Extraction and DNA Cleanup Micro Kit, following the instructions of the manufacturer. DNA sequencing was performed with 3130 xl Genetic Analyzer (Applied Biosystem, Life Technologies) by Genomed S. A. (Poland), and then the nucleotide sequences were analyzed using BLAST against the nucleotide database at the NCBI website.

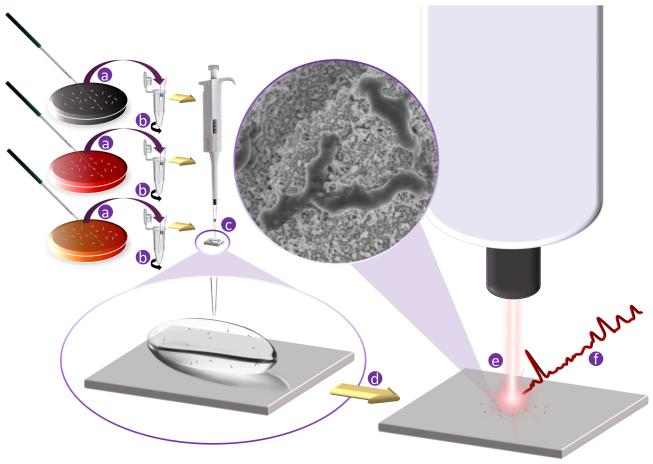


FIGURE 2 Scheme representing the surface-enhanced Raman scattering (SERS)-based detection of *Campylobacter* spp.; The procedure can be divided into several steps based on A, collecting bacterial colonies from solid culture media (from the top: modified charcoal-cefoperazone-deoxycholate agar (mCCDA), Skirrow agar, Columbia blood agar), B, suspending bacterial cells in saline solution, preceded by three centrifugation processes in different baths of saline solution C, placing 2.5 µL of bacterial solution over the SERS substrate, D, drying, E, performing SERS measurements, and F, generating SERS spectrum by a dedicated software

2.5 | Bacteria sample preparation for SERS measurements

All types of bacteria colonies were collected via 1 µL loop. Afterwards, bacteria cells were suspended in sterile saline solution (500 µL) and centrifuged for 3 minutes at 1070×g. The supernatant was discarded and the bacterial cells were resuspended in sterile saline solution. The centrifugation process was repeated three times to obtain a solution of bacterial cells without additional contaminations from culture media. After final centrifugation, the pellet of bacterial cells was resuspended in saline solution (~5–10 µL). About 2.5 µL of such bacterial solution was placed over freshly prepared SERS substrate and dried for a few minutes in sterile conditions. The scheme of sample preparation for SERS measurements is depicted in Figure 2.

2.6 | SERS substrates

In order to enhance the Raman signal of bacterial cells we used polycrystalline silicon covered with silicon nitride and Ag nanostructures. This previously developed SERS substrate [43] is based on PV panels obtained from Bruk-Bet Solar Tarnów, Poland as a post-production residue.

In order to prepare such SERS substrate the PV cells were divided into pieces of ~0.5 cm × 0.5 cm, placed in a beaker filled with acetone, and put in a shaker. After 40 minutes of shaking at room temperature, acetone solution was replaced with isopropyl alcohol and then with Millipore water, and the process was repeated. Finally, the SERS substrates were dried with compressed pure nitrogen and sputtered with 100 nm layer of silver via Physical Vapor Deposition (PVD) device (Quorum Q150T ES). The sputtering process conditions were as follows: (a) 25 mA current, (b) argon plasma, (c) 10^{-2} mbar of working pressure. The silver targets of 4 N purity, used in sputtering process, were provided by Mennica Metale (Warsaw, Poland). The substrates were used immediately after their preparation. The reproducibility of SERS-active platforms is presented in Figure S1.

2.7 | Instrumentation and software

2.7.1 | Raman and SERS spectroscopy

Measurements were carried out with a Renishaw inVia Raman system equipped with a 300 mW diode laser emitting 785 nm light, which was used as an excitation source. The light from the laser was passed through a line filter and focused on a sample mounted on an X–Y–Z translation stage with a ×20 microscope objective, NA = 0.25. The beam diameter was approximately 5 µm, and the laser power at the sample was 1.5 mW. The microscope was

equipped with diffraction grating with 1200 grooves per mm, cut-off optical filters, and a 1024 × 256 pixel Peltier-cooled RenCam CCD detector, which allowed for registration of the Stokes part of Raman spectra with 5 to 6 cm^{-1} spectral resolution and 2 cm^{-1} wavenumber accuracy. The experiments were performed at ambient conditions using a back-scattering geometry.

The spectra were recorded immediately after placing the analyzed sample on the SERS substrate surface. During the period of about 10 minutes, SERS spectra were repeatedly recorded for different hot spots on the SERS platforms. At the same time, the focus of the laser beam was readjusted. The laser power at the sample was 1.5 mW. The time required for completing a single SERS spectrum was about 4 seconds.

2.7.2 | Scanning electron microscopy

In order to attach the SERS substrate to aluminum scanning electron microscopy (SEM) pin stubs we used Micro to NanoEM-TecAG42 (Ted Pella, Inc.). Observations were performed under high vacuum using the FEI Nova NanoSEM 450. The accelerating voltage was in rage from 2 kV up to 10 kV. The SEM images of bare Ag/PV surface and Ag/PV surface covered with *C. jejuni* NCTC 11351 cells are presented in Figure 3.

2.7.3 | SERS spectra preprocessing

The preprocessing was performed using the OPUS software (Bruker Optic GmbH 2012 version). The spectra were smoothed with Savitzky–Golay filter (9 points) [49], the background was removed using baseline correction (concave rubberband correction; number of iterations: 10, number of baseline points: 64), and then the spectra were normalized using a Min-Max normalization. All the spectra presented in this work are averaged from at least 25 SERS measurements. The spectra were obtained from three different experiments (in each experiment different pieces of SERS substrate and different bacterial cell colonies were used) conducted on different spots of one SERS substrate.

2.7.4 | Chemometric analysis of spectral data

All preprocessed data were transferred to the Unscrambler software (CAMO software AS, version 10.3, Norway),

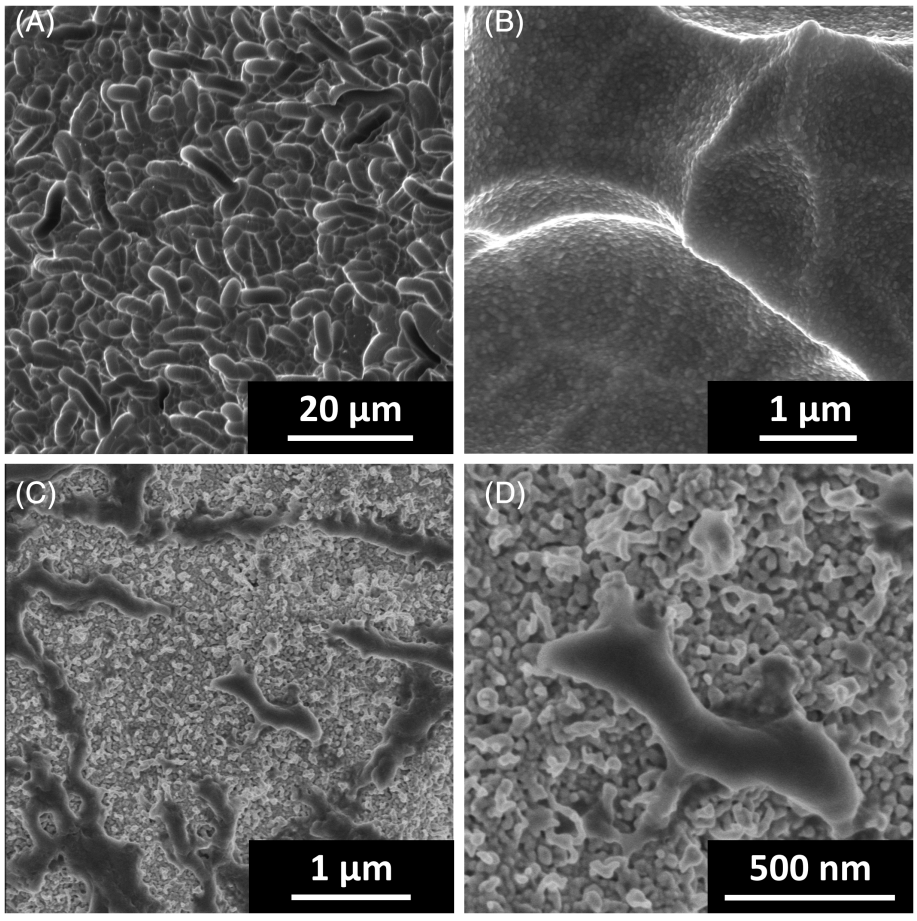


FIGURE 3 Scanning electron microscopy (SEM) images, at different magnifications, of A,B, bare surface of surface-enhanced Raman scattering (SERS) substrate and C,D, *C. jejuni* National Collection of Type Cultures (NCTC) 11351 cells on SERS substrate

used for the three-dimensional (3D) PCA and the cluster analysis.

3 | RESULTS AND DISCUSSION

3.1 | SERS and PCA of thermotolerant *Campyobacter* control strains cultured on mCCDA

In the present study, the SERS technique was introduced into ISO 10272-1:2017 procedure for identification of pathogenic bacteria, namely *Campylobacter* spp., in food samples. As shown in Figure 1, in the second step of ISO procedure, applied after enrichment in Bolton (procedure A) or Preston (procedure B) broths are next cultured on mCCDA. By applying the SERS-PCA technique, the bacteria colonies which have grown on the mCCDA after cultivation in microaerobic conditions can be analyzed within several minutes, in contrast to biochemical methods.

The selective medium used for the detection and enumeration of *Campylobacter* spp. enables the growth of other, non-campylobacter bacterial species. As all colonies which may grow on this type of medium have similar, slightly pale color, the further identification procedures completely depend on the experienced microbiologists who makes the selection of suspect colonies. It means that in the process of selection of typical or suspect colonies of *Campylobacter* spp., the presence of a qualified personnel is necessary. Finally, the procedures based on microbiological tests are time-consuming. To complete the entire identification procedure, up to 8 days are needed in the case of procedure A or 7 days in the case of procedure B. The SERS-PCA method allows to shorten the procedure to 4 days (procedure A) or 3 days (procedure B), as the whole spectroscopic analysis takes only a few minutes. Thus, one of the aims of the present study was to create a database of four thermotolerant *Campylobacter* species which may cause food poisoning in humans.

Although the SERS spectra of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* show many similarities, for example, the most intense bands are located at around 730 cm^{-1} (FAD, NAD), 1335 cm^{-1} (C–H deformation), and

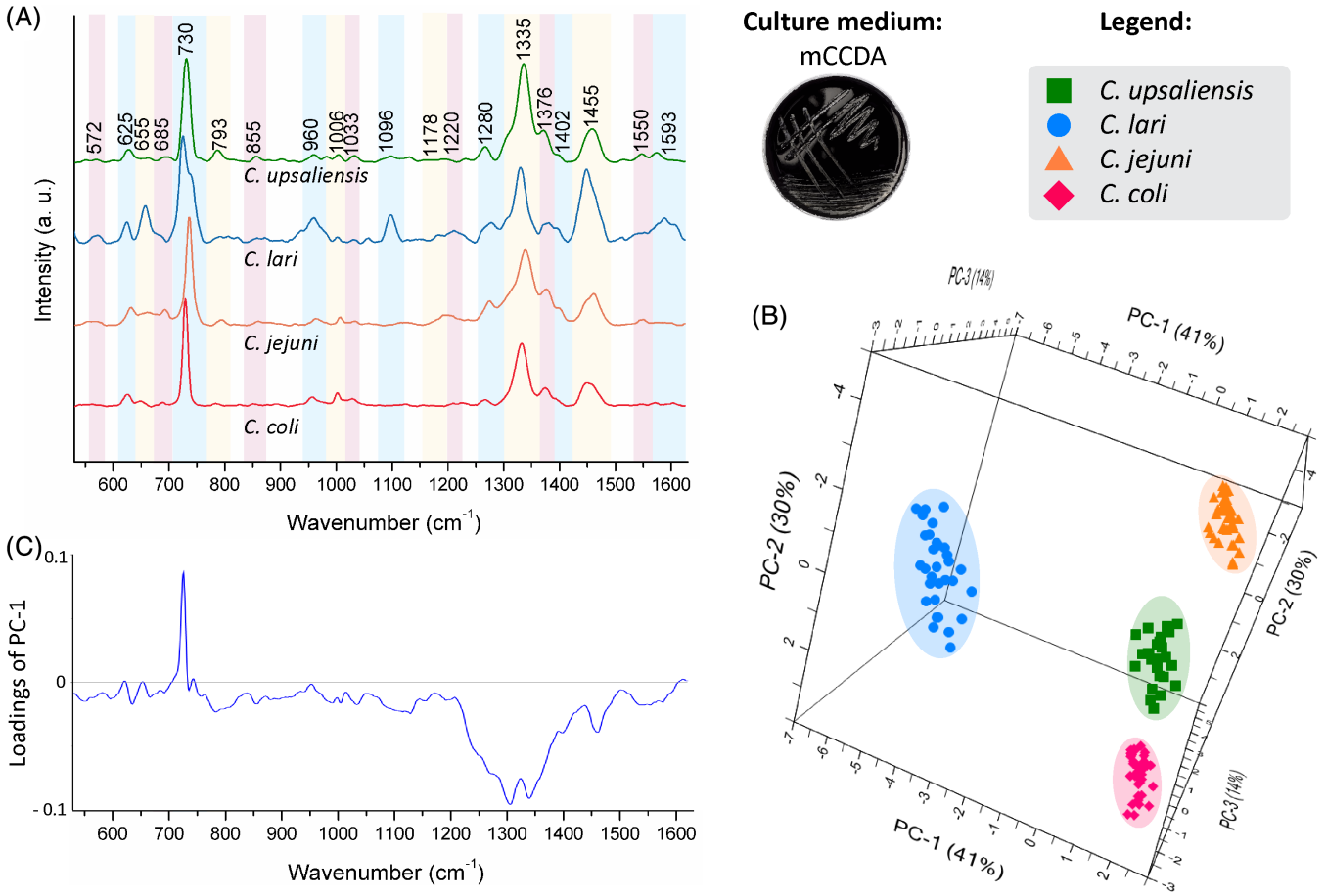


FIGURE 4 Surface-enhanced Raman scattering (SERS) and principal component analysis (PCA) results obtained for *Campylobacter* spp. cultured on modified charcoal-cefoperazone-deoxycholate agar (mCCDA); A, averaged SERS spectra, B, 3D-PCA and C, loadings plot of the first principal component (PC-1)

1455 cm^{-1} (CH_2 deformation) (see Figure 4A), there are still some differences appearing in the form of additional bands and/or changes in band intensities and shapes. The most characteristic spectrum is the one of *C. lari*: it reveals quite intense bands at around 650, 960, 1100 and 1590 cm^{-1}. These bands are absent or weak in the case of three other species.

The band which also deserves attention is the one at around 722 cm^{-1} with shoulder at around 732 cm^{-1}. Interestingly, the presence of such shoulder was also observed in the SERS spectra of *C. lari* cultivated on mCCDA, Skirrow and Columbia blood agar media (see Figures 4A, 5A and 6A).

The band at 730 cm^{-1} corresponds to in-plane ring breathing mode of adenine or from other adenine-bearing molecules, for example, flavin adenine dinucleotide (FAD) or nicotinamide adenine dinucleotide (NAD) [50]. The last two molecules are important in the process of cellular respiration taking place in the bacterial cell membrane and demonstrate a close interaction between SERS-active platform and cell wall/membrane of bacteria. It should also be noticed that metabolites of purine degradation may also contribute to the intensity of the band at 730 cm^{-1} [51].

The comparable spectra of *C. coli*, *C. jejuni* and *C. upsaliensis* could be distinguished due to the slight changes in intensities of the bands at around 793, 1335, 1400, 1455 and 1550 cm^{-1}. Additionally, in the spectrum of *C. upsaliensis* one can observe weak bands at ca. 975 and 1580 cm^{-1} which are absent in the case of *C. coli* and *C. jejuni*. All these SERS bands originate mainly from the components of the bacterial cell wall and membrane. The tentative assignments of the recorded SERS bands are presented in Table S1. All these features are responsible for group separation in PCA. It was found that for bacteria species grown on mCCDA the principal components PC-1, PC-2 and PC-3 together accounted for 85% of the variance in the data (see Figure 4B). Moreover, the analysis of the loadings plot of PC-1 leads to the information about the most significant diagnostic variables in the data set, which were indicated by the highest loadings value. Such value was attributed

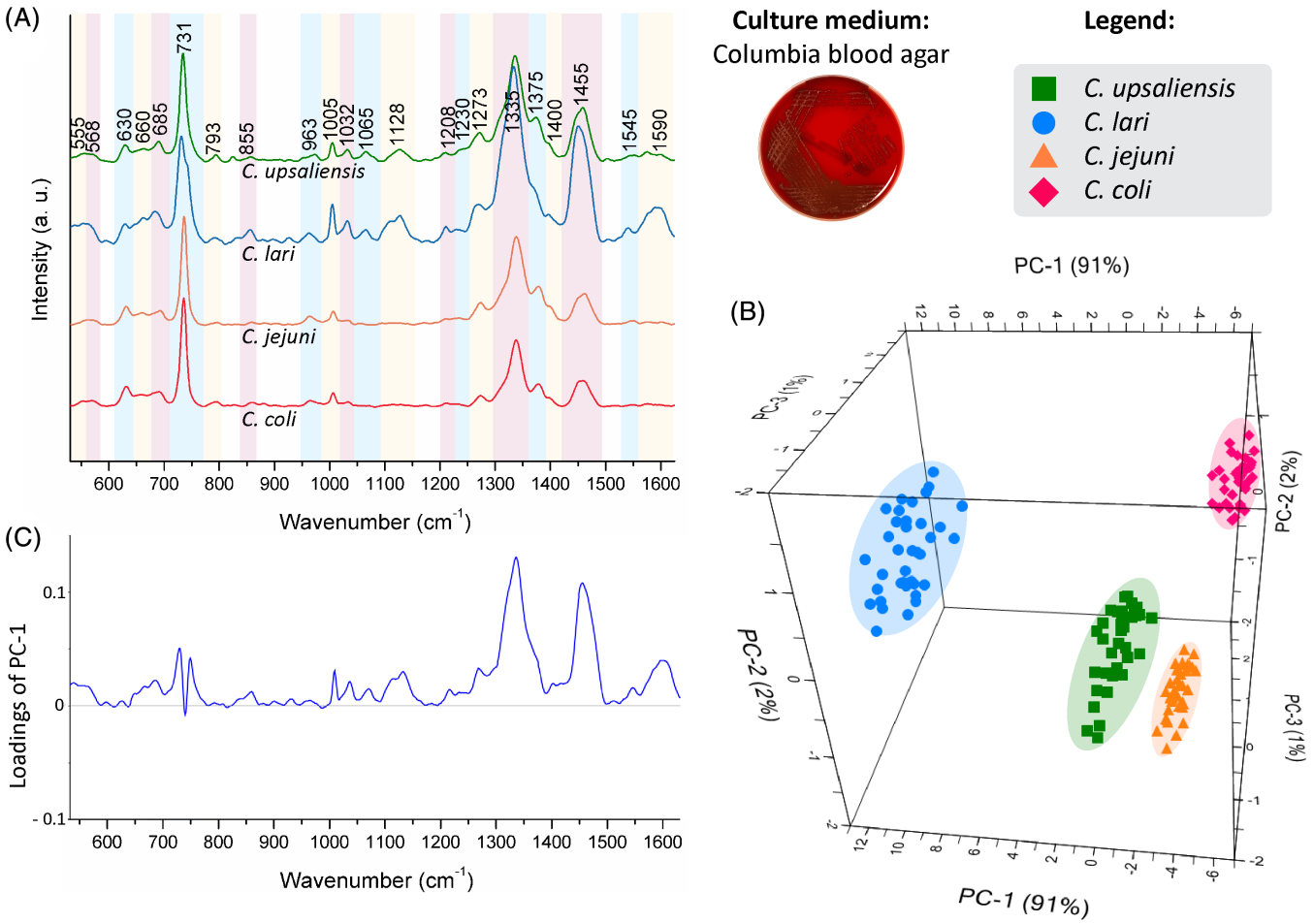


FIGURE 5 Surface-enhanced Raman scattering (SERS) and principal component analysis (PCA) results obtained for *Campylobacter* spp. cultured on Skirrow agar; A, averaged SERS spectra, B, 3D-PCA and C, loadings plot of the first principal component (PC-1)

to the band at around 730 cm^{-1} and the region between 1300 and 1350 cm^{-1} (see Figure 4C).

3.2 | SERS and PCA of *Campyobacter* control strains cultured on Skirrow agar

In the ISO 10272-1:2017 procedure, in order to detect *Campylobacter* spp. according to procedure A (see Figure 1), two types of selective media should be applied, one of which being mCCDA. The document does not regulate exactly which type of second medium should be used, but it has to be any other solid selective *Campylobacter* medium using selection principles different from those in mCCDA. Here, the Skirrow agar was used as a second selective medium.

The results of SERS measurements obtained for bacteria grown on Skirrow agar were comparable to those from mCCDA (Figure 5A). As before, the most distinct spectrum was obtained for *C. lari*, characterized by intense bands at around 570, 650, 960, 1100, 1130, 1245 and 1585 cm^{-1}. The shoulder at ca. 735 cm^{-1}, specific for this bacterial species, is also visible. In the case of three other strains, the spectroscopic differences are minor, but not unnoticeable: they appear as, for example, changes in the intensity ratio of the bands at ~630 and ~655 cm^{-1} or in the changes of intensity of the band at ca. 1550 cm^{-1}. This is the reason why *C. upsaliensis*, *C. coli* and *C. jejuni* are located on the right part of PCA cube, while *C. lari* is on the left side (see Figure 5B). As one can see, the PCA allowed to divide these four bacterial strains into four clusters. Moreover, PC-1 together with PC-2 and PC-3 accounted for 96% of variability. As expected, the most important diagnostic variables in the analyzed data set were located at around 655, 730 and 1330 cm^{-1} (Figure 5C).

3.3 | SERS and PCA of *Campyobacter* control strains cultured on Columbia blood agar

To confirm the results obtained on selective culture media in procedures A and B, at least one typical or

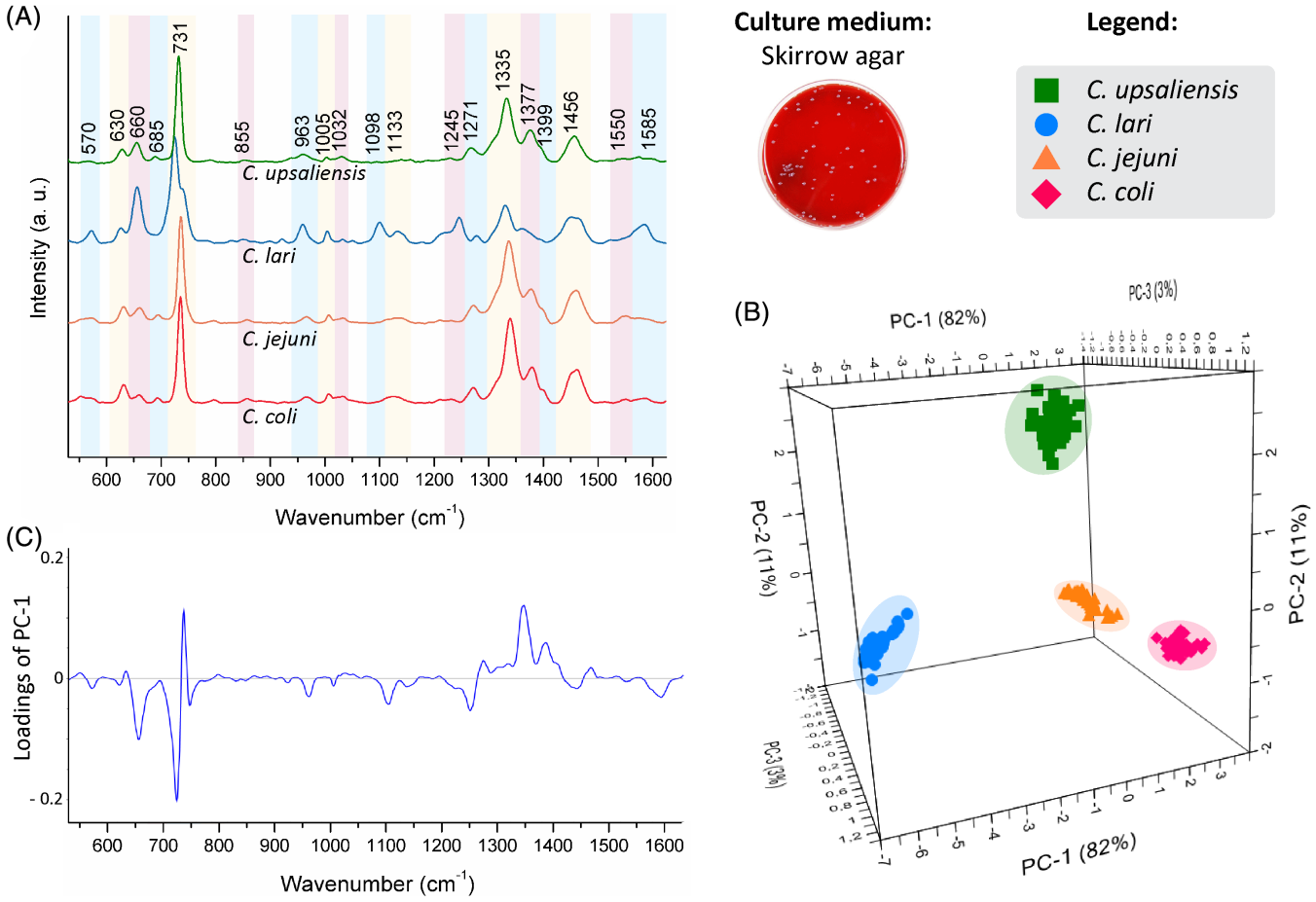


FIGURE 6 Surface-enhanced Raman scattering (SERS) and principal component analysis (PCA) results obtained for *Campylobacter* spp. cultured on Columbia blood agar; A, averaged SERS spectra, B, 3D-PCA and C loadings plot of the first principal component (PC-1)

suspect *Campylobacter* colony which has grown on mCCDA and/or for example, Skirrow agar has to be selected. If the first colony is negative, up to four more suspect colonies should be selected. Next, each of the selected colonies should be streaked onto a non-selective blood agar plate (eg, Columbia blood agar) to allow the development of well-isolated colonies. These colonies are then subjected to further analysis which may take at least two more days. The purpose of SERS-PCA-based identification is to make such confirmation shorter.

In Figure 6A, the SERS spectra of four *Campylobacter* strains cultivated on Columbia blood agar are depicted. The shoulder in the region of 735 cm^{-1} in the case of *C. lari* is reduced, but still present. Interestingly, new spectral features, for example, bands at ~1065 and 1208 cm^{-1} in the spectra of *C. lari* and *C. upsaliensis* can be observed. The differences between *C. coli* and *C. jejuni* are almost invisible. However, a watchful eye may notice a lack of the band at 1550 cm^{-1} in the spectrum of *C. coli* and its presence in the case of *C. jejuni*.

These results enabled to divide the investigated strains into separate groups in PCA. The calculated PC-1, PC-2 and PC-3 reached together the value of 96% of total variance, presenting a distinct possibility of *Campylobacter* strains differentiation with high accuracy (Figure 6B). As can be concluded from Figure 6C, the bands most important for group separation in this chemometric analysis are located at around 1335 and 1455 cm^{-1}. These bands correspond to protein vibrational modes, which suggests that the spectral differences between the examined species may be connected with distribution of different proteins in the bacterial cell wall.

3.4 | Summary of SERS and PCA results obtained for *Campylobacter* control strains

The main bands observed in SERS spectra of *C. lari*, *C. upsaliensis*, *C. coli* and *C. jejuni*, cultured on three different solid culture media recommended by ISO, namely mCCDA, Skirrow agar and Columbia blood agar, are listed in Table S1. Table 1, in turn, shows the values of PCA scores calculated for analyzed thermotolerant *Campylobacter* species cultured on mCCDA, Skirrow agar and Columbia blood agar media.

In order to check which *Campylobacter* species are most similar to each other, the cluster analysis (CA) over SERS results was performed. In this type of analysis, the spectra are grouped in such a way that objects in the same group are more similar to each other than to those in other groups. The obtained results revealed interesting information. As one can see in Figure 7, the species most distinct from the rest is *C. lari*, regardless of what type of solid medium was used. For this reason, *C. lari* is located on the top of each diagram. As the spectra of *C. coli* and *C. jejuni* measured after cultivation on Skirrow agar and Columbia blood agar show high level of spectroscopic similarity, the relative distance between them in CA is small. Only in the case of mCCDA, the situation is slightly different—here the spectra of *C. coli* were more similar to *C. upsaliensis* than to *C. jejuni*. However, the relative distance between the most closely located groups from mCCDA is larger than the distance between the most distant groups in the case of Skirrow and Columbia blood agar media, which indicates that all four species can be effortlessly differentiated from each other.

These results correspond well with the information obtained from phylogenetic tree, which shows the evolutionary relationships among various biological species. The most closely related species are *C. coli* and *C. jejuni*, which also has been firmly confirmed by the SERS results obtained for bacteria cultured on Skirrow and Columbia blood agar.

TABLE 1 Values of PCA scores calculated for analyzed *Campylobacter* species

	Scores [%]			
Culture medium	**PC1**	**PC2**	**PC3**	**Total**
mCCDa	41	30	8	85
Skirrow	82	11	3	96
Columbia	93	2	1	96

Abbreviations: mCCDa, modified charcoal-cefoperazone-deoxycholate agar; PC, principal component; PCA, principal component analysis.

3.5 | Detection of *Campylobacter* spp. cells in poultry samples

In order to check whether the ISO-SERS-PCA-based method can be applied in the case of real food samples, 10 samples of poultry, purchased in different retail stores, were tested for the presence of campylobacters. Thus, homogenized meat portions were placed in flasks with Preston broth and cultivated in microaerobic conditions at 41.5°C (see procedure B, Figure 1). After 24 hours of incubation, the mCCDA plates were inoculated with ~10 μm of obtained mixture and cultured for 48 hours.

In the case of each plate the surface of mCCDA was covered, to high extent, with bacterial cells. It is worth reminding, that not only campylobacters, but also other types of microorganisms may grow on this type of medium. These include *i.a.* cefoperazone-resistant *Pseudomonas* spp, *Enterobacteriaceae*, and some streptococci

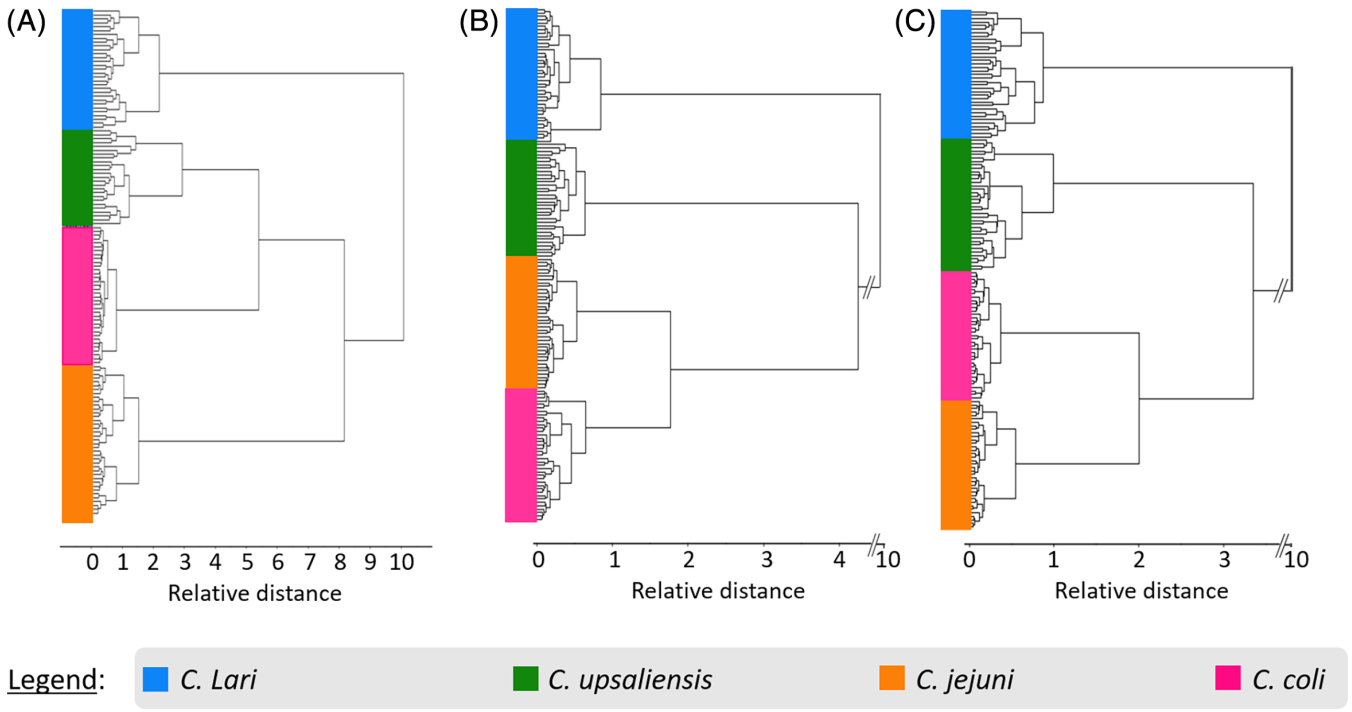


FIGURE 7 Cluster analysis of surface-enhanced Raman scattering (SERS) spectra of *Campylobacter* spp. obtained after cultivation on A, modified charcoal-cefoperazone-deoxycholate agar (mCCDA), B, Skirrow agar and C, Columbia blood agar

and yeasts. For this reason, all the colony types (showing even slight differences in morphology) were selected, suspended in sterile saline solution and subjected both to SERS and genetic analysis. The aim of genetic examination, and more precisely, of *16S rRNA* gene sequencing, was to identify the selected isolates and to confirm the results obtained in the SERS experiments. As a result, three different bacterial species were identified: *Proteus mirabilis*, *Escherichia coli*, and *C. jejuni*, however, the last species was found only in one food sample. Additionally, as *Pseudomonas* spp. may also be detected in poultry samples and can grow on mCCDA, *P. aeruginosa* ATCC 27 853 was cultured in microaerobic conditions for 48 hours and also analyzed via SERS method. The SERS measurements and PCA results obtained for the mentioned bacterial species are depicted in Figure 8. The sample labeled as *Campylobacter* sp. (Figure 8A) or as a violet star (Figures 8B and 9A-D) represents the averaged SERS spectrum of *Campylobacter* isolate, which was detected in poultry sample.

As one can see in Figure 9A, the SERS spectrum of *Campylobacter* isolate found in poultry sample is most similar to the spectrum of *C. jejuni* NCTC 11351 (Figure 8A). Moreover, the SERS spectrum of *P. mirabilis* is also comparable to spectrum of *Campylobacter* sp. (Figure 8A). On the contrary, the spectra of *E. coli* and *P. aeruginosa* show many unique features, which allow clear distinction between them and the other investigated species, for example, the bands at ~650 and 1455 cm^{-1} are relatively strong in the case of *E. coli*, while the one at 720–730 cm^{-1} is weak or absent in the spectra of *E. coli* and *P. aeruginosa*, respectively. Additionally, the spectrum of *P. aeruginosa* shows only few bacterial bands—the rest probably comes from pigments which these bacteria produce.

These findings were confirmed by PCA—the violet star in 3D-PCA was found in close proximity to the group of *C. jejuni* NCTC 11351 and *P. mirabilis* and at a greater distance to *E. coli* and *P. aeruginosa* (see Figures S2, 8B and 9A,B). The PC-1, PC-2 and PC-3 values yielded 89% of total variance in the case of mentioned strains (Figure 8B), while the variables which had the biggest impact on group separation are located at around 730 and 1455 cm^{-1} (Figure 8C).

As in 3D-PCA the *Campylobacter* isolate from poultry sample was found in close proximity not only to *C. jejuni*, NCTC 11351 but also to *P. mirabilis*, the another chemometric analysis was done. In Figure 9A, one may observe the same scores as in Figure 8B, but with selected violet region for which the separate PCA is shown in Figure 9B. As one may observe in Figure 9B, the violet star is on the same side of the PCA cube as *C. jejuni* NCTC 11351, which indicates that the analysis of PC-1, responsible for 72% of separation, is sufficient to identify unknown strain from poultry sample (see also 2D-PCA, Figure S3).

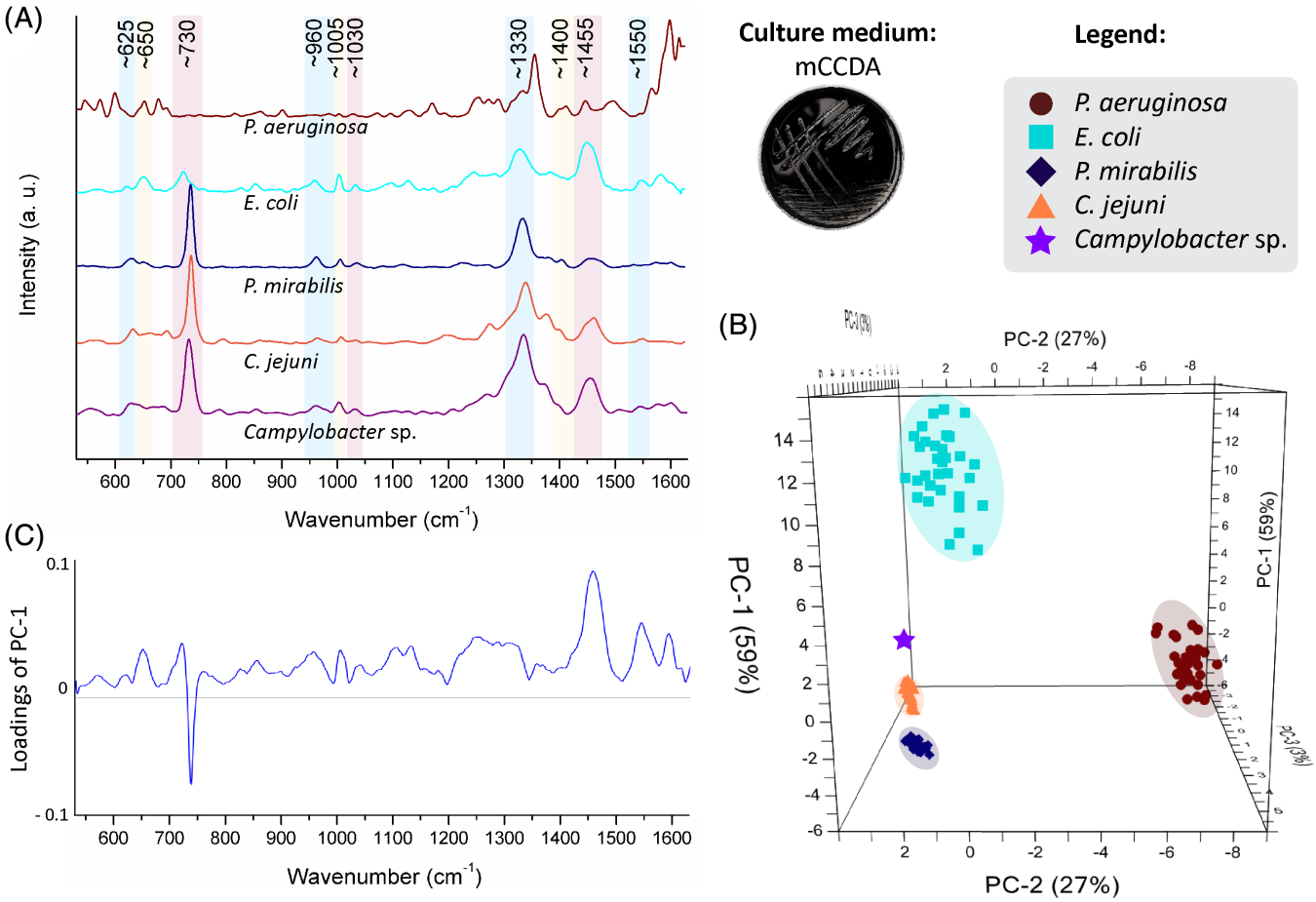


FIGURE 8 Surface-enhanced Raman scattering (SERS) and PCA results obtained for different bacterial species present in poultry samples and detected on modified charcoal-cefoperazone-deoxycholate agar (mCCDA); A, averaged SERS spectra (the SERS spectrum of *C. jejuni* National Collection of Type Cultures (NCTC) 11351 was added as a control and was given for comparison), B, 3D-PCA (the points described as *C. jejuni* NCTC 11351 were added to PCA as a control), and C, loadings plot of the first principal component (PC-1). The *Campylobacter* sp. spectrum from image A and violet star from image B represent the averaged SERS spectrum of *Campylobacter* strain isolated from poultry sample

The reason why the violet star representing *Campylobacter* isolate from poultry sample is not grouped together with *C. jejuni* NCTC 11351 is most probably connected with the fact that both investigated campylobacters represent two different strains. This in turn may lead to some differences in their SERS spectra, which has been already proven in several scientific works showing SERS experiments conducted on bacterial strains belonging to the same species [52–55].

The star representing the spectrum of *Campylobacter* isolate, which was detected in poultry sample, was also found in very close proximity to *C. jejuni* NCTC 11351 in the PCA performed for all four thermotolerant *Campylobacter* control strains cultured on mCCDA (see Figure 9c). In order to confirm the obtained results in accordance to ISO standards (see procedure B, Figure 1), the *Campylobacter* isolate from poultry sample was also cultured on Columbia blood agar. As previously, the strain was located very closely to *C. jejuni* NCTC 11351, which was cultured on Columbia blood agar (see Figure 9D).

4 | CONCLUSIONS

SERS spectroscopy is a powerful and noninvasive technique. Although SERS is rather used in qualitative measurements than quantitative studies, especially in the case of the samples of biological origin, this ultrasensitive and label-free technique ensures reproducible results in relatively short time.

The results obtained in the present study demonstrate that SERS is a powerful technique for the detection and identification of pathogenic bacteria in food samples and can be introduced into ISO standards as an alternative method. This strategy enables avoiding

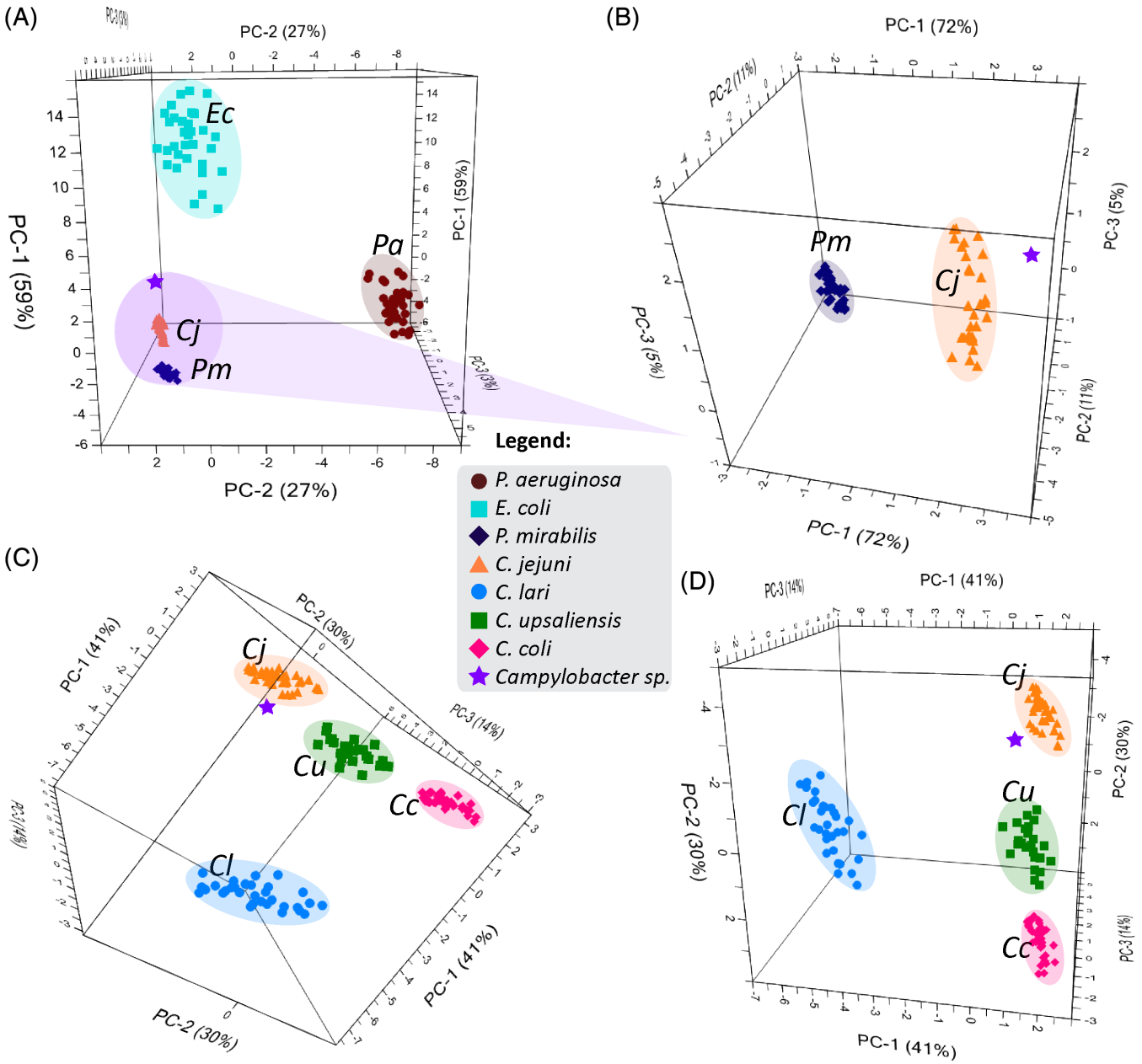


FIGURE 9 The three-dimensional principal component analysis (3D-PCA) results obtained for A, all strains from poultry samples growing on modified charcoal-cefoperazone-deoxycholate agar (mCCDA), B, additional PCA of violet region grouping together *C. jejuni* National Collection of Type Cultures (NCTC) 11351, *Campylobacter* isolate, and *P. mirabilis*, C-D, all thermotolerant *Campylobacter* control strains and *Campylobacter* isolate cultured on C, mCCDA, and D, Columbia blood agar. The violet star represents the averaged Surface-enhanced Raman scattering (SERS) spectrum of *Campylobacter* sp. strain, which was detected in poultry sample

the time-consuming methods routinely used in the laboratory and reducing the time of analysis at least by a half.

The PCA calculations were performed to demonstrate the opportunities offered by SERS strategy and its applicability in detection and identification of food-borne bacteria found in poultry samples, namely *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*. The chemometric analysis allowed for differentiation of all thermotolerant *Campylobacter* species with 85% (mCCDA medium) and 96% (Skirrow agar medium and Columbia blood agar) of accuracy. It also enabled to differentiate *C. jejuni* isolate, found in poultry sample, from other species (*E. coli*, *P. mirabilis* and *P. aeruginosa*) detected on mCCDA medium with the accuracy of 89%. The research presented here should open a new path in microbiological diagnostics. However, further research and validation with other types of samples and bacteria species are

needed before SERS can become a robust tool for pathogen identification and quantification.

Besides many advantages of the SERS technique, there are also some shortcomings of the proposed approach. The technique requires further automation so that it could be applied in microbiological laboratories and hospitals. Additionally, the inclusion of the methods of bacteria multiplication in the microfluidic system would additionally improve the SERS-based technique. The aforementioned issues are the topic of our future investigations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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