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DOI: 10.37722/ANAFS.2023105

Research Article | Advances in Nutrition and Food Science | ANAFS-252
ISSN 2641-6816

The potential of the Confocal Raman Microscopy for characterization of biofilms monobacterial and mixed

Samia Hamma.Faradji[1*], Amel Ait Meddour[1], Farida Bendali[1], Hamideh Salahi[2], Fréderic Cuisinier[2] and Djamila[1] Sadoun

[1] *Laboratory of Applied Microbiology, Faculty of Natural and Life Sciences, University of Bejaia, 06000, Algeria.*
[2]*Bio-health laboratory and Nanoscience, UFR of Odontology, University of Montpellier 1, France.*

Received Date: 02 November, 2023; **Accepted Date:** 27 November, 2023; **Published Date:** 09 December, 2023;

[*]**Corresponding author:** Samia Hamma.Faradji, Applied Microbiology Laboratory, Nature and Life Sciences Faculty, University of Bejaia, 06000 Bejaia, Algeria.

Abstract

Raman spectroscopy is emerging as an important nondestructive, noninvasive analytical technique for the analysis of biologic materials. This study presents biofilm monobacterial of foodborne pathogens Gram negative *E. coli*, *P. aeruginosa*, Gram positive LAB *En. faecium*, *Lb. paracasei* and mixed of *En. faecium* + *E. coli* consists of a structured community of bacterial cells, enclosed in a self-produced polymer matrix. This multilayered biofilm is often referred to EPS. The chemical analysis of those biofilms and according to the Raman bands of reference samples, water was an important component in the biofilms matrix. Additionally EPS reveals a range of macromolecules, including carbohydrates (polysaccharide) which are one major component of EPS matrix; proteins exhibit the typical broad bands (amide I, amide III, ring vibrations from aromatic residues of tyrosine, tryptophan and phenylalanine). Presence of lipids can be found. From this it clear that EPS play a major role in the formation and maintenance of the biofilm structure.

Keywords: Raman spectroscopy; confocal microscopy; biofilms matrix; lactic acid bacteria and target strains.

Introduction

Biofilms represent a ubiquitous form of microbial life in the natural environment. They can occur at solid–liquid, solid–air, liquid–liquid, and liquid–air interfaces **(Costerton *et al.*, 1995)**. Biofilms are communities of microorganisms, which are embedded in a matrix formed by extracellular polymeric substances (EPS). Depending on the biofilm type and microorganisms involved, up to 90% of the particulate fraction of the biofilm can be EPS **(Staudt *et al.*, 2004;** Karayiannis **et *al.*, 2020)**. EPS are biopolymers of microbial origin such as polysaccharides, proteins, glycoproteins, nucleic acids, lipids, and phospholipids. EPS play a major role in the formation and maintenance of the biofilm structure **(Neu *et al.*, 2001, Shivani et *al.*, 2021)**.

Confocal Raman Microscopy (CRM) is an analytical technique combining confocal microscopy with an analysis of Raman spectral bands capable to rapidly to identify/ discriminate different biological systems (bacteria, yeast, pollen), since all biologically associated molecules such as proteins, nucleic acids, carbohydrates, and lipids exhibit distinct spectral features, for the localization of solutes within the biofilms **(Marcotte *et al.*, 2004)** and for the characterization of the chemical composition of biofilms **(Sandt *et al.*, 2007)**. The aim of the study reported in this paper was to further explore the applicability of CRM for nondestructive chemical characterization of biofilm including microbial constituents and EPS. We analyzed spectra from monospecies and multispecies biofilms matrix including microbial constituents. Thus, we demonstrate that CRM can provide detailed information about chemical composition of complete biofilm.

Materiel and methods

Bacterial strains and growth conditions

Tow LAB strains were used in this study; *L. paracasei* isolated from a milk tank and *En. faecium* isolated from a milking machine. These strains were identified phenotypically and genotypically by sequencing the gene coding the 16S rRNA. LAB were grown in de Man-Rogosa and Sharpe (MRS, Merck, Germany) medium and incubated at 30°C for 24 to 48 h. All the strains were stocked at -20°C in the same medium with the addition of 30% (vol/vol) glycerol (Merck)**.** *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (Pasteur Institute of Algeria) were included as target strains. The later were maintained as stocks at 4°C in nutrient agar (NA, Fluka, Spain) before use.

Biofilm culture

All the strains were transferred to fresh TSB-YE (TSB supplemented with 0.6% Yeast Extract, Difco, France) periodically to maintain viability and, prior to use, they were activated by two successive 24 h transfers in the same medium at 30 and 37 °C for LAB and target strains respectively . Bacterial cell were harvested by centrifugation at 4°C (8000*g*x 20 mn) and washed twice and resuspended in the TSB-YE to give 10^8CFU ml^{-1} for LAB strains and 10^6CFUml^{-1} for target strains. 15 milliliters of the bacterial suspension was poured into a Petri dish containing fluorine lame ($CaCF_2$) and stored at 30°C for 7 days. The medium was replaced with fresh broth each 24 h. The no adherent bacteria were rinsed by 20 ml of PBS (Sigma, France).

Raman Data Acquisition

The Raman spectra were collected using a Witec Confocal Raman Microscope System alpha 300R (Witec Inc., Ulm, Germany). The excitation for the confocal Raman microscope was provided by a frequency doubled Nd: YAG laser (Newport, Evry, France) at a wavelength of 532 nm, with 50 mW laser output power in a single longitudinal mode. The light was carried to the microscope by a multimode fiber (diameter of 125 µm). The incident laser beam was focused onto the sample through a 60× water immersion objective with a numerical aperture of 1.0 and a working distance of 2.8 mm (Nikon, Tokyo, Japan). The Raman backscattered radiation mixed with the Rayleigh scattered light were then passed through an edge filter to block the Rayleigh light. With the assumption that most of the Rayleigh light was filtered, the entire light was passed through to a single mode fiber (25 µm) directed to the Electron Multiplying Charge Coupled Device (EMCCD) camera (DU 970N-BV353, Andor, Hartford, USA). The EMCCD chip size was 1600×200 pixels, the camera controller was a 16 bit A/D converter operating at 2.5 MHz, and the camera was cooled by a Pelletier system. The acquisition time of a single spectrum was set to 0.5 s. For experiments, 150 x 150 points per image were recorded using a piezoelectric table, leading to a total of 22.500 spectra for one image, with each spectrum corresponding to a spatial unit defined as a voxel. The data acquisition and processing were performed using the Image Plus software from Witec. The data acquisition time for an image was approximately 1.5 hour. The laser power on the sample was estimated to be less than 15mW.

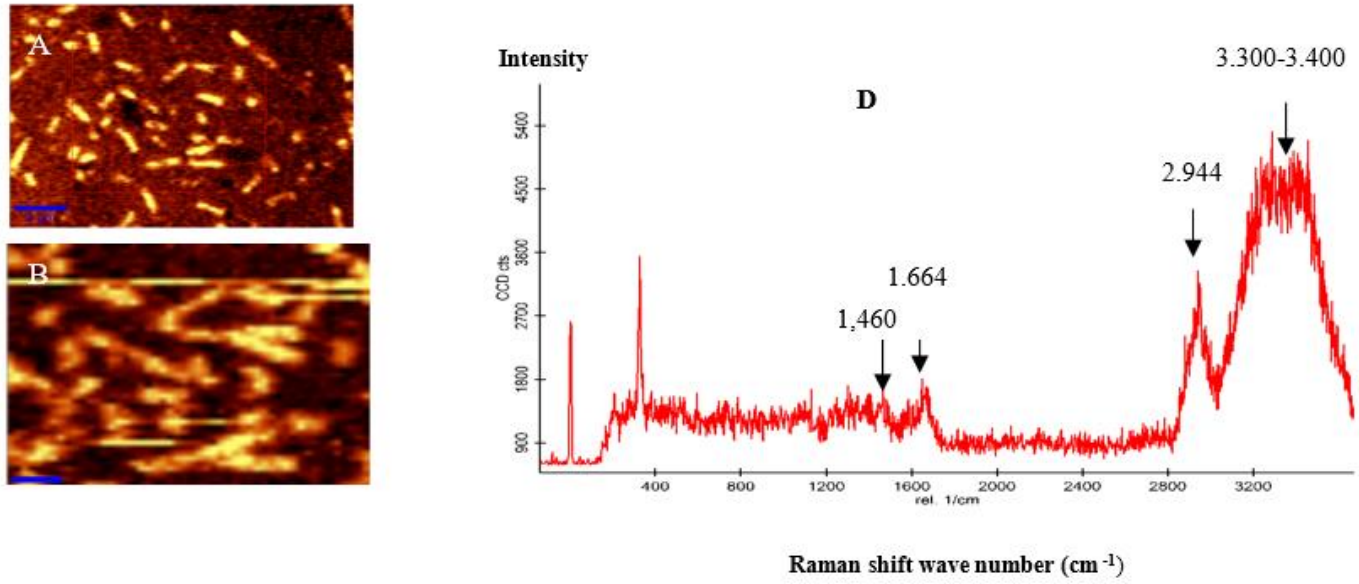
Results

Raman microscopic analysis of biofilms

The chemical information about biofilms monobacterial and mixed is desired to improve the understanding of the composition and structure of this. EPS are a complex mixture of different polymers with a large number of potential binding sites, the staining of the total EPS is complicated.

E. coli

Observations of *E. coli* biofilm on fluorine lame by CRM is shown in Fig.1. The different images (A, B and C) obtained allowed us to observe the adhesion potential of this trains and the contamination levels of the hydrophilic supports. Adhesion of *E. coli* can be noted with the presence of aggregates. The Raman spectrum of an aggregate of cells (Fig.2.D) are characterized by intensies bands at 3.300-3.400 cm^{-1} witch characterized the O-H vibration of the water, the bands at ≈2.940 indicated the C-H vibration, this signal represents the total amount of organic compounds in the cells. Not only is the C-H vibration characteristic of one special component. We observed weak bands at 1.664 cm^{-1} which can be assigned to amide I (presence of protein) **(Schuster *et al.*, 2000** and **Harz *et al.*, 2005)**, the position at 1.460 cm^{-1} can be assigned to δCH_2 deformation that originate in carbohydrate, lipids or protein of the biofilms matrix **(Neugebauer *et al.*, 2007, Darakshan *et al.*,2018)**.



The results of the cluster analysis (Fig.1.E) showed four spectra acquired with a significant differences in the regions between (1.000 - 1.500 cm^{-1}). The Fig.1.F. Illustrated that the red spectrum with strong bands at ≈789 cm^{-1} characterized the C, U from DNA/RNA (**Notingher et *al.*, 2003**). The pink one with characterized band at ≈459 cm^{-1} indicated carbohydrates (slime) (**De Gussem *et al.*, 2008**); the green and the yellow one concerned the PBS and fluorescence spectrum (Fig.1.F).

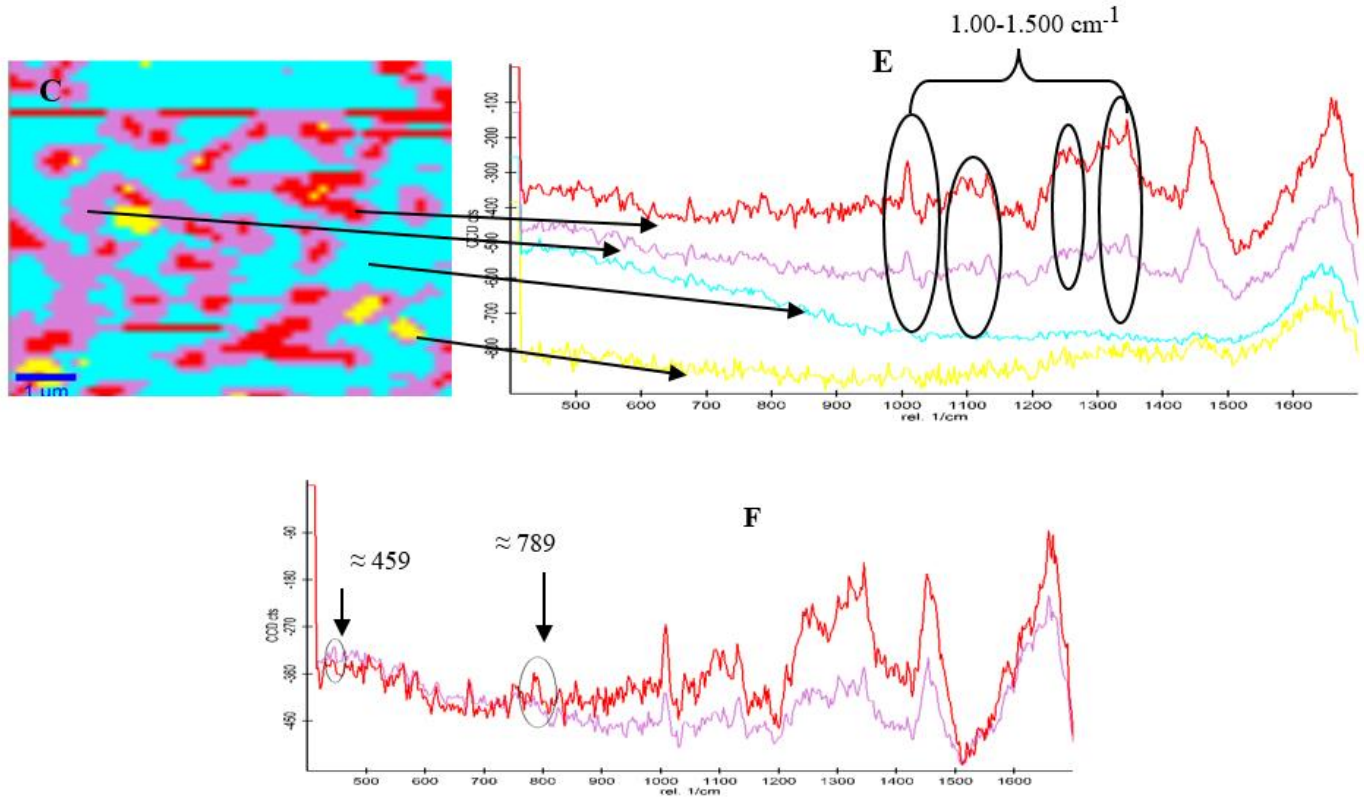
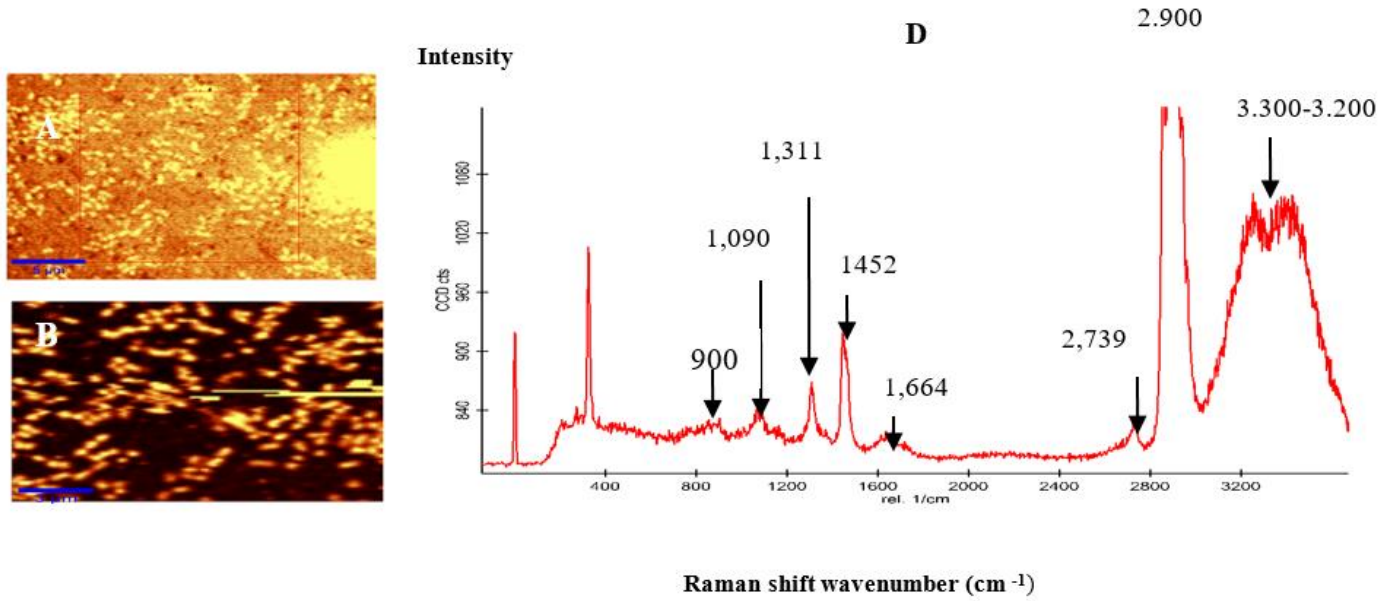


Fig.1. (A): optical image; (B): Raman image (C): cluster analysis image (D): Raman spectrum; (E and F): cluster analysis spectrum of *E. coli* biofilm.

P. aeruginosa

The figure illustrates the Raman spectra of *P. aeruginosa* in biofilm (Fig.2.D). These spectra show bands in corresponding to C-C skeletal and C-O-C stretch from glycosidic link (carbohydrates), PO_2 from DNA/RNA and lipids at ≈1,090 cm^{-1} (**Maquelin *et al.*, 2002**), at ≈ 900 cm^{-1} we can attributed the vibration to C–C and C–O–C 1,4-glycosidic link from carbohydrates. Additionally, O-H vibration of the water at ≈ 3.300-3.200 cm^{1}. The bands at 2.900 cm^{-1}, 2.739 cm^{-1} arising mainly from proteins and lipids (C-H), band at 1.452 cm^{-1} can be assigned to CH_2 deformation. The bands at 1.664 cm^{-1} corresponded for amid I vibration (presence of the protein) (**Schuster *et al.*, 2000**). According to **Sandt *et al.* (2009)** the moderate bands intensities at 1.311 cm^{-1} were attributed to pigments secreted from the cells and associated with the extracellular polymeric substance, these peaks, which occurred at 1.130 1.175, 1.233, 1.311 and 1.585 cm^{-1}.



The results of the cluster analysis showed four spectra acquired and significant differences in the regions between (1.000 cm^{-1}) and (1.500 cm^{-1}) (Fig.2.E). According to **Sandt *et al.* (2009)**, these frequency ranges were characteristic for polysaccharides and associated predominantly with glycosidic ring vibrations (green and pink spectrum). So they indicate a substantial production of EPS matrix in the form of polysaccharides by the *P. aeruginosa* which were attributed to pigments secreted from the cells and associated with the extracellular polymeric. The red and the bleu ones present the bacteria and PBS respectively.

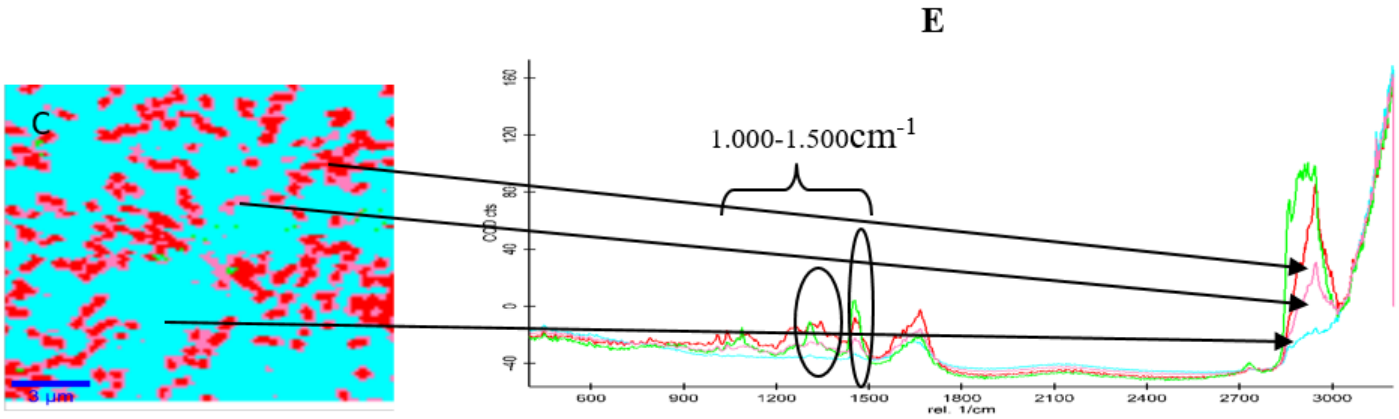


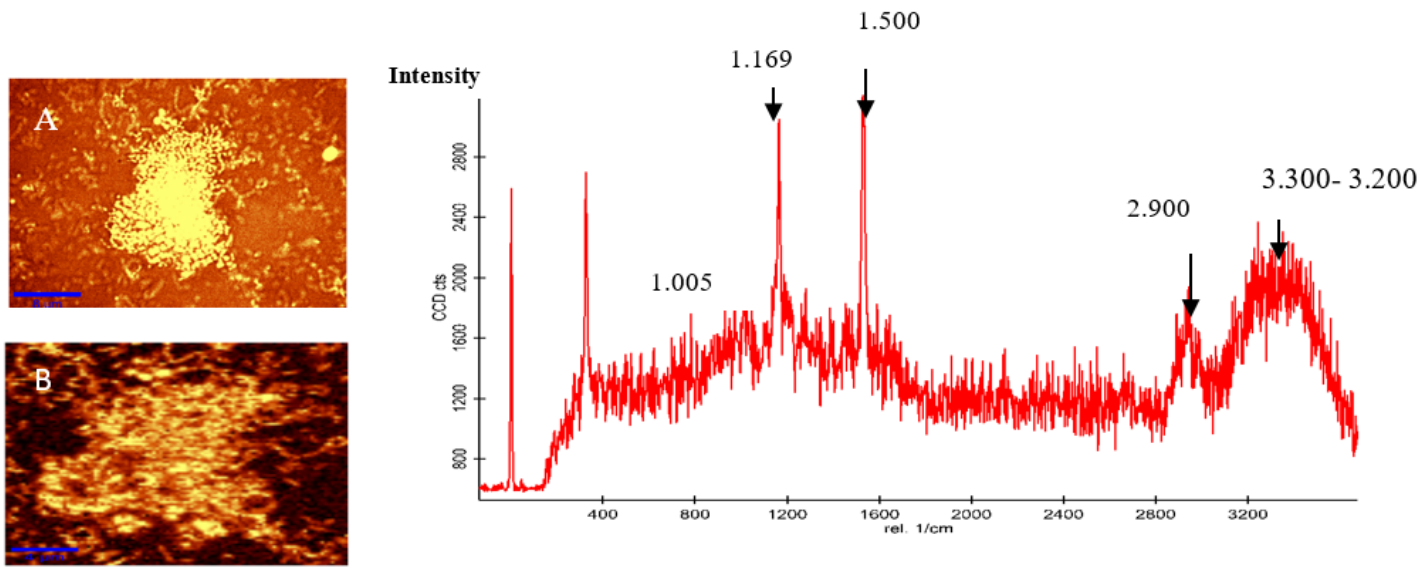
Fig.2. (A): optical image; (B): Raman image (C): cluster analysis image; (D): Raman spectrum; (E): cluster analysis spectrum of *P. aeruginosa* biofilm.

The Raman analysis of the tow biofilms formed by the Gram negative bacteria, *E. coli* and *P. aeruginosa* shows only little similarity. Differences were observed in the region at ≈1.460-1.452cm^{-1} *P.aeruginosa* exhibit a moderate intensity while lowest for *E. coli*. This finding can be attributed to additional peaks that occur at≈1.090 cm^{-1}, ≈ 905 cm^{-1}and 1.311 cm^{-1} specific compound synthetized observed in the Raman spectrum of *P. aeruginosa*.

En. faecium

The Raman spectrum of *En. faecium* (Fig.3.D), reveals O-H bands of the water at ≈3.300- 3.200 cm^{1}, C-H bands around 2. 900 cm^{-1} witch is characteristic of biomass, at ≈1,500cm^{-1} and 1.169 cm^{-1} we noted the higher intensities of this band indicated the =C-C= constituents which can be assigned to a carotenoid structure in biofilms matrix (**Rösch *et al.*, 2005**). According to **Wagner, (1986)** bands near 1.160 cm^{-1} (C-C) and 1.500 cm^{-1}(C=C) were specific to *Enterococci* genus. At ≈ 1,005 cm^{-1}the phenylalanine confirming the presence of the protein.

D



Raman shift wavenumber (cm $^{-1}$)

The results of the cluster analysis showed four spectra with significant differences in the regions between (1.000cm^{-1}) and (1.500 cm^{-1}). According to **Sandt *et al.* (2009)**, these frequency ranges are characteristic for EPS matrix (green and pink spectrum), the red and the bleu ones present the bacteria (in comparison with single bacteria spectrum, data no shown) and PBS respectively. The comparison between the two spectrums of EPS matrix (Fig.3.F) (green and pink spectrum) respectively relived that pink one with intensities bands at ≈1,500- 1.160 cm-^{1} characterized the carotenoid (**Maquelin *et al.*, 2002**).

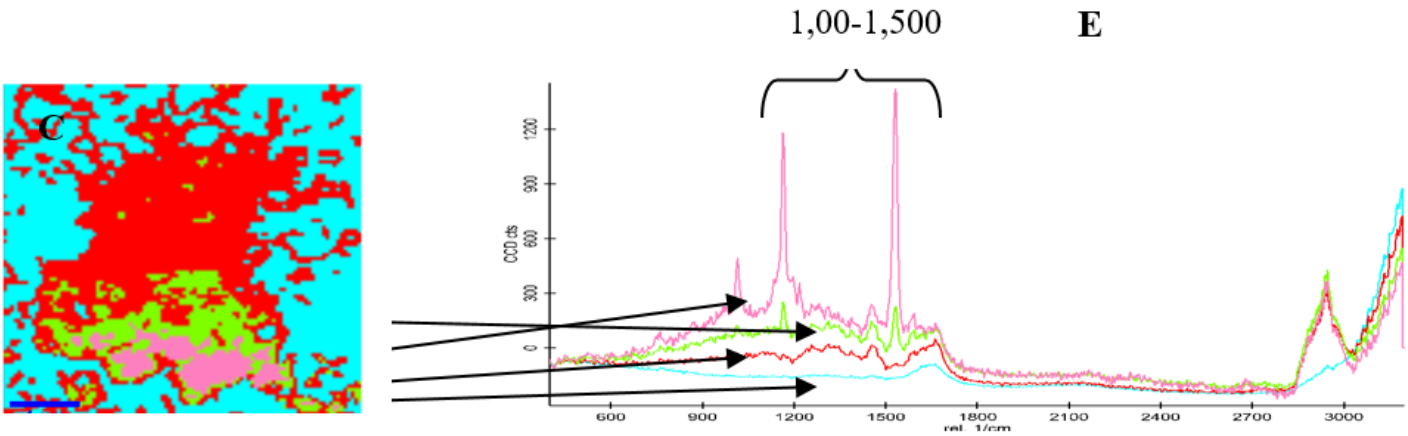
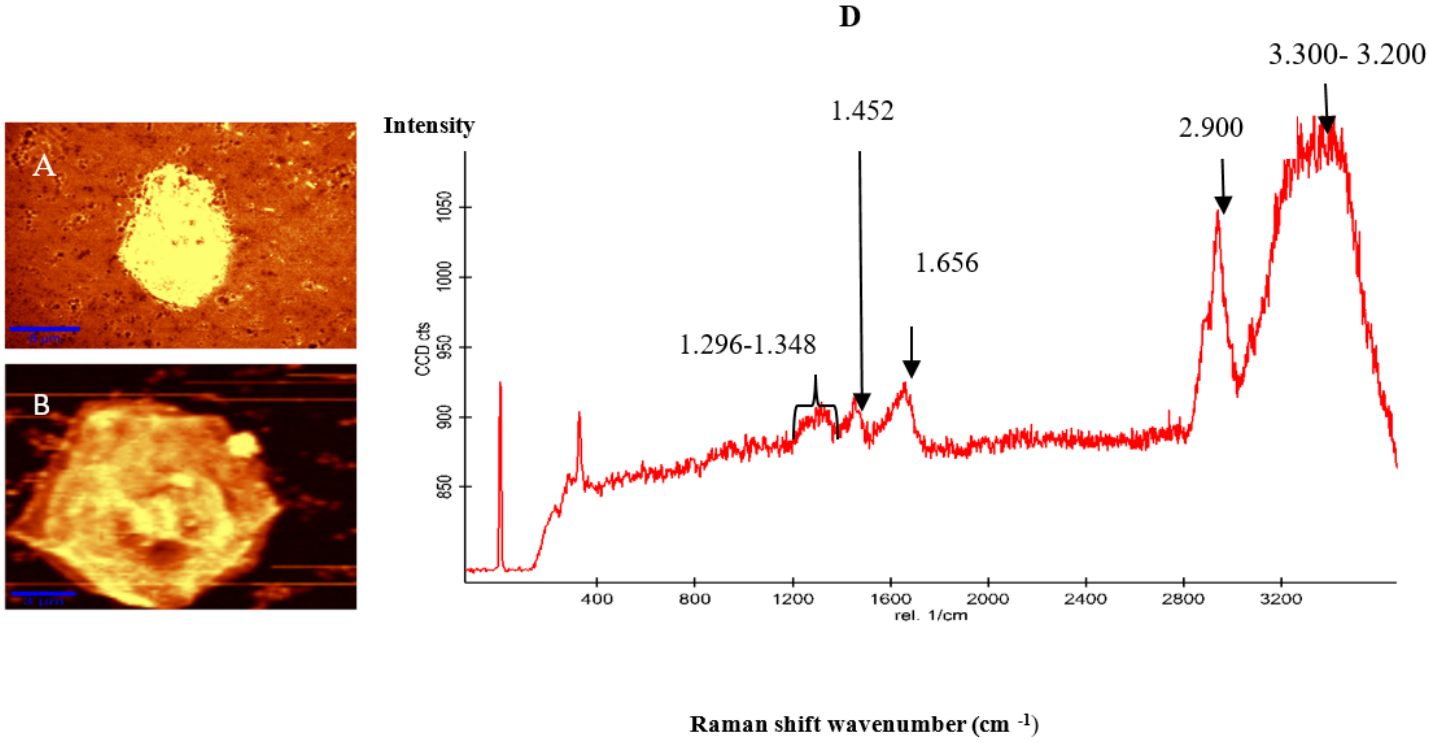


Fig.3. (A): optical image; (B): Raman image (C): cluster analysis image; (D): Raman spectrum; (E): cluster analysis spectrum of *En. feacium* biofilm.

L. paracasei

L. paracasei exhibit spectra with characteristic bands near 1.656 cm−1 (C=O, C=C) from carbohydrate (Himmelsbach and Akin, 1998), amid III near 1.296-1.348 cm−1 (C–N stretch, 40%; N–H deformation, 30%; and C–H deformation). Ring vibrations from aromatic residues of tyrosine, and phenylalanine in the region between 1.606 cm−1 and 1.302 cm−1. The bands at 1.452cm−1 found the δCH2 deformation in carbohydrate, lipids and proteins (Neugebauer et *al.*, 2007). ≈937 cm−1 C–C, C–O–C 1.4-glycosidic link indicted carbohydrate (Harz et *al.*, 2005), C-H band characteristic of biomass at 2,900cm−1 and vibration of water at 3.300- 3.200 cm^{-1} (Fig.4D).



The comparison between the two spectrums of the cluster analysis (Fig.4.E) (green and pink spectrum) we noted that no differences between them, they indicated the bacteria in biofilm in comparison with the spectrum of *L. paracasei* (single bacteria, data no shown)

but those present differences with the green spectrum in the region at 1.200 -1.500 cm^{-1} witch can be assigned to δCH_2 deformation in carbohydrate, lipids or protein of the biofilms matrix

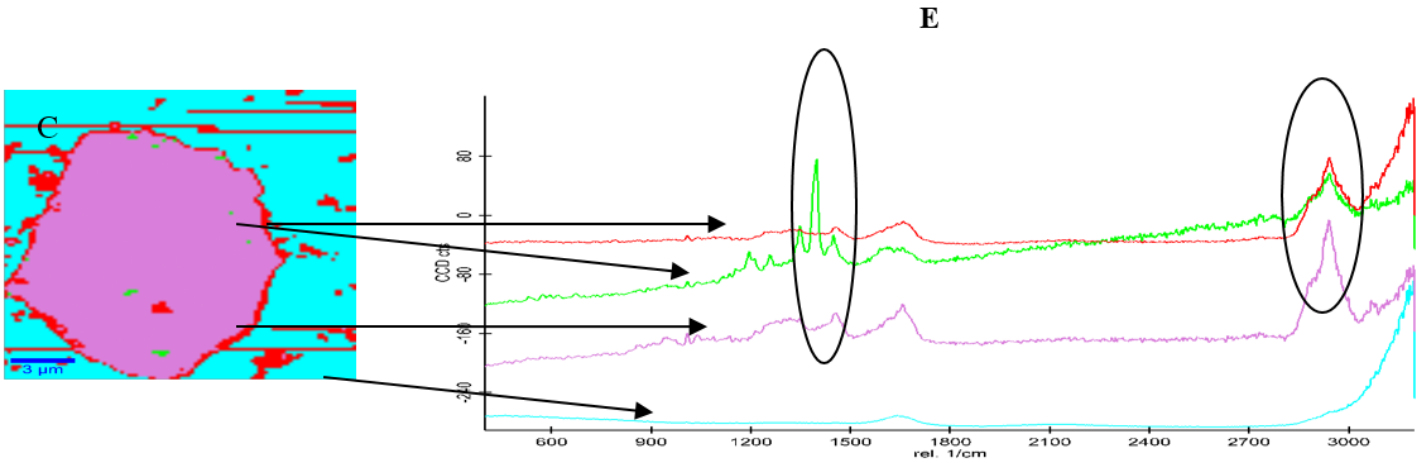
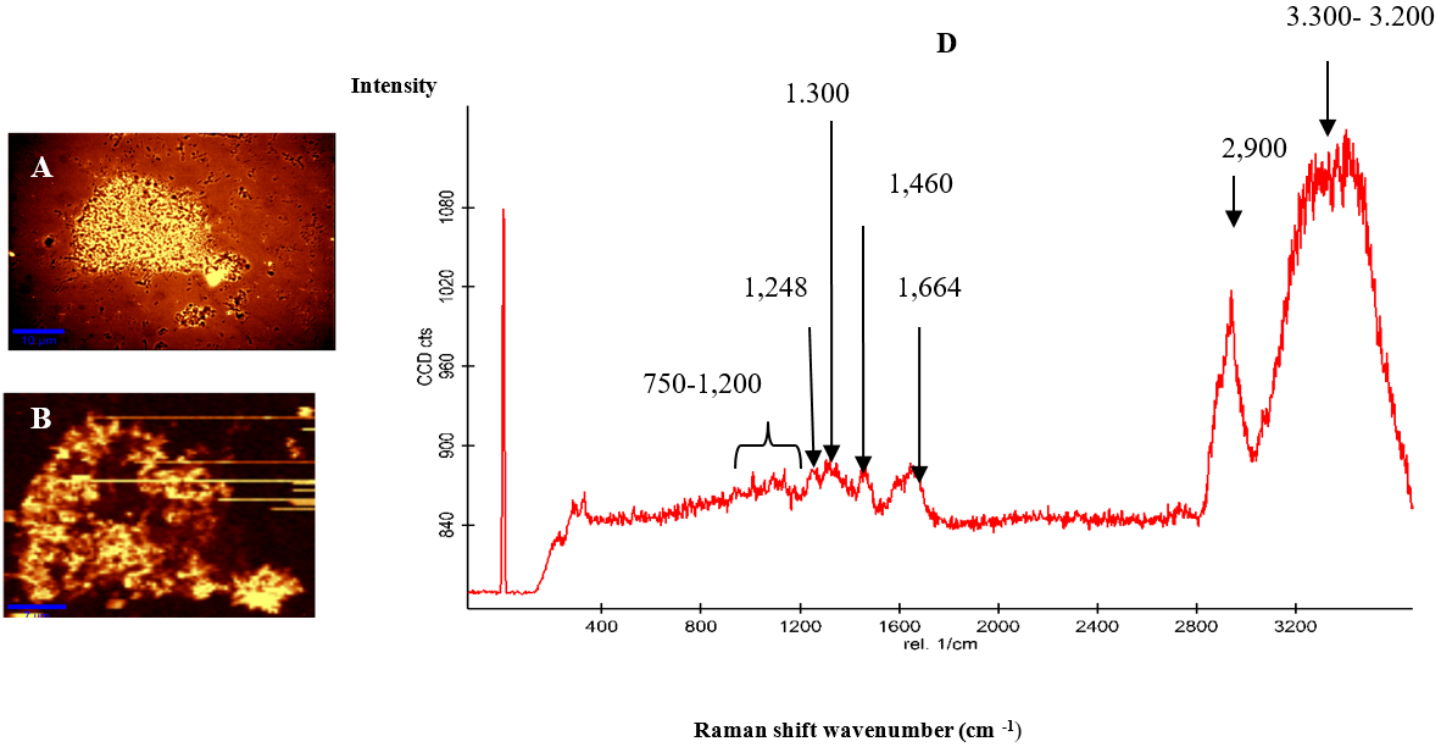


Fig.4. (A): optical image; (B): Raman image (C): cluster analysis image (D): Raman spectrum; (E): cluster analysis spectrum of *Lb. paracasei* biofilm.

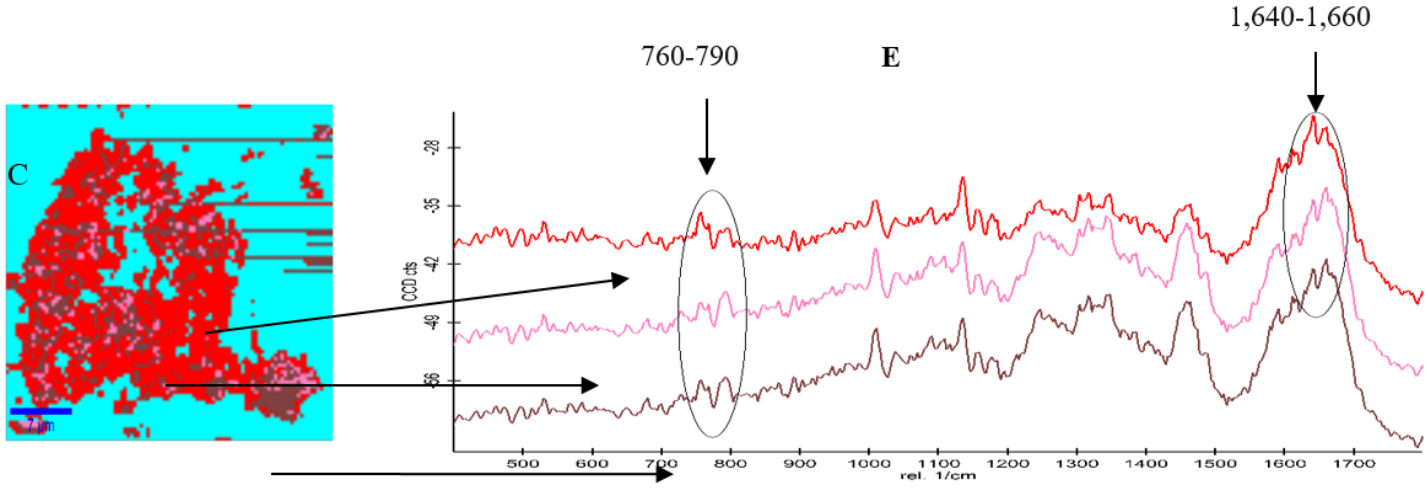
The Raman spectra of LAB strains *En. faecium* and *L. paracasei* show little similarities, their spectra differ greatly in peak in the region between 1.000-1.500 cm^{-1} with peak characteristic at 1.656 cm^{-1}, 1.296-1.348 cm^{-1}**,** 1.606 cm^{-1} and 1.302 cm^{-1}, 1.452 cm^{-1} and ≈937 $cm^{-1}$for *L. paracasei* and 1. 160cm^{-1}, 1.500cm^{-1} for *En. faecium*.

Mixed biofilm from *En. faecium* and *E. coli*

After analysis of pure biofilms of each species, we analyzed the biofilms mixed (Fig.5). The most characteristic band in spectra is due to the presence of carbohydrate, lipids and protein. The bans of amid I and amid III at 1. 664 cm^{-1}, 1.315 cm^{-1} – 1.248 cm^{-1} respectively and phenylalanine at ≈1.008 cm^{-1}. At 1.460 cm^{-1} present the assigned to δCH_2 deformation that originate in carbohydrate, lipids or protein **(Neugebauer *et al.*, 2007)**, 1.300 cm^{-1} CH_2 in lipids, ≈1.142 cm^{-1} =C-C= acid in lipids, 2.900 cm^{-1} C-H and ≈760 indicated the tryptophan .



The comparison between the two spectrums of the cluster analysis (Fig.6.E) (pink and brown spectrum), we noted that no differences between them, but this present differences with the red one at the regions: 1.640-1.660cm^{-1} (amid I) and ≈760-790cm^{-1} (tryptophan in proteins and C, U in DNA/RNA respectively.



Discussion

As a reaction to environmental stress, bacteria can produce EPS, these compounds may remain either unattached (as slime) or covalently linked (often by a phosphodiester or lipid link) to macromolecules on the cell surface **(Whitfield and Roberts, 1999; Nguyen et *al.*, 2020)**. In the latter case, the material can be attached firmly to the surface. This can make the separation of the EPS from bacteria very difficult **(Ivleva *et al.*, 2009)**. As illustrated in Fig.1 adhesion of *E. coli* was noted with the presence of aggregates. This structure does not be observed in monospecies biofilms of LAB strains and mixed (*En. faecium* and *E. coli*). The principal characteristic of this biofilms was the presence of the bans in the region at ≈450 cm^{-1}. According to **Ivleva *et al.* (2009)** the spectra of aggregates are also characterized by bands at ≈450 cm^{-1}. The bands resemble the spectrum of agarose with bands at 456, 840, and 890 cm **(Schuster *et al.*, 2000)**. The Raman spectrum of *P. aeruginosa* biofilm is characterized by the specific band at ≈1,311 cm^{-1} attributed to pigments secreted from the cells. Carotenoid compounds in region at 1500-1160 cm^{-1} is present only in the spectrum of *En. faecium* biofilm and not in

the other. Raman spectrum of *L. paracasei* biofilm identified several region characteristic for proteins, polysaccharide and lipids and little similarities were observed for this tow LAB strains. Concerning biofilms mixed, the CRM was limited to resolving distributions of cells and were incapable of distinguishing between the two species, (differentiations of the structure and the chemical composition of the bacteria in biofilms) . The biofilms antiquated the presence of water witch constitute a major component, lipids, proteins accompanied with the polysaccharides, nucleic acids as well as weak bands of polysaccharides in the glucosidic ring region.. The polysaccharides synthesized by microbial cells canvary greatly in their composition and hence in their chemical and physical properties. Which polysaccharides are produced depends on both intrinsic and extrinsic factors. Intrinsic factors arise in accordance with the genetic profile of the microbial cell, whereas extrinsic influences include the physicochemical environment in which the microorganisms are located **(Sutherland, 2001)**. In addition to organic components of the biofilm community, inorganic substances could also be found.

Conclusion

In the presented work, we have shown the potential of vibrational Raman spectroscopy in the differentiation of the LAB and target biofilms from different sources and strain collections. The results reflect the high discriminatory power of the Raman technique that allows accurate differentiation of closely related bacterial biofilms. The most prominent features observed in the Raman spectra of each biofilms can be broadly into four groups – proteins, DNA/RNA, carbohydrates, and lipids from this it is clear that this compounds constitute the biofilms composition.

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Citation: *Hamma Faradji S., AitMeddour A, Bendali F, Salahi H, Cuisinier F, Sadoun D (2023) The potential of the Confocal Raman Microscopy for characterization of biofilms monobacterial and mixed. Adv in Nutri and Food Sci: ANAFS-252.*