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Raman spectroscopy applied to early detection of Clostridium infection in milk

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ABSTRACT

Detecting *Clostridium* in milk presents a significant challenge for the dairy industry, given that traditional methods are time-consuming and not specific for these bacteria. Microbiological techniques are expensive and require qualified personnel. *Clostridium*, in the form of spores, can withstand pasteurization and revert to its vegetative form during cheese aging. Those gas-producing bacteria are known for their production of carbon dioxide and hydrogen, causing the formation of slits, cracks, and irregular eyes in hard and semi-hard cheeses. However, gas analysis in the vial headspace of appropriate culture can be exploited to specifically detect *Clostridium* presence, since the closest competing bacterial *Bacilli* produces only carbon dioxide. The aim of this paper is to present a Raman-spectroscopy based instrument for a rapid, inexpensive identification of *Clostridium* in milk with a limit of detection (LOD) of 29 spores/l. The proposed measurement procedure is analogue to that routinely used, based on Most Probable Number (MPN). The Raman-based instrument speeds up the detection of a vial's positivity. A test conducted with *Clostridium* spores demonstrated its effectiveness in almost halving the time needed for the measurement campaign compared to the traditional method.

INTRODUCTION

The early identification of *Clostridium* in milk still remains a problem of fundamental importance in the dairy industry. Their endospores are heat-resistant[2] and therefore they can survive the pasteurization process and become active again when exposed to more favorable conditions such as during cheese ripening.

Clostridium can transform lactic acid into butyric acid and gas (hydrogen and carbon dioxide), through butyric acid fermentation[3]. Four species *C. butyricum, C. sporogenes, C. beijerinckii* and *C. tyrobutyricum* have been frequently isolated from milk and cheeses, whereas the last one species is considered the primary cause of the late-blowing defects[4,5]. Since the produced gas solubility in cheese matrix is very low, (especially for hydrogen), the large amount of gas leads to a creation of irregular eye formation, cracks and slits. This problem is particularly relevant for hard or semi-hard cheeses such as Grana Padano[6]. In addition to the altered appearance, the flavor is also compromised and becomes rancid due to the butyric acid presence.

Rapid identification of *Clostridium* in milk is necessary to avoid an economic loss that is twofold, both because aged cheese suffering from late blowing is not of interest to the market, and because clostridium-infected milk can be consumed in liquid form or used for unaged cheese [3,7].
Several methods have been proposed to prevent Clostridium spoilage. The most common techniques appear to be microfiltration of milk and bactofugation, but

this approaches also alter the flavor and remove some of the protein content respectively[8].

The utilization of additives[9], such as nitrate, is not permitted since it was banned by decision of the European Food Safety Authority (EFSA, 2010).

Although there is currently no international standard method for the enumeration of spores in milk, the most commonly used ones rely on microbiological methods based on MPN[1,10]. Indirect measures, such as the detection of gas production through the raising of a paraffin plug has been used since 1921[11]. Since that time, efforts have been pursued to find *Clostridium* exclusive culture broths.

The main problem in the MPN approach is represented by the lack of selectivity as it suffer cross-talk interference between bacterial species. In particular, the closest competitive bacterium, *Bacilli,* is able to produce enough carbon dioxide to raise the paraffin plugs.

Furthermore, MPN methods take several days and only return a quantity estimation of spores within a large confidence interval. In the end, the resulting value should be considered as indicative[3].

Other investigation approaches are molecular methods; they satisfy the demand for fast and selective analyses. Common molecular methods involve DNA extraction from spores and subsequent amplification by Polymerase Chain Reaction (PCR). This methodology can be applied for possibly real time analyzes[12]. However, extracting DNA from a complex matrix is a difficult process and requires several steps[13]. Finally, its subsequent classical analysis does not reflect biological activity, as it cannot discriminate between live and dead cells[1]. A significant concern is the frequent occurrence of false negative results. This happens because certain food substances and chemicals used in the extraction process can interfere with the PCR reaction[14]. In addition, the main drawback of this approach is related with costs: primarily due to the requirement for specialized equipment and trained personnel. For this reason, molecular methods are currently too expensive for everyday use in the dairy industry.

A further analysis methodology is immunological, adopted for its specificity in *Clostridium* detection. It relies on the use of antibodies capable of specifically binding to their molecular target. The most widely used is enzyme-linked immunosorbent assay (ELISA), capable of providing quantitative and qualitative detection of a specific target[3]. The main issues with these techniques are the generation of a suitable antigen or antibody, which is costly, and the low sensitivity. The typical LOD for immunological methods is attested to be about thousands of cells/ml when a concentration of tens of spores per liter can cause late blowing effects[15].

For these reasons, a *Clostridium* specific instrument for a sensitive and fast diagnostic has been developed. It is based on spontaneous Raman spectroscopy, which is a multi-gas non-invasive spectroscopic technique. Since the hydrogen production is correlated only with the *Clostridium* metabolism; the headspace gas analysis in culture vials can be exploited for its unique identification.

So, the proposed instrument is able to distinguish between *Clostridium* and other non-hydrogen productive bacteria, such as *Bacilli.*

To meet the demands of the dairy industry, the instrument has been designed in order to provide fast and reliable analysis, by implementing automated tests without requiring specialized personnel. The instrument's components, mechanical and optical, have been chosen to reduce the overall economic impact when compared to other techniques.

MATERIAL AND METHODS

Clostridium preparation

The spores of a strain of *Clostridium butyricum* and a strain of *Clostridium tyrobutyricum* were prepared as follows: a colony was inoculated in Reinforced Clostridium Broth (RCM, Biokar Diagnostics, France) modified by the addition of 1.8% Na-lactate, pH 6.1 at 37°C for 48h, then 2 ml were transferred into 200 ml of RCM-lactate broth under anaerobic conditions at 37°C for 7 days. At the end of the incubation

the spores were collected and purified by centrifugation for 10 min at 16000g and resuspension in 50 ml of physiological solution.

A milk sample was contaminated with a mixture of the spores of the two Clostridium strains. The quantity of spores inoculated was determined in order to have as an average 20 positive vials out of 24 on a total milk quantity of 72 ml, calculated with the MPN method in an estimated spore value of 600 spores/L [16].

Samples preparation

For the Raman-based method: RCM-lactate broth was used to inoculate spore contaminated milk. 3 ml of milk was added to 25 ml of RCM broth in 24 test vials, the resulting liquid was covered with 2 ml of vaseline oil and heat treated at 85°C for 15 min in order to eliminate vegetative cells. Vials were incubated at 37°C.

For the reference method: RCM-lactate broth was used to inoculate spore contaminated milk. 1 ml of milk was added to 9 ml of RCM broth in 24 test vials, overlayed with paraffin (approximately 1.5 cm in height in each tube) and heat treated at 85°C for 15 min in order to eliminate vegetative cells. Vials were incubated at 37°C.

EXPERIMENTAL

Raman gas analyzer *setup*

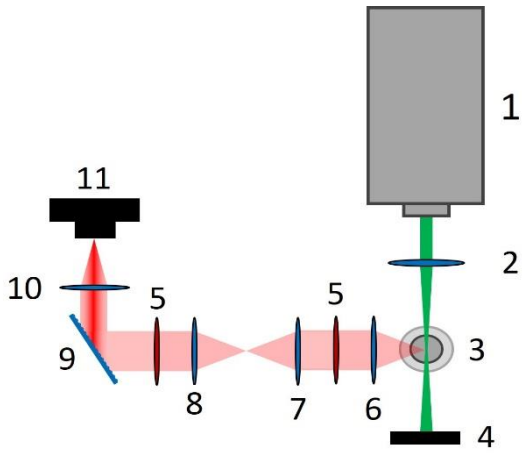

Figure 1: Instrument layout

Figure 1 shows a schematic of the proposed instrument.

For the Raman scattering excitation, a Nd:YAG solid-state pumped laser with a wavelength of 532 nm and a power of 1 W was used (1), its diameter at the aperture ($1/e^2$) is 0.70±0.05 mm. The laser beam is focused by a f = 50 mm lens (2) in the central region of the vial (3) to be tested, where the excitation spot size is ≈120 µm. The beam then is stopped by a light trap (4). The scattered radiation is collected by a custom-made spectrometer positioned orthogonally to the laser propagation.

The Raman source is focused on the entrance plane of the spectrometer by a combination of two Hastings Achromatic Triplets (6-7, Thorlabs, USA), on the vial side with focal length f = 40 mm and on the spectrometer side with focal length f = 20 mm. Their overall effect is to halve the source image into the spectrometer entrance slit with a magnification factor of 0.5, giving a final image size of ≈60 µm. Between the two triplets, where the collected light is collimated, a 550 nm long-pass interferometric filter is placed (5, Thorlabs, USA) to cut-off the Rayleigh component of the scattered light

The compact f/2.8 spectrometer consists of an objective lens (8, Edmund, USA), an additional long-pass filter (5), a diffraction grating (9, Edmund, USA), a focusing lens (10, Edmund, USA) and an CMOS detector (11, Basler). Given the small size of the image, the spectrometer is operated without an entrance slit. However, a 4-mm-wide entrance aperture is placed on the input plane to reduce the diffused light entering the spectrometer.

The use of two long pass filters is necessary because of the high amount of Rayleigh scattered light due to the non-ideal optical vial structure. The two filters are not positioned in cascade configuration to prevent any residue of green stray light entering the spectrometer through the entrance aperture from reaching the detector.

With only one filter the hydrogen vibrational spectral line at (4156 cm^{-1}) is not visible but

hidden by the Rayleigh diffused light. The use of a third filter has been tested but no improvements have been recorded.
The detector employed in the spectrometer is a BASLER industrial-grade camera based on an uncooled CMOS sensor (Sony, 1920 x 1200 px) with a square pixel with 5.86 x 5.86 µm. The diffraction element is reflective grating with 1200 grooves/mm density. To achieve higher intensities and greater image compression, a vertical and horizontal binning operation were carried out on the acquired images on a 4 pixel window, thus obtaining an image of 480x300 pixels.
The overall system is designed to analyze a range of 157 nm with a resolution of 14 nm/mm, i.e. 12 cm^{-1}/px.
Particular effort was spent to find an inexpensive vial with good optical qualities, causing reduced fluorescence with a lower cost compared to quartz ones, and routinely used in chemical/biological laboratories. In particular, the cap must be leak-proof for headspace gases because during bacteria proliferation the headspace pressure can exceed 2 bar. In addition, the vial's volume must be a compromise between containing a good number of spores and limiting the waste of broth required for the test. For this reasons, a commercial 42-ml 27.5-mm-diameter transparent borosilicate glass vial type with a screw cap and polytetrafluoroethylene (PTFE) silicone septa has been chosen.
It has been verified that the laser spot size does not change when the vial is inserted in the optical path, therefore the effect of the glass curvature on the laser beam propagation is negligible. In addition it has been verified that the spectral shape of the Raman spectrum is not affected by the vial. Finally, beyond fluorescence, the effect of the glass on the spectrum is a small decrease of the Raman intensity (about 15%) due to the glass absorption. The test tubes are placed in an appositely realized 3d-printed vial holder, which can simultaneously accommodate six vials. An automated routine actuates a linear translator driven by a stepper motor to positioning each vial one at time in the spectrometer focal plane, where the laser is focused.

The positioning of the vial along the laser propagation direction is not particularly challenging given the long focusing lens and low beam divergence (<1.5 mrad) that give a Rayleigh length on the focus of about one centimeter. The resulting uncertainty on the longitudinal positioning of the vial is ±5 mm.
Similarly the uncertainty on the transversal direction positioning is ±6 mm, that is the lateral displacement causing a 10% reduction of the optical path inside the vial.
These alignment precision requirements are well satisfied even by common automation-grade linear translation stages on the market.

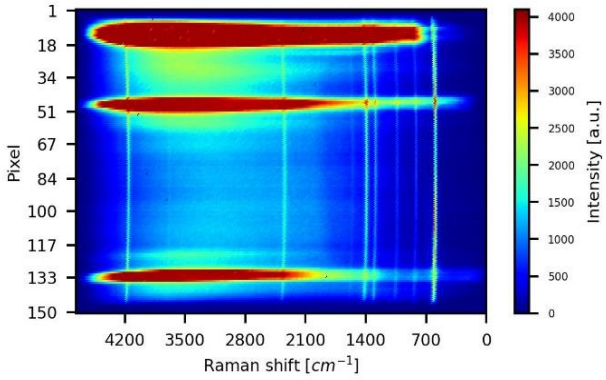

Figure 2: Image acquired without complete detector saturation

In Figure 2 a typical image, acquired in case of milk infected by Clostridium, is presented, that show the presence of H_2 (at 4156 and 587 cm^{-1}), H_2O (3657 cm^{-1}), N_2 (2331 cm^{-1}), O_2 (1555 cm^{-1}), CO_2 (1388 and 1285 cm^{-1}).

The camera frame is cropped to show only the region of interest of the acquired image, 150 rows and 480 columns.
The main challenge in adopting Raman spectroscopy for gas analysis in vials is to prevent detector saturation in case of an elastic scattering sources on the glass surface crossed by the laser beam. Moreover, given the high source power, fluorescence phenomena could be triggered by spurious particles along the laser path.
Detector saturation causes may be the presence of dust, dirt, condensation drops, milk agglomerates. To overcome this issue, the

system is equipped with a pulley that enables the vial to be rotated; this arrangement permits to rotate from a fully saturated image to a useful one. Once a valid image is recognized by the system, the routine acquires the camera frame, save the data into the memory and then the real-time gas composition analysis is triggered (more details are provided in the next paragraph). After the gas composition calculation, the linear translator moves the vials holder in order to analyze the next vial.
To comply safety regulation the instrument is mounted into a laser safety enclosure box. In addition, to prevent the condensation formation an electric heater is controlled by a PID module to maintain its internal volume at a constant temperature of 37 degrees.

Spectral analysis

In this paragraph, spectral elaboration applied to the valid camera frames is presented.
As a first step, an image with the laser turned off (dark) is acquired. The dark is then subtracted from each acquired image.
The laser propagation direction is imaged along the vertical camera axis, while the spectral dispersion takes place over the horizontal camera axis. Thus, the Raman spectra are obtained by averaging each column to generate a row of spectral intensities.
Only the proper rows are selected during the spectral array generation since some horizontal frame regions are completely saturated as shown in Fig.2. An appropriate rows selection algorithm was developed to maximize the spectral SNR while rejecting the saturated data. The row selection is based on the observation of the spectral region close to the nitrogen Raman emission peak (2331 cm^{-1}). Nitrogen is not involved in the bacterial metabolism; its partial pressure remains constant over time. So, its Raman signal is a key factor in determining unsaturated rows. For each row, the ratio factor between the nitrogen peak and the average value in 15-pixel window before and after the peak is calculated. Once the vector of 150 ratios is obtained, a row is taken into consideration for spectral generation if its ratio factor is larger than 85% of its maximum value over all the rows.
The threshold at 85% was chosen after fine tuning optimization. It is a compromise between selecting as much rows as possible, since the SNR is proportional to the number of rows used for the spectral generation, while rejecting the saturated rows.
This method has the advantage to process independently every single row of the frame. In this way, rows can be selected from several non-saturated areas of the image.
The obtained Raman spectra were finally subdivided into spectral regions of interest, for example the carbon dioxide region. For each region, the baseline was subtracted by fitting with a third-degree polynomial limited to the spectral zones free from Raman features of interest.

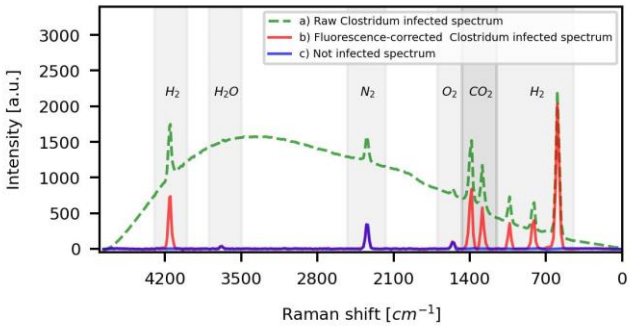

Figure 3: Trace a) raw spectrum of an infected sample, including the fluorescence contribution. Trace b) fluorescence-corrected spectrum from the infected sample shown in trace a. Trace c) fluorescence-corrected spectrum from a non-infected sample

As an example, Figure 3 shows the raw spectrum obtained from the frame shown in Fig. 2 averaging over 112 rows, the same spectrum after the baseline correction and a spectrum in case of no infection.
Clostridium infection is clearly visible as hydrogen and carbon dioxide are present, while the other gases (water vapour, nitrogen and oxygen) are present with concentrations comparable to those in the case without infection, i.e. air.
Once the net hydrogen and carbon dioxide spectra were obtained, they were used to determine the positivity of a vial.

MPN Method

The MPN method is used to estimate the most probable number of colony forming units (CFU) spores in a sample, provided the quantity of inoculation, the number of vials used and the number of positive vials.
The limit of detection (LOD) of MPN method depends on the number of vials and the amount of inoculation per vial. In this case, given 12 vials for both methods but with different fillings, the standard method (1 ml milk per vial) gives an LOD of 87 CFU/l, while the method based on Raman spectroscopy (3 ml per vial) gives 29 CFU/l[16]. Obviously, if the quantity of inoculant and tubes is the same for both methods then the LODs are equal.
The advantage of proposed method is the capability of identify positive vials in a shorter time than the standard method and discriminate the presence of *Clostridium* infection against non-hydrogen forming bacteria.
The way positivity is determined between the two methods is completely different. For the traditional method: a positive vial is indicated by a lift of the paraffin layer (more than 2 cm over the sample surface). For the Raman-based method: a vial is considered positive if the hydrogen signal is larger than a positivity threshold set equals to the mean background integral plus 5 times the background integral standard deviation, as described in the following paragraph.

Raman based method positivity threshold

Since hydrogen is not present in non-infected vial headspace, the detection of this gas is sufficient to determine the positivity of the vial, provided the hydrogen concentration is the fixed threshold but regardless of its actual concentration. The positivity threshold is determined based on the background noise in absence of hydrogen. The background noise, to be representative of the real experimental noise, must be calculated from vials compliant with the measurement, i.e., filled with the mixture of culture broth, milk and vaseline oil. The background noise has been measured on the spectra obtained after 24 hours when the hydrogen had not yet developed, on the spectral region around the hydrogen vibrational line (587 cm^{-1}) . In this way we are sure that all the possible sources of noise give contribution to the spectrum, e.g. possible condensation, agglomerates or dirt on vials walls. The average noise has been calculated, after the fluorescence correction, from 78 spectra. The obtained values follow a Gaussian distribution centered around 9 a.u. with a standard deviation equal to 19 a.u. It should be noted that values are also negative and this is due to the residuals after fluorescence subtraction. To limit the number of false positives to a probability of $3 \cdot 10^{-7}$, the positivity threshold has been set to 5 standard deviations above the mean, that is 104 a.u. for hydrogen detection.

To determine the carbon dioxide positivity threshold, the same procedure was performed, in the CO_2 spectral region around 1388 and 1285 cm^{-1}. The average noise distribution is 11 a.u. ± 35 a.u. (σ), giving a positivity threshold of 186 a.u. for CO_2 detection.

To quantify the hydrogen concentration corresponding to the positivity threshold, a batch of vials filled with a calibrated mixture of 4.8% hydrogen in nitrogen has been acquired, giving a mean hydrogen signal of 440 a.u.. Therefore the threshold as above identified corresponds to about 5.7% of hydrogen at ambient pressure.

TEST

To compare our method with the standard method, a test was carried out by preparing 24 vials to be analyzed with the Raman based instrument and 24 vial for reference, analyzed with the traditional method.
In its context of use, the proposed instrument is intended to work with 12 vials for each

measurement campaign, the use of 24 vials in this test was chosen to enlarge the dataset.

Measurement procedure

The 24 vials were kept on an incubator at a constant temperature of 37°C ± 0.2°C and were kept out from the incubator and put on the instrument only for the time requested for the measurement, that is few minutes for a batch of 6 vials hosted in the translation stage.
Starting 24 hours after inoculation, sampling was done approximately every couple of hours. For each vial, several frames at different rotation position were acquired until enough unsaturated images are found. In general, by acquiring 4 images, at least 3 valid frames are found, therefore 3 valid spectra are obtained. Each image is acquired with an integration time of 2 seconds.

RESULTS AND DATA ANALYSIS

A vial is defined *Clostridium*-infected if at least one of the valid obtained spectra has an hydrogen signal higher than the positivity threshold. The results of the measurement are presented in figure 4, where the number of vials found *Clostridium*-positive with the two methods is shown.

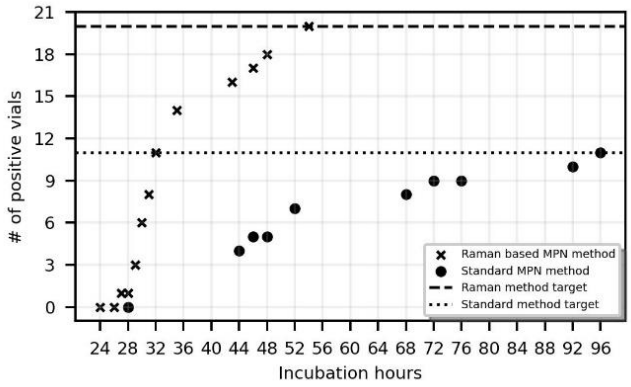

Figure 4: Methods comparison

Given the different filling level of the vials and the same quantity of injected spores (600 CFU/l), 11 infected vials are expected for the standard method and 20 for the Raman spectroscopy based method. In both cases, the actual total number of infected vials is in perfect agreement with predictions.

The data clearly show how the proposed instrument allows infected vials detection much more quickly than the traditional method. In particular, the first infected vial is identified by the Raman method after 27 hours from incubation, while the standard method requires 44 hours. In addition, the campaign is to be considered completed after 54 hours with the proposed method compared to the 96 hours required by the traditional method.

For *Clostridium*-non-infected vials also the carbon dioxide signal is always below than its positivity threshold, thus confirming the absence of non-hydrogen-producing bacteria.

CONCLUSIONS

The comparison test between the Raman based method and the reference one shows that the analysis of gases produced in the headspace can be effectively exploited to reduce (almost halve) the time required to identify contaminated vials. The use of the Raman analysis here discussed allows to save ≈42 hours on the total time required by the measurement campaign and to identify the first infected vial 17 hours earlier than the traditional method. In addition, it makes it possible to exclude contamination by other bacteria such as the main competitor *Bacilli*, that is producing only carbon dioxide.

In the future, samples with different spores concentrations will be tested to find spores/l limit of detection. Furthermore, the technique will be validated with raw milk from different dairy producers. Moreover, the instrument will be made portable to allow its use in the real operational context. The whole instrument will be enclosed in a safety box, thus the operator will not have access to the vials as long as the laser is on. This makes the final instrument as safe as using a class-1laser.

Further analyzes will be carried out to reduce the scattered light as much as possible in order

to allow integration for a longer time without saturating the detector, this will allow the increase of signal to noise ratio especially in view of the study of the dynamics of the gaseous species as a function of incubation time and different culture broth.

Conflict of Interest

The Authors declare that there is no conflict of interest.

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CAPTIONS:

- Figure 1: Instrument layout
- Figure 2: Image acquired without complete detector saturation
- Figure 3: : Clostridium infected and non infected headspace gas Raman spectrums comparison
- Figure 4 : Methods comparison