Near Infrared Spectroscopy for Bacterial Detection in the Dairy Industry

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***Abstract*—This article discusses the use of near-infrared (NIR) spectroscopy combined with multivariate classification methods for detecting bacterial contamination in milk in the dairy industry. In the first experiment, the study found that NIR was accurate and reliable in detecting the presence of biofilms in milk. Our results showed that the technology was effective in distinguishing between contaminated and uncontaminated samples with an area under the receiver operating characteristic (ROC) curve (AUC) greater than 99%. It was also effective in classifying the samples belonging to different strains. In a second experiment, we used the same methodology to assess their effectiveness in detecting bacterial contamination proportions in milk. Our results showed that the technology was effective in classifying milk samples contaminated with four different bacteria and uncontaminated controls with an AUC greater than 97%. Moreover, results were still good when data from all bacteria were analyzed together, even at low bacterial concentrations, obtaining an average precision of 70%. These results demonstrate the potential of this technology to be used as a rapid and accurate method for identifying bacterial contamination in the dairy sector.**

***Index Terms*— Bacteria, biofilm, dairy industry, milk contamination, spectroscopy.**
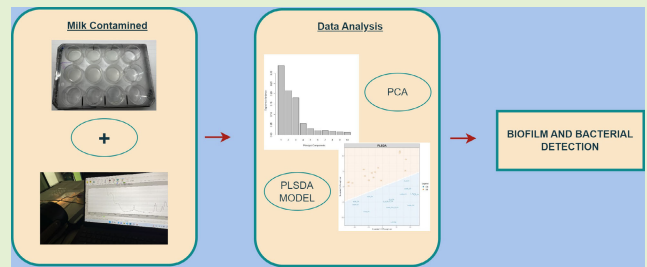
I. INTRODUCTION

MILK has been a major part of the human diet for millennia since the domestication of cattle allowed easy access to this nutritious food. Later on, fermentation and ripening processes led to the development of milk-derived products that can be stored for longer periods of time and are easier to digest by adult humans. Today, dairy products remain as important as ever, although their manufacture will likely face some challenges in the near future in order to meet the needs of an ever-changing, and increasingly large, world population. Indeed, the dairy sector will have to adapt to satisfy consumer demands, who have become more concerned about both their health and the environment. In that sense, consumers favor foods manufactured according to sustainable, traditional, and natural methods, while retaining the quality and safety standards that can be achieved through the implementation of modern technologies.

In the dairy sector, many microorganisms play crucial roles, with some being necessary for fermentation, while others can lead to food spoilage or health concerns for the end consumers. Therefore, the ability to detect the presence of bacteria at different stages along the dairy chain is essential to guarantee both quality and safety [1].

Near-infrared (NIR) spectroscopy is a technique that uses light in the infrared wavelength to determine the physical, chemical, or structural properties of materials. It is a type of infrared spectroscopy that measures the interaction of infrared radiation with matter by absorption, emission, or reflection covering the spectral region in a wavelength range from 780 to 2500 nm. This technology has demonstrated its reliability to detect and quantify microorganisms, becoming a non-destructive alternative to molecular biology techniques [2].

This technology combined with multivariate classification methods has been proposed as a method to efficiently and quickly identify bacterial contamination in cow's

milk [3], [4], [5], [6]. Other techniques for detecting microbial contamination in dairy products include principal component regression (PCR) applications using nanoparticles [7] or chemometric techniques such as clustering and classification [8].

The detection of bacteria in milk is important for a number of reasons. First and foremost, milk contamination with pathogens can lead to food poisoning and other illnesses if consumed [9], [10]. Bacterial contamination can also cause spoilage of milk, resulting in a decrease in shelf life [11], [12]. Additionally, the presence of bacteria in milk can also affect the taste, odor, and quality of the product, making it less desirable for consumers. Finally, bacterial contamination can also lead to the spread of disease and infection to other dairy products. Therefore, it is essential to detect the presence of bacteria in milk in order to ensure the safety and quality of the final product [13], [14], [15].

Bacteria can also form biofilms on surfaces of equipment and piping used for milk processing and packaging [16], which can increase the risk of contamination with potentially harmful pathogens [17]. These biofilms constitute a protective layer for bacteria, allowing them to survive and colonize dairy environments. Indeed, the use of biofilm-contaminated equipment is often the cause of the presence of bacteria in raw or pasteurized milk, a risk that increases with higher storage temperatures [18].

This study aims to determine the ability of NIR to detect the presence of bacterial contamination in milk; first, by analyzing the ability of this technology to determine the presence of biofilms formed in milk by two different bacterial strains, and then by inoculating four different bacterial species in liquid milk at different concentrations.

II. MATERIAL AND METHODS

A. Biofilm Contamination Experiments

Biofilms were formed as described by [19] with some modifications. Briefly, overnight cultures of two *Staphylococcus aureus* strains, Sa7 and 15 981 [20], were diluted in fresh TSBg (Tryptic Soy Broth supplemented with 0.25% w/v D-(+)-glucose) or skim UHT milk (Central Lechera Asturiana, Siero, Spain) to obtain cell suspensions containing 10^6 CFU/mL. Then, 2 ml aliquots of these suspensions were used to inoculate each well of a microtiter plate (Thermo Scientific, NUNC, Madrid, Spain). The control wells were inoculated with 2 ml of bacteria-free TSBg or milk. These microtiter plates were incubated for 24 h at 37 °C, and then the planktonic phase was removed and analyzed using NIR.

B. Bacterial Contamination Experiments

The strains used in this study included *Escherichia coli* CECT 434, *Lactiplantibacillus plantarum* 55-1 [21], *S. aureus* 15981, and *Lactococcus lactis* MG1363 [22], which were grown in Luria Broth (LB), De Man, Rogosa and Sharpe medium (MRS), Tryptic Soy Broth (TSB), and GM17 (M17 supplemented with glucose), respectively. *E. coli* and *S. aureus* were incubated at 37 °C under shaking, while the other two strains were grown at 32 °C in a static

TABLE I
TOTAL SPECTRAL SIGNATURES FROM THE EXPERIMENTS

Biofilm Contamination		Bacterial Contamination	
Medium	Bacterial Strains	Concentration	Bacterial Strains
TSBg	*S. aureus* Sa7 48 s. signatures	C0-C10	*E. coli* CECT 434 96 s. signatures
Milk	*S. aureus* Sa7 48 s. signatures	C0-C10	(*S. aureus*) 96 s. signatures 15981
TSBg	*S. aureus* 15981 48 s. signatures	C0-C10	*L. lactis* MG1363 96 s. signatures
Milk	*S. aureus* 15981 48 s. signatures	C0-C10	L. (*L. plantarum* 551 96 s. signatures

incubator. About 20 ml overnight cultures of all bacteria were centrifuged and resuspended in 2 ml of Ringer to prepare cell suspensions that were subsequently diluted in UHT milk (Central Lechera Asturiana, Siero, Spain). The resulting suspensions contained tenfold dilutions of bacteria ranging from 10^{-1} to 10^8 CFU/ml. Control samples contained milk without bacterial contamination. The degrees of contamination were assigned alphanumeric codes in descending order from highest to lowest concentration, being C10 the code for the maximum concentration of each bacterium, and C0 for the uncontaminated control samples. Next, 2 ml from each sample were placed into each well of a Nunclon plate (Thermo Scientific, NUNC, Madrid, Spain). These samples were then subjected to NIR analysis.

There were duplicate samples for each bacterium and concentration that were divided into two sets (training and test). The first set of samples was used to train the classification model and to differentiate the spectral data of the set of contaminated samples and uncontaminated samples. The second set was used to validate the model and to determine whether it was able to accurately classify the samples.

In Table I, we provide the count of total spectral signatures from the two experiments, each associated with different strains and samples. The number of available samples was constrained by the experimental protocols for biofilm formation and bacterial contamination. The limited amount of data used for training the classification models led to overfitting because the general features of the data could not be learned by the models. In spite of this, they were chosen to maximize the utility of the available samples and considering the following guidelines [3], [4], [23], [24], [25] regarding the minimum number of samples per classes to obtain meaningful insights and draw reliable conclusions from data.

C. Spectral Acquisition

The samples were analyzed using NIR to determine the ability of the technique to detect the presence of planktonic bacteria and/or biofilms in milk. The NIR spectra of the contaminated samples were compared to the spectra of a control sample of uncontaminated milk, and the changes were analyzed to determine the effectiveness of NIR in detecting the presence of bacteria.

All measurements were taken with an analytical spectral device (ASD) LabSpec[1] 4 Standard-Res (provided by Bonsai Advanced Technologies, S.L, Madrid, Spain) with a wavelength accuracy of 1 nm, a spectral range of 350–2500 nm, and a spectral resolution of 3 nm at 700 nm, and 10 nm at 1400 and 2100 nm. Although the spectral range of the instrument included the visible spectrum (350–780 nm), this was not considered in the analysis based on preliminary experiments. Before scanning the samples, a white Spectralon[1] panel was used to optimize the scans.

The instrument begins to acquire spectral information by using the contact probe, equipped with an attenuated total reflectance (ATR) cell that undergoes 50 reflections to measure the electromagnetic spectrum at different points in each sample. The contact probe is placed in contact with the sample container and emits electromagnetic radiation, which is then detected by the probe and collected by the instrument. The instrument then processes the data and provides the spectral information of the sample.

D. Data Analysis

Statistical analysis of the generated spectral data was carried out with R (https://www.r-project.org/) and the MixOmics package for R [26]. In the first step, preprocessing of the raw data was performed in order to improve the signal-to-noise ratio by spectral smoothing. Two different techniques were used for this purpose: in the first place, standard normal variate (SNV), which is a preprocessing technique that corrects any variation in the spectral data due to differences in sample handling or instrumentation. And second, Savitzky–Golay filtering, which is another preprocessing technique that reduces spectral noise and makes spectral data easier to interpret. As a result, the quality of spectral signatures from NIR data in this study was improved.

To analyze the NIR spectral data in this study, we have selected the combination of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to address the challenge of high-dimensional spectral data, aiming to reduce dimensionality, extract informative features, and enhance the effectiveness of sample classification [27]. PCA is an unsupervised technique that is used to identify and extract the most important components of the data. It is based on the concept of finding the linear combination of variables that explain the most variance in the data. PLSDA is a supervised classification method that uses linear combinations of variables to construct predictive models. It uses a set of labeled data points and a set of input variables to build a predictive model that can be used for classifying new data points.

It is important to note that these models have certain limitations, primarily related to the selection of the most significant variables and the risk of overfitting. For more information about the PCA and PLSDA underlying mathematical principles, please refer to [28], [29], [30], and [31]. To evaluate the accuracy of the model we used leave-one-out (LOO) cross-validation. This technique works by training the model on all
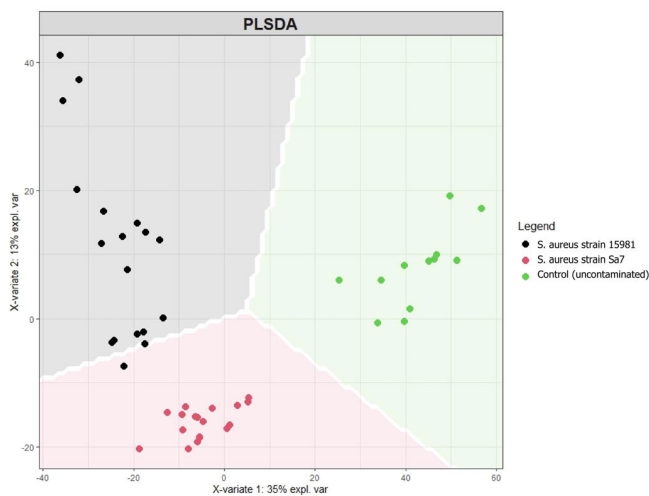
[1]Registered trademark.

Fig. 1. Model validation results for biofilm detection in TSBg medium.

but one of the observations in the dataset and then testing the model on the remaining observations. This is repeated for each observation in the dataset. Confusion matrices were used to calculate the accuracy of the classification model by comparing the predicted results from the model with the actual results from the data. Accuracy is computed by dividing the sum of true positives and true negatives by the total number of cases.

Finally, we also employed the Kappa coefficient, sensitivity, and specificity concepts. The Kappa coefficient is a statistic that measures the agreement between two raters. It is often used in the context of confusion matrices because it takes into account the agreement due to chance and is a more accurate measure than simply looking at the percentage of agreement. It is generally used in the field of spectral analysis to measure the accuracy of a classification model. The Kappa coefficient ranges from −1 to 1, with values closer to 1 indicating higher levels of agreement between the raters. Values of 0 indicate chance agreement, and negative values indicate less agreement than expected by chance. Sensitivity and specificity analyses were used to evaluate the model's ability to correctly identify true positives and true negatives in classification, respectively. The receiver operating characteristic (ROC) curve is a graph that represents the relationship between sensitivity (true positive rate) and specificity (true negative rate) of the model for different classification thresholds. Sensitivity is represented on the y-axis, while specificity is represented on the x-axis. The area under the ROC curve (AUC) is utilized to measure the discriminative ability of the models. An AUC value of 1 signifies a flawless classification, whereas an AUC of 0.5 indicates a random classification.

III. Results and Discussion
A. Detection of Biofilms Formed in Milk

The first analyses were carried out to verify the ability of the NIR technique to detect the presence of biofilms grown in the TSBg medium.

The statistical analysis resulted in a predictive and stable PLSDA model with the first four main components. The model was able to predict the outcome of the dataset with 100%
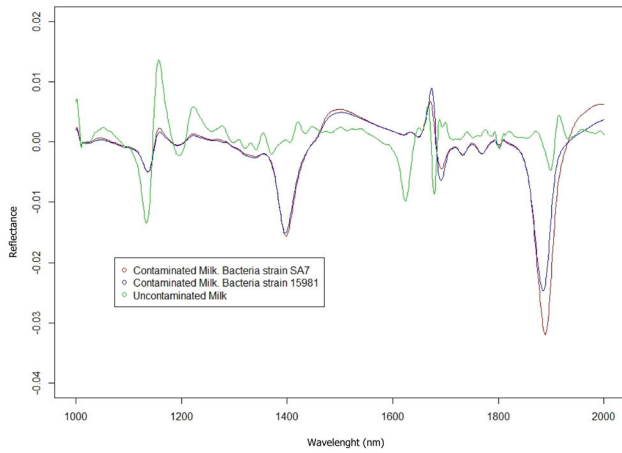
Fig. 2. Spectral data of biofilms grown in milk and non-contaminated control samples.

Fig. 3. Tuning for the PLSDA model.
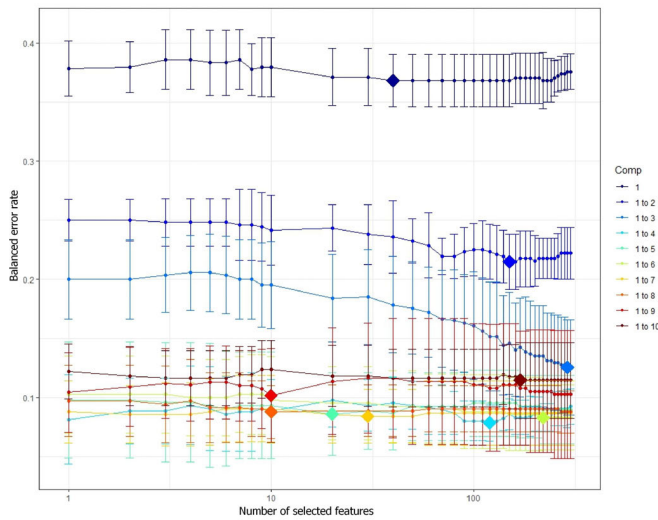

Fig. 4. ROC plot for a model containing four components. The outcome reveals the AUC values.

Fig. 5. PCA of data from different bacteria.

accuracy and a Kappa coefficient of 1. This indicates that the model is highly accurate and reliable.

The graph showing the model validation result with the data set is in Fig. 1. The results showed that the technique was able to accurately detect the presence of biofilms and could also differentiate between the two different bacterial strains based on their spectral profiles.

In the second phase, the analyses were repeated with biofilms grown in milk. The resulting spectral data shown in Fig. 2 indicate significant differences between the biofilm samples compared to the control samples.

In the PCA, the first four components explained more than 80% of the variance in the data and allowed obtaining a stable predictive model, with more than 95% according to the confusion matrix and a Cohen's Kappa coefficient of 0.9363.

The performance metrics such as overall error rate or BER are evaluated on the validation set for different combinations of hyperparameters and the combination that yields the best performance is chosen as the optimal value. Fig. 3 shows that we achieved the optimal results for the PLS-DA model with four components and 120 variables where the diamond indicates the optimal number of variables to keep for a given
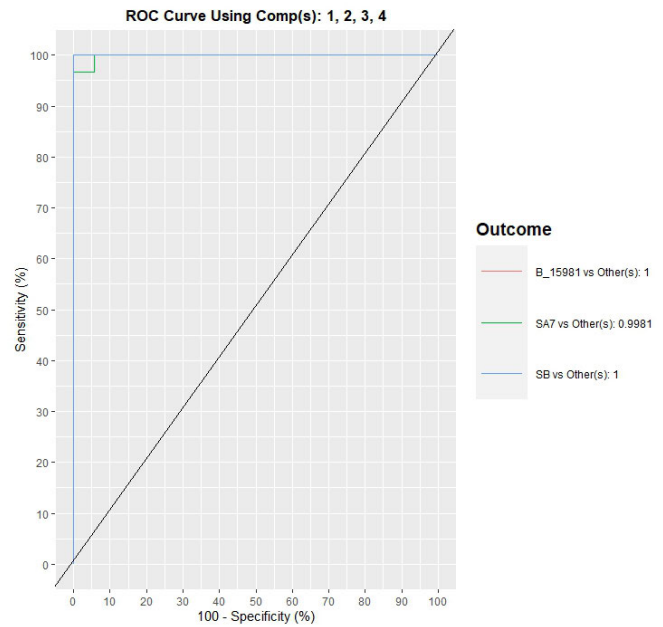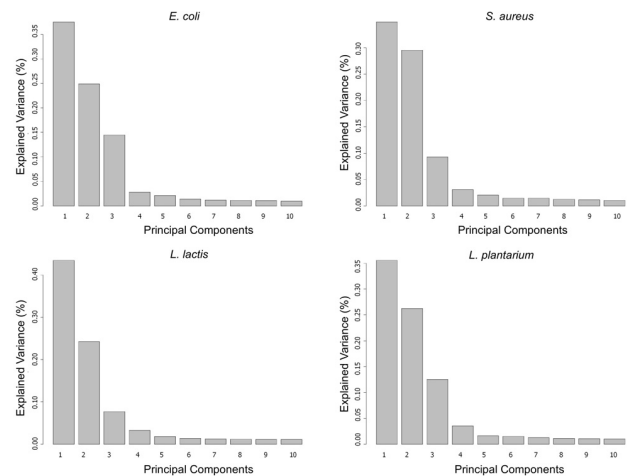
component, selected for the lowest classification error rate. The error bars indicate the standard deviation across the repeated, cross-validated folds.

The ROC curve is shown in Fig. 4 and represents the relationship between the sensitivity (true positive rate) and specificity (true negative rate) of the model.

The results (AUC > 99.8%) showed that the technique was also able to accurately detect the presence of biofilms developed in milk and to differentiate between biofilms formed by different strains.

B. Bacterial Detection in Milk

First, data from different bacteria were analyzed separately in order to determine the ability to detect each species in milk.

The results of PCA indicate that the data is highly condensed in the first three principal components, which means that these components explain the majority of the variance in the data. These results are shown in Fig. 5.
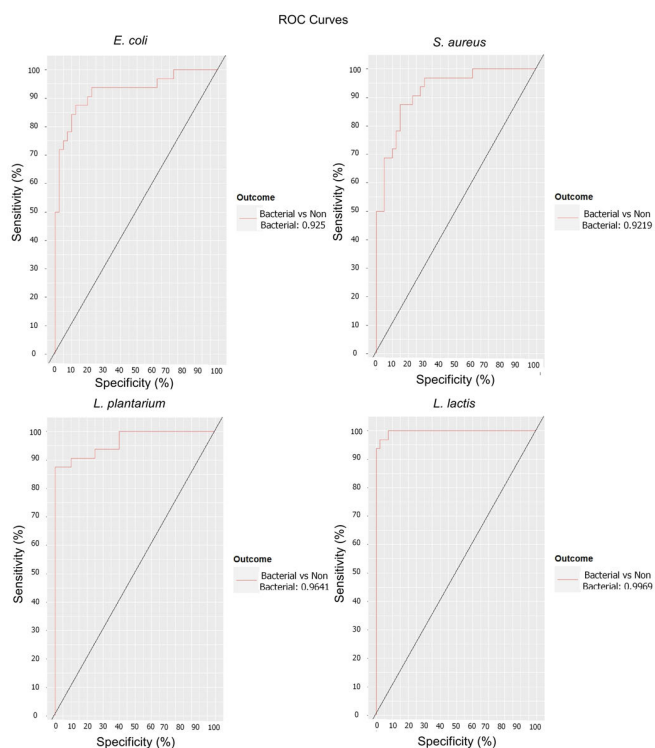
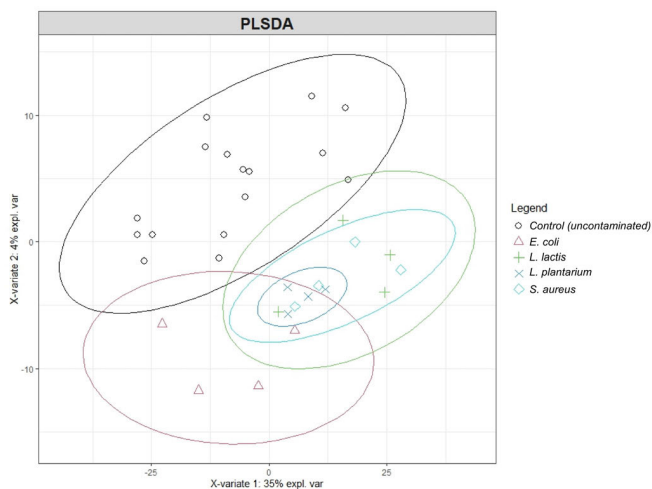Fig. 6. ROC curve and AUC values from PLSDA models.

Fig. 7. Separability among different bacterial strains in milk for concentration level C5.
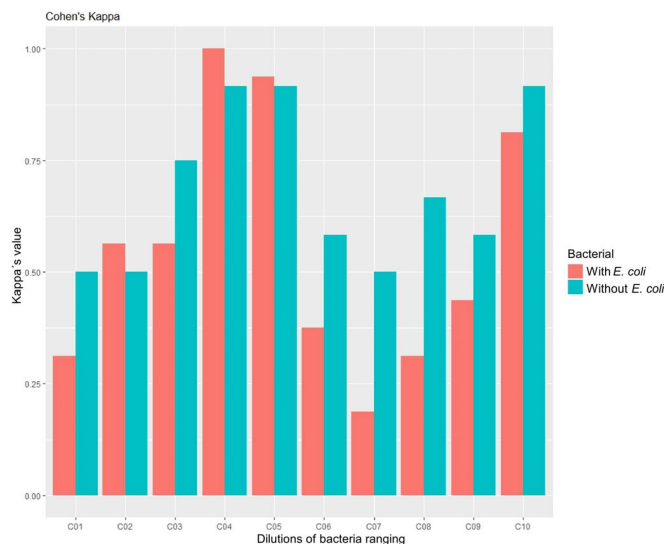

Fig. 8. Values of Cohen's Kappa for the different concentrations of bacteria in milk.
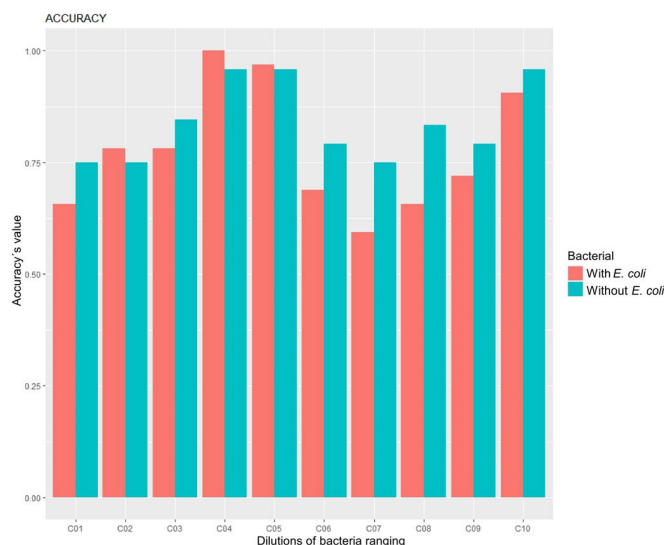
Fig. 9. Accuracy values for the different concentrations of bacteria in milk.

A PLSDA model was built using the training data and selecting a set of predictor variables obtained as principal components in PCA analysis. The optimal results for the PLS-DA models were attained with different configurations for each bacterial species. Specifically, the *E. coli* strain exhibited optimal performance with three components and 20 features, while the S. *aureus* strain required two components and 80 features. For the *L. plantarum* strain, three components and 180 features yielded the best outcome, whereas the *L. lactis* strain required three components and 20 features for optimal results.

The validation process involved making predictions on the test dataset using the models and comparing the predictions with the actual values to evaluate how well the model generalizes to test data.

The results of the validation analysis indicate that the most easily detected bacterium in milk was *L. lactis*, with an accuracy of 0.89 (Cohenś Kappa = 0.77). For the other bacteria, the accuracy values were: 0.88 for *E. coli* (Cohenś Kappa = 0.75), 0.86 for *S. aureus* (Cohenś Kappa = 0.72), and 0.81 for *L. plantarum* (Cohenś Kappa = 0.60). The resulting ROC curves are shown in Fig. 6.

We used the same methodology to assess the effectiveness of this technology in classifying contaminated and uncontaminated milk samples when the type of bacteria causing the contamination was unknown. We tested this technique on a set of samples with different degrees of contamination by the four bacteria studied. This enabled us to determine the scope of the technology for this purpose.

Statistical analysis of the data obtained with concentration C5 yielded the results shown in Fig. 7. The graph shows that the inclusion of the *E. coli* data in the analysis decreases the accuracy of the classification model to distinguish between

uncontaminated and contaminated milk and milk samples. This suggests that the spectral signature corresponding to *E. coli* samples might be too different from those of other bacteria, making the model unable to group them together and distinguish them accurately from the control samples.

The results of the validation analysis comparing the models that included *E. coli* data with those that did not are shown in Figs. 8 and 9, which respectively depict Cohen's Kappa coefficient and accuracy as calculated from the confusion matrix.

As can be observed in Figs. 7 and 8, non-inclusion of *E. coli* spectra improves Cohen's Kappa coefficient and yield more consistent outcomes, although the confusion matrices of the models may not show a significant difference in accuracy.

Confusion matrix analysis showed worse results in terms of model precision for concentrations C6, C7, and C8. This may be due to errors or distortions at the time of spectral recording due to the location of the samples in the containing plate.

IV. CONCLUSION

The results of this study suggest that NIR technology can be used to accurately classify contaminated and uncontaminated milk samples, regardless of the type of bacteria causing contamination, even at low concentrations (C2). However, spectral analysis does not appear to be capable of distinguishing between different contaminating bacteria.

Here, we show that the use of Near IR Spectroscopy detection is a promising non-destructive method for detecting bacterial contamination in milk. The spectral instrument and data processing methods used in this study allow for distinguishing between contaminated and control samples based on the qualitative determination of bacterial contamination by analyzing changes in spectral patterns. Similarly, NIR can detect biofilms formed in milk, as well as it does when biofilms grow in a culture medium. This finding is important because it reflects more accurately the surface-attached bacteria found in the dairy industry, where milk would be the main nutrient source.

Overall, rapid and noninvasive testing for the detection of bacteria is an important goal in the context of the dairy sector. Conventional methods for detecting bacteria in milk can be time-consuming and require skilled personnel, whereas NIR spectroscopy can provide accurate results in real-time without the need for sample preparation or destruction and could reduce the time and cost associated with traditional bacterial detection methods. However, further research is still needed to optimize the technique and validate its accuracy on a large scale before it can be widely adopted in the industry. In addition, it is necessary to consider the possibility of errors or distortions in the spectral recording of the samples and review the acquisition protocol in order to improve the accuracy of the models.

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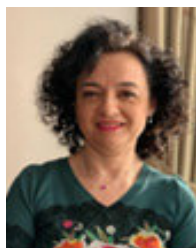
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