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Rapid Detection of Bacterial Contaminants in Cow's Milk Using Near Infrared Reflectance Spectroscopy

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ABSTRACT

Milk, a nutrient-rich food of animal origin, is essential for human nutrition. However, its high nutritional value also increases the risk of it serving as a vector for food-borne diseases. Traditional methods for detecting contamination in milk are often time-consuming, complex, and require additional reagents. To streamline this process, near-infrared reflectance spectroscopy (NIRS) has been developed as a rapid and straightforward alternative. NIRS technology generates unique spectral signatures based on the molecular composition of examined samples. In conjunction with multivariate analysis methods, particularly principal component analysis (PCA) and linear discriminant analysis (LDA), NIRS offers a robust approach for identifying bacterial contamination in milk. This study investigates the efficacy of NIRS combined with PCA and LDA in distinguishing between contaminated and sterile milk samples. The findings indicate that by integrating NIRS with PCA and LDA, we can achieve a 100% accuracy rate in classifying bacteria-contaminated milk compared to sterile milk. This advancement not only enhances the efficiency of contamination detection but also showcases the broader potential of NIRS technology in rapid food safety assessments and sustainable microbiological practices. While these results are promising, further validation involving a wider diversity of samples is necessary.

1. INTRODUCTION

Cow's milk, a nutrient-rich liquid produced by cows, is essential for human health due to its high content of proteins, fats, vitamins, and minerals. Its beneficial properties have led to significant increases in its production and consumption globally, particularly in developing countries [1, 2]. However, the high nutritional value also makes milk an ideal medium for microbial growth, which can lead to contamination through various sources such as equipment, floors, soil, water, and feces. Common contaminants include Staphylococcus aureus, Campylobacter spp., Salmonella, Listeria monocytogenes, and Escherichia coli [3].

Escherichia coli (*E. coli*) and Listeria monocytogenes are notable pathogenic bacteria that frequently contaminate food, causing foodborne diseases [4]. *E. coli*, typically a commensal organism in the intestines of vertebrates, can become an opportunistic pathogen leading to various intestinal and extra-intestinal infections [5]. In particular, certain strains like *E. coli* O157:H7 are infamous for causing severe food poisoning outbreaks. Listeria monocytogenes, on the other hand, is pervasive in nature and can thrive at refrigeration temperatures due to its psychotropic characteristics. This bacterium is capable of forming biofilm-structured communities that adhere to surfaces and can be resilient against sanitization processes, thus making milking equipment a potential vector for contamination and posing significant risks as a foodborne pathogen [3, 6].

The prevalence of these bacteria in milk poses a substantial threat to public health. *E. coli* infections can lead to symptoms ranging from mild gastroenteritis to life-threatening conditions such as hemolytic uremic syndrome. Listeria infections, which are particularly dangerous to pregnant women, newborns, the elderly, and immunocompromised individuals, can result in severe outcomes, including meningitis, septicemia, and fetal loss.

Traditional bacterial detection methods are labor-intensive and time-consuming, involving the growth of bacteria in culture media, isolation using selective media, and subsequent identification based on morphological, physiological, and biochemical characteristics [7, 8]. While these conventional methods are reliable and capable of detecting small amounts of bacteria and recovering live cells for further analysis, their complexity and environmental impact due to the use of chemicals are notable drawbacks.

Near-Infrared Reflectance Spectroscopy (NIRS) has emerged as a potential alternative for microbial identification due to its speed, cost-effectiveness, ease of use, and ability to

provide clues about the chemical content and physical properties of samples. These advantages have led to its widespread application in the agri-food industry for both on-site and laboratory analyses [9-11]. By utilizing near-infrared optical wave technology, NIRS can estimate sample quality parameters through spectral analysis, which reveals information about the chemical composition of the object [12].

NIRS has proven to be a promising tool in the agricultural sector for the rapid and accurate analysis of food compositions and quality control. Its benefits include high-speed operation, simplicity, non-destructive analysis, and the simultaneous measurement of multiple constituents [13]. These characteristics have made NIRS a preferred method for various applications, including monitoring livestock health and product quality [14].

Over the decades, NIRS technology has been recognized as one of the most effective non-destructive analysis methods. It is widely utilized in fields such as milk inspection due to its straightforward sample preparation, quick processing, and environmentally friendly nature, as it does not involve chemical use. Numerous studies highlight the application of NIRS in assessing food quality, predicting the authenticity of oils [15, 16], evaluating food composition and quality [10, 12], and identifying bacteria [9, 17, 18]. The integration of NIRS with chemometric techniques further enhances its potential as an alternative method for food safety testing and surveillance.

Building on these previous studies, we hypothesize that NIRS technology could be an effective tool for classifying bacterial species in milk. This research aims to develop a qualitative model for classifying *E. coli* and *L. monocytogenes* in milk using NIRS technology, coupled with multivariate analysis via Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) methods.

2. MATERIALS AND METHODS

2.1 Milk samples

Milk samples collected from dairy farms in Aceh Besar were stored in sterile bottles and transported in designated storage boxes under controlled temperature conditions to maintain sample integrity during transit. Upon arrival at the laboratory, strict aseptic procedures were followed to prevent any potential contamination.

The sterilization process involved subjecting 300 ml of each milk sample to autoclaving at 121℃ for 3 minutes. This thorough sterilization method effectively eliminated all microorganisms, including bacterial spores, ensuring that the milk was rendered sterile and free from any initial contamination.

Post-sterilization, the sterile milk was divided into three distinct portions: the control group of sterile milk, milk designated for contamination with *E. coli*, and milk designated for contamination with *L. monocytogenes*.

To prepare for the contamination process, bacterial suspensions of *E. coli* and *L. monocytogenes* were cultured and prepared to meet a 0.5 McFarland turbidity standard, equivalent to a concentration of approximately 1.5×10^8 CFU/ml. Specific volumes of the bacterial suspensions were then carefully measured: 0.6ml for *E. coli* and 0.5ml for *L. monocytogenes*.

Following preparation, each bacterial suspension was thoroughly mixed to ensure uniform distribution of the bacteria. Subsequently, 1 ml of each bacterial suspension was added to its respective portions of sterile milk to initiate the contamination procedure.

The contaminated milk samples were then divided into subsets: 8 samples for milk contaminated with *L. monocytogenes*, 7 samples for milk contaminated with *E. coli*, and 4 samples of sterile milk serving as controls. In total, 20 milk samples were meticulously prepared for further analysis using Near Infrared Reflectance Spectroscopy (NIRS) to facilitate accurate examination and evaluation.

These detailed steps in the sample preparation process aim to uphold consistency, accuracy, and reproducibility in the subsequent analysis and assessment of the milk samples for bacterial contamination.

2.2 NIR spectral acquisition

Diffuse reflectance spectra in the wavelength range 1000-2500 nm will be obtained with a self-developed diode-array NIR instrument (PSD NIRS i16). The spectral bandwidth of that instrument can be adjusted up to 4nm. For the sake of standardization, the acquired spectra were interpolated to a 0.2nm step using a piece-wise linear function. Spectra will be acquired with an integration time of 1s. The analysis was carried out at room temperature (25±1℃).

2.3 Spectral data analysis

The entire data is used as a dataset to build a classification model for bacterial contamination in milk. An outlier test was carried out first to see outlier data using the PCA and Hotelling T^2 methods. Data analysis was processed using the principal component analysis (PCA) and linear discriminant analysis (LDA) methods using Unscrambler X version 10.3 software to enable the classification of sterile and contaminated milk samples, as presented in Figure 1. PCA is a dimensionality reduction technique applied to identify underlying patterns and principal components in high-dimensional spectral data.

Principal component analysis (PCA) is a technique for simplifying data by changing data into new variables. PCA can also be used to classify or differentiate products from other products. PCA works by finding new variables, called principal components (PC), that explain most of the variability in the data. This PC allows us to describe information with far fewer variables than originally existed.

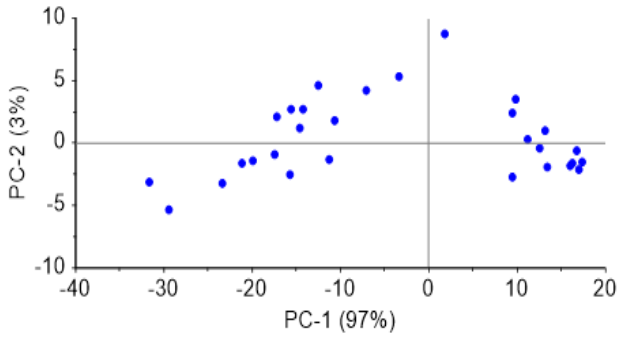

Figure 1. Spectral data analysis for the classification based on bacteria species and benefits

LDA is a commonly used technique for data classification and pattern recognition as well as dimensionality reduction.

LDA will classify objects into one of two or more groups based on various features that describe classes or groups of data [10]. In this case, the use of LDA for data classification is applied to the classification of sterile milk with milk contaminated with *E. coli* and *L. monocytogenes* bacteria.

3. RESULTS AND DISCUSSION

3.1 Spectra features

The NIRS spectrum is formed due to the absorption of NIRS light by the material, which causes the molecules to vibrate and then form peaks and valleys in the spectrum [19, 20]. Figure 1 shows typical NIRS diffuse reflectance spectra for sterile milk samples and milk contaminated with *E. coli* and *L. monocytogenes* bacteria.

When performing spectral analysis of milk and bacteria, there are several key NIR spectral regions to consider: Overtone bands and combinations associated with OH bonds around 1450nm and 1940nm. This is mainly related to water content. After 1350 nm, the absorbance of all samples changes drastically caused by the absorption of OH and water [10]. The main band formed is defined as containing information on carbohydrate content around 1483-1490nm and 2100nm, protein content information around 2050-2060nm, 1500-1530nm; fat content information 2070nm [17]. The NH bond absorption is around 2100nm which can provide information about the protein content in bacteria. The structure of the bacterial cell membrane and the ratio of lipids, proteins, and polysaccharides (IR active molecular bonds CH, NH, OH) depend on the bacterial species. These changes can appear in the IR vibration spectrum.

Correction of the spectrum of milk contaminated with bacteria with absorbance transformed into % transmittance is seen in Figure 2.

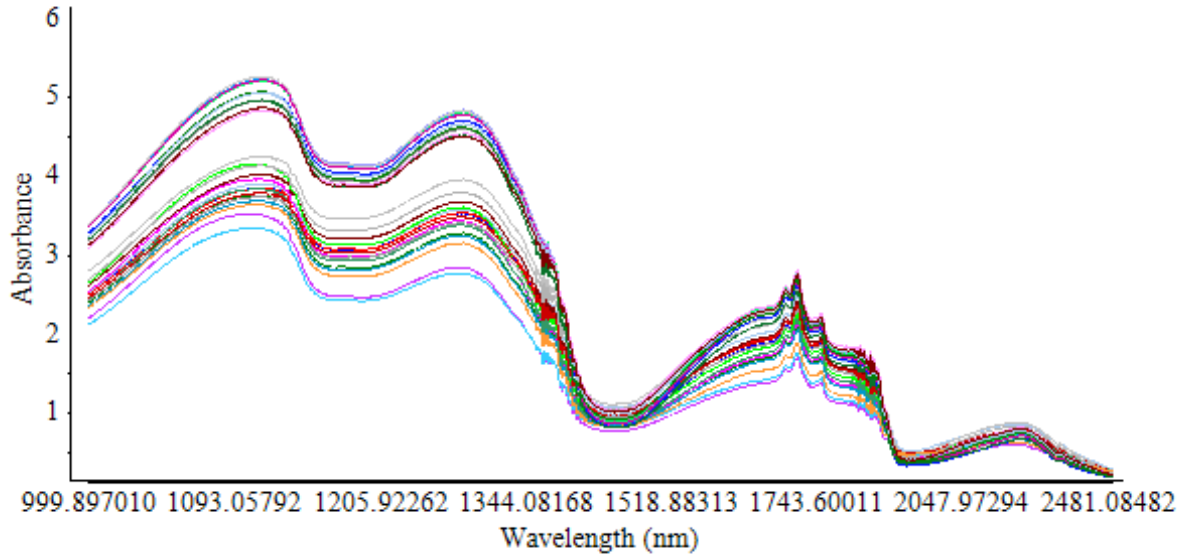

Figure 2. Typical spectrum of *E. coli* and *L. monocytogenes* bacteria in NIR region

When light originating from a light source falls on the biological components of the sample, an interaction between the biological components and the light will occur, giving a response in the form of reflection, absorption, and transmission [11]. Each spectrum result obtained will be different for each sample because it is influenced by the interaction of infrared rays with the biological content contained in the sample so that different spectrum shapes will be obtained between samples, as presented in Figure 2. Although only three different species are distinguished, it can be seen that the NIR spectrum contains information about the chemical composition of the sample.

Information on chemical content in spectrum data cannot be seen just by observing the spectrum but must be analyzed further using multivariate analysis. According to previous research [21], this is due to the complex infrared spectrum pattern, making direct and visual interpretation difficult.

NIRS shows potential in classifying different bacterial species accurately based on the spectral patterns formed. By analyzing the reflectance or absorption of NIRS Light by the biological components of bacteria, NIRS can identify the structure of certain molecules. This spectral profile can be used as reference data in building classification models that enable rapid identification of bacterial contamination.

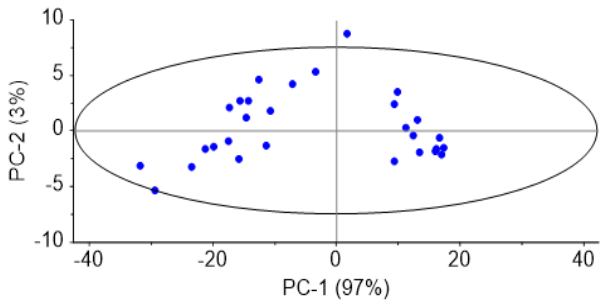

Figure 3. Outlier data examination with PCA method and *Hotelling* T^2 *ellipse*

Examination of outlier data shows that no data is outside the ellipse line, so no data needs to be discarded or removed, so all 20 samples can be used for analysis using PCA and LDA (Figure 3). The data used for further analysis is the data within

the ellipse [20]. If the data is outside the circle of the ellipse, the data is marked as outlier data.

3.2 PCA and LDA classification models

Data processing using the PCA method aims to make it easier to visually see the formation of data classification clusters. A good classification model, built using the PCA method, must have a latent variable (LV) smaller than 9.

The classification results obtained based on Figure 4 show very good results because the data is classified at 100%, which indicates that all data is collected into their respective clusters. The PCA analysis carried out resulted in a total PC (PC-1 and PC-2) of 100%, indicating that PCA was able to collect all the data in two PCs to carry out a classification process, which showed that all the data held could be collected well.

PCA is a statistical method that transforms an original dataset into a new coordinate system. The axes of this new system, called principal components, are orthogonal and linear combinations of the original variables. Each principal component represents a certain percentage of the total variation in the dataset. Two PCs were selected by examining the eigenvalue plot, outliers were detected and removed from the NIRS data after confirming their presence by spectral analysis [21]. In the context of bacterial classification, PCA can be used to generate a reduced set of variables that still captures most of the patterns in bacterial data. These variables, or principal components, can be visualized in a scatter plot to help differentiate between bacterial species.

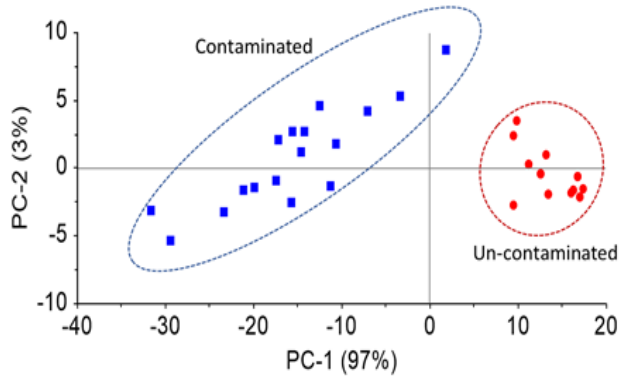

Figure 4. Classification based on NIR spectral data of milk samples using PCA method

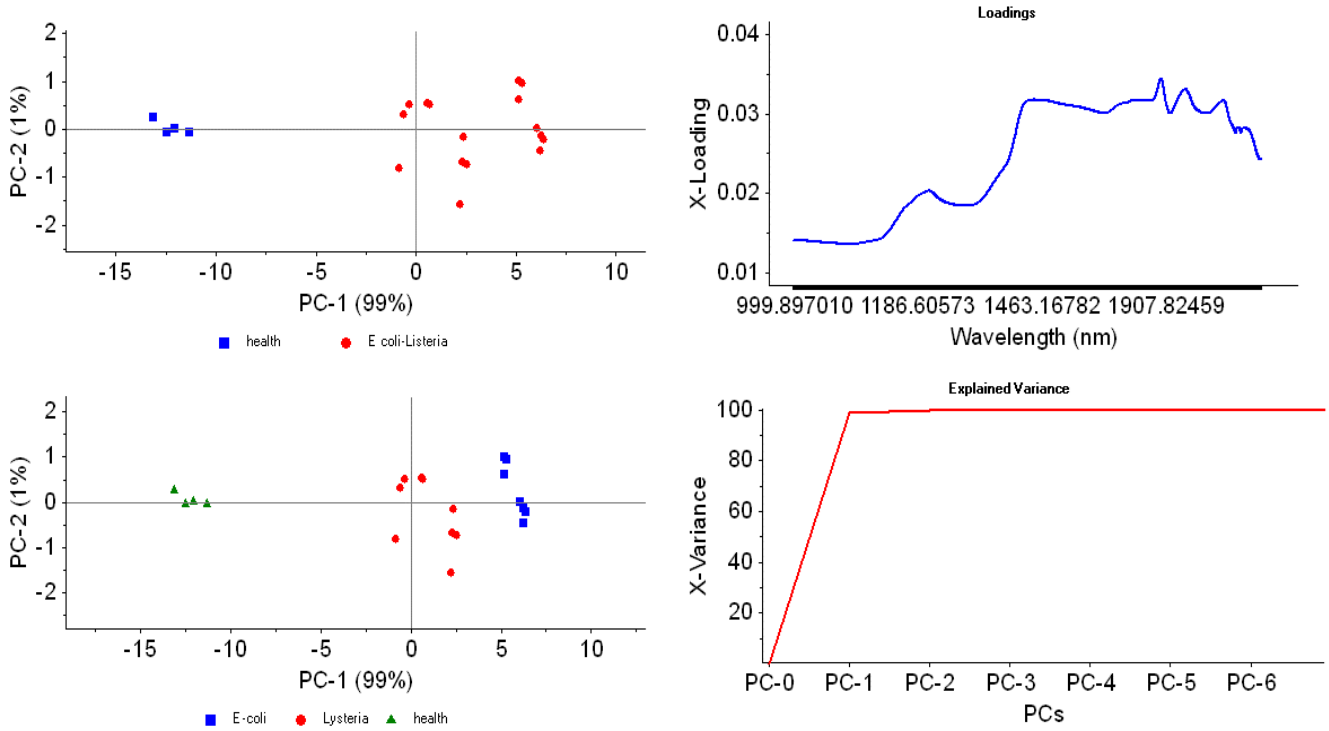

Figure 5. Classification based on NIR spectral data of bacteria samples using the LDA method

After PCA reduces the dimensionality of the NIR data to 20 dimensions, linear discriminant analysis (LDA) is applied to extract discriminant features from the training set. For LDA, the sum of eigenvectors and eigenvalues is usually the category number minus one [2]. The classification plot using LDA can be seen in Figure 5. It shows that the LDA classification results succeeded in classifying milk contaminated with *E. coli* and *L. monocytogenes* bacteria by producing a validation accuracy rate of 100%, which was projected using 2 components. This method is able to detect all sample treatments correctly. In the category description, 2 types correspond to the treatment carried out in this study.

Data processing using the LDA method aims to make it easier to see the level of success of the classification model. This method maximizes the ratio between class variance to within-class variance in any data set, thereby guaranteeing maximum separation [22]. Linear discriminant analysis (LDA) classification has been most widely used for NIRS because of its excellent performance reflected by the fast-learning rate and good classification performance. In applying classifiers, dimensionality reduction or feature selection methods are generally used because the number of NIRS feature vectors is usually larger than the dataset.

This score plot illustrates the separation of milk samples

into two distinct groups: healthy milk (blue squares) and contaminated milk (red circles). The x-axis represents Principal Component 1 (PC-1), which accounts for 99% of the variance in the dataset, while the y-axis represents Principal Component 2 (PC-2), contributing only 1% of the variance. The clustering demonstrates that PCA effectively reduces the dimensionality of NIRS data while preserving essential information for classification. The clear separation between healthy and contaminated samples signifies that PC-1 captures most of the variability associated with the contamination status, making it a dominant feature for distinguishing between these groups.

The loading plot provides insight into how different wavelengths contribute to PC-1, which captures nearly all the variance in the dataset. Peaks in this plot correspond to wavelengths that have a significant influence on the separation of sample groups. These wavelengths likely represent spectral features associated with specific chemical or biological properties of milk contaminated with *E. coli* and *Listeria monocytogenes.*

The results from the PCA analysis demonstrate a robust classification, with 100% of the data allocated to their respective clusters, indicative of successful data collection within two principal components. Subsequent LDA application, as shown in Figure 5, further enhances classification accuracy, achieving a validation rate of 100% by differentiating milk contaminated with *E. coli* and *L. monocytogenes* bacteria. LDA simplifies the assessment of the classification model's success by optimizing the ratio of class variance to within-class variance, ensuring effective separation within the data set.

The use of Linear Discriminant Analysis (LDA) in conjunction with NIRS technology has proven to be highly effective in classifying bacterial contaminants in milk, such as *Escherichia coli* and *Listeria monocytogenes*. This section delves into the specifics of how LDA enhances the classification model's success and its role in maximizing separation within datasets.

LDA classification, renowned for its fast-learning rate and superior classification performance, is widely employed in NIRS applications due to its ability to maximize separation efficiency within datasets. Dimensionality reduction or feature selection techniques enhance the classification process by managing the extensive NIRS feature vectors commonly present in datasets. This is also in agreement with previous works and literature [6, 12, 18]. Moreover, it operates by maximizing the ratio of class variance to within-class variance in any dataset, thereby ensuring maximum separation between different classes or groups of data. This is particularly beneficial in NIRS applications, where the number of feature vectors (spectral data points) often exceeds the number of samples. By optimizing this variance ratio [23, 24].

Future research in the field of NIRS offers a multitude of avenues for enhancing the sensitivity and specificity of contaminant detection in milk, thereby augmenting overall food safety measures. One particularly promising area for exploration involves the optimization of detection limits within NIRS methodologies. By delving into techniques that can enhance the sensitivity of NIRS to identify bacterial contaminants at even lower concentrations, researchers can pave the way for more precise and early detection capabilities, bolstering the method's utility in safeguarding milk quality.

Expanding the application of NIRS to encompass a wider spectrum of contaminants beyond the current focus on *E. coli* and *L. monocytogenes* presents another fruitful research direction. By exploring the detection of additional contaminants, such as diverse pathogenic bacteria strains, toxins, or chemical residues in milk samples, researchers can develop a more comprehensive contamination screening platform. This extension of capabilities could significantly enhance the overall efficacy of NIRS in ensuring the safety and integrity of milk products.

One key area involves optimizing the detection limits within NIRS methodologies. By developing techniques that enhance the sensitivity of NIRS, researchers can identify bacterial contaminants at even lower concentrations. This would enable more precise and early detection capabilities, bolstering the method's utility in safeguarding milk quality. Techniques such as improving spectral resolution, enhancing signal processing algorithms, or integrating NIRS with other spectroscopic methods could be explored to achieve this goal.

Expanding the application of NIRS to detect a broader range of contaminants beyond *E. coli* and *L. monocytogenes* is another fruitful research direction. This could involve exploring the detection of additional pathogenic bacteria strains, toxins, or chemical residues in milk samples. By developing a more comprehensive contamination screening platform, researchers can significantly enhance the overall efficacy of NIRS in ensuring the safety and integrity of milk products. For instance, detecting antibiotic residues, as explored in other studies using NIR-II fluorescence-based methods, could be integrated into NIRS platforms for a more holistic approach to milk safety [25].

Moreover, the integration of advanced algorithms, such as machine learning and artificial intelligence, holds significant promise for enhancing the analytical capabilities of NIRS. Through the development and implementation of sophisticated algorithms, researchers can refine the accuracy and specificity of NIRS-based contaminant detection in milk samples. This innovative approach has the potential to revolutionize the precision and efficiency of contaminant identification, paving the way for more robust food safety practices within the dairy industry.

Another promising avenue is the exploration of multimodal spectroscopy approaches, combining NIRS with other spectroscopic techniques like Mid-Infrared (MIR) spectroscopy or Surface-Enhanced Raman Spectroscopy (SERS) [26, 27]. These combinations could leverage the strengths of each method to provide more comprehensive and accurate detection of contaminants. For example, MIR spectroscopy is excellent for the quantitative identification of organic functional groups, while SERS offers high sensitivity and specificity for molecular fingerprinting [28, 29].

Furthermore, future outlook could delve into the feasibility of implementing real-time monitoring systems utilizing NIRS technology. By exploring the development of systems that allow for continuous monitoring of milk quality throughout the production process, researchers can enable timely interventions in response to contamination events. Investigating the integration of NIRS into on-field applications in dairy farms or processing facilities could provide a practical solution for the efficient and rapid screening of milk samples for contaminants, ensuring the preservation of milk quality and safety at every stage of production.

4. CONCLUSION

The research findings significantly highlight the efficacy of Near Infrared Reflectance Spectroscopy (NIRS) in conjunction with Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) for accurately discerning bacterial contamination by *L. monocytogenes* and *E. coli* in milk samples. The PCA analysis revealed a remarkable achievement, where all data points were classified into their designated clusters with an outstanding rate of 100%, indicating the robustness and precision of the classification process. Similarly, the LDA analysis further reinforced the study's credibility by showcasing a validation accuracy rate of 100%, underscoring the reliability and accuracy of the NIRS technology in identifying specific bacterial contaminants in milk.

As evidenced by the study outcomes, the application of NIRS technology emerges as a rapid and efficient method for detecting bacterial contamination in milk with minimal sample preparation requirements. This technology not only streamlines the process but also significantly reduces the time and resources traditionally associated with examining milk contamination, making it a valuable tool for expedited and high-throughput analyses in scenarios demanding swift and large-scale assessments.

While the PCA and LDA analyses yielded exceptional classification results across diverse sample treatments, it is imperative to acknowledge the importance of continued evaluation under varied conditions. Future research endeavors should encompass a broader spectrum of samples and various bacterial strains commonly found in animal-derived food products, ensuring the robustness and versatility of the NIRS technology in real-world applications. Furthermore, the study's outcomes underscore the immense potential of integrating NIRS with sophisticated multivariate analyses for assessing bacterial contamination in milk, hinting at its profound implications on enhancing veterinary public health practices and ensuring the safety and quality of dairy products across the industry.

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