

FOURIER TRANSFORM INFRARED MICRO-SPECTROSCOPY FOR RAPID DETECTION OF *Bacillus Cereus* AND *Pseudomonas Aeruginosa* SPOILAGE IN MILK

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Abstract

Conventional methods of detecting milk spoilage and quantification of microbes in milk are time-consuming. Thus, the aims of this study were to utilise Fourier transform infrared (FT-IR) microspectroscopy to detect *B. cereus* ATCC 11778 and *P. aeruginosa* ATCC 15442 spoilage in ultra-high temperature milk and to develop a predicted model for quantification of the total viable count (TVC) using partial least squares (PLS) analysis from the FT-IR spectra. Actual TVCs of bacteria either grown singly or in co-culture were carried out for model calibration of the predicted TVCs from PLS. The FT-IR spectra were acquired at absorbances ranging 4,000-700 cm⁻¹. The results from the FT-IR analysis revealed the proportionally significant changes in milk lipid and fatty acids as indicated by the wavelength number in the regions of the lipid (2,957, 2,923, and 2,852 cm⁻¹) and fatty acids (1,745 cm⁻¹) over the increasing incubation times in mono-cultures of *B. cereus* and *P. aeruginosa*. Furthermore, the present study also used the FT-IR spectra coupled with PLS to develop a model to predict the TVC in milk spoilage. We found that the coefficients of determination (R²) representing the accuracy of the model fit of *B. cereus* were 0.972 and 0.982 and of *P. aeruginosa* were 0.971 and 0.981 for the mono-cultures and co-culture, respectively. Interestingly, the predicted TVC by FT-IR through the PLS analysis provided a high accuracy of 94-99%. To summarise, FT-IR could be applicable in rapid detection of milk spoilage and bacterial load and could be an alternative approach for total plate count assay, particularly for *B. cereus* and *P. aeruginosa* quantifications.

Keywords: Bacterial spoilage, milk, FT-IR, rapid detection, *Bacillus cereus*, *Pseudomonas aeruginosa*

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Introduction

Cow's milk is much favoured by the world's population. Since pasteurised milk comprises the majority of processed milk, the dairy industry has concern over the microbial quantity in milk as a high temperature may not destroy all the foodborne pathogens in milk (Oliver *et al.*, 2005). Spoilages of milk products caused by microbial contaminations have a large impact on the loyalty towards, and the acceptance by consumers of, dairy products (Rysstad and Kolstad, 2006). The spoilage in dairy products can be perceived by physical or organoleptic properties including taint in product packaging, bad odour, and flavour changes (Nicolaou and Goodacre, 2008). The following group of bacteria are prone to contamination of dairy products: aerobic psychrotrophic gram-negative bacteria, heterofermentative lactobacilli, and spore-forming bacteria (Ledenbach and Marshall, 2009). Many dairy factories attempt to extend the shelf-life of their dairy products by detecting and eliminating contaminating microbes. Doing a total plate count (TPC) immediately after processing cannot predict the shelf-life, because it cannot discriminate between the contaminating bacteria and bacterial survival after the pasteurisation process. The shelf-life determination depends on the number of contaminants after pasteurisation, particularly bacteria that can grow rapidly at a low temperature in a refrigerator (e.g. gram-negative psychrotrophs, especially the pseudomonads) (Robinson, 2002).

Detection of milk spoilage caused by contamination of microorganisms is important. The standard plate count method is traditionally used to investigate psychrotrophic bacteria in milk. This technique requires plates to be incubated at 7°C for 7-10 days. This method is time-consuming and labour-intensive. Therefore, rapid and straightforward culture-independent methods are required to detect psychrotrophic bacteria in milk, which are considered to be the most important spoilage microbes. Molecular biological, biochemical, and immunological techniques have been applied for rapid and

specific detection of microorganisms to replace the TPC. Molecular-based methods to analyse the DNA or RNA are among the most precise techniques in identification of contamination of microbes in milk (Nollet and Toldra, 2010). Real-time PCR is one of the most popular techniques for detection of the spoilage microorganisms. This technique is versatile, rapid, and sensitive (Martínez *et al.*, 2011). The dairy industry is still seeking a novel technique to determine viable bacteria that can provide the results within 2-3 h.

Fourier transform infrared (FT-IR) microspectroscopy has previously been employed to detect a variety of foodborne pathogens and spoilage microorganisms because it is rapid, sensitive, and relatively inexpensive (Lin *et al.*, 2005; Nicolaou and Goodacre, 2008; Wang *et al.*, 2011; Schmidt *et al.*, 2012). It has also been used to rapidly monitor meat spoilage (Ellis *et al.*, 2002; Panagou *et al.*, 2011; Papadopoulou *et al.*, 2011). In addition, the FT-IR technique has been able to detect heat-induced conformational rearrangements of protein, protein-protein, and protein-lipid interactions in UHT milk, which was studied with accelerated shelf-life protocols (Grewal *et al.*, 2017).

The objectives of this study were to investigate the applications of FT-IR microspectroscopy for rapid detection of the bacterial load in milk and to develop a predicted model by partial least squares (PLS) analysis from FT-IR spectra for estimation of the total viable counts (TVC) of *B. cereus* and *P. aeruginosa* spoilage in ultra-heat treated or ultra-high temperature (UHT) whole milk.

Materials and Methods

Culture Media and Materials

Tryptic soy agar (TSA) and nutrient broth (NB) were purchased from Merck Pte Ltd., Singapore, while sterile plastic petri dishes and materials were obtained from Regional Medical Science Center 9, Nakhon Ratchasima, Thailand.

Bacterial Strains

Bacillus cereus ATCC 11778 and *Pseudomonas aeruginosa* ATCC 15442 were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Bangkok, Thailand.

Milk Samples

One hundred percent UHT whole milk of Thai-Denmark® (lot number EXP. 08/12/16 03:38:56 17) used in this experiment was supported by the Dairy Farming Promotion Organization of Thailand (Muaklek, Saraburi, Thailand).

Standard Curves of Bacterial Suspension

The standard curve method was used to select the bacterial suspension with a known total viable count. An eighteen-hour culture was collected by centrifugation at 8,000 rpm and the pellets were washed twice and resuspended in 10 ml of sterile 0.85% NaCl. The cell suspensions were adjusted to obtain different optical densities (ODs) (0.05, 0.1, 0.15, 0.2, 0.25) at a wavelength of 500 nm. Suspensions of each OD were 10-fold diluted with sterile normal saline to enumerate the growing colonies. One hundred microlitres of each dilution were spread and incubated at 37°C for 20 h. The growing colonies were counted, calculated, and plotted as a standard curve (Liu *et al.*, 2000; Eumkeb *et al.*, 2010).

Sample Preparation

A single colony each of *B. cereus* and *P. aeruginosa* was inoculated in NB medium, and then incubated for 18 h at 37°C. *B. cereus* and *P. aeruginosa* were collected, washed, and adjusted with sterile 0.85% NaCl to obtain approximately 1×10^6 cfu/ml in accordance with the bacterial suspension standard curve for each strain. Two millilitres of adjusted bacteria were added into the flasks containing 498 ml of UHT milk (final inocula approximately 4×10^3 cfu/ml). The experiments were designed as follows: 1) *B. cereus* ATCC 11778 mono-culture, 2) *P. aeruginosa* ATCC 15442 mono-culture, 3) co-culture of *B. cereus* and *P. aeruginosa*, and 4) bacterial free control. All milk samples were shaken at 200

rpm at 37°C for 24 h. Two aliquots of samples were taken from distinct time points (0, 60, 120, 150, 180, 210, 240, 300, 360, 420, 480, 720, 960, and 1,440 min) (Nicolaou *et al.*, 2011). One millilitre was used to determine the TVC, while another aliquot was equally added into 3 Eppendorf tubes and preserved at -70°C for further FT-IR microspectroscopy experiments (Nicolaou *et al.*, 2011).

Total Viable Counts (TVC)

The TVC were determined according to Nicolaou *et al.* (2011). One ml of each sample was transferred to 9 ml of phosphate-buffer and was vortexed for 1 min before spreading on the TSA medium. The experiment was carried out delicately for each dilution which was incubated for 20 h at 37°C. The growing colonies were counted and calculated for the TVC per ml.

Statistical Analysis

All total numbers of bacterial counts in cfu/ml were converted to log cfu/ml before statistical analysis and then all statistical analyses were conducted using SPSS software (Statistics Package for the Social Sciences, version 18). Statistical analysis of microbial data was expressed as means $\pm$ the standard error of means (SEM) and was determined by one-way analysis of variance (ANOVA) followed by a Fisher-Hayter *post hoc* test. Statistically significant differences of the results were considered when $p < 0.05$. Data from principle component analysis (PCA) and PLS were plotted by SigmaPlot, version 12.0 (Systat® Software Inc, Chicago, IL, USA)

FT-IR Microspectroscopy with Focal Plane Array detector

Sample Preparation

The frozen samples (-70°C) from the previous experiment were slowly defrosted on ice. A sample from each time point was vortexed for 1 min. One microliter was used and then air-dried on MirrIR low-e glass microscope slides (Kevley Technologies, Chesterland, OH, USA) which were used for FT-IR microspectroscopy analysis (Osawa *et al.*, 2002). The slides were desiccated under

vacuum for several hours and stored in a desiccator to form suitable films before analysis. The spectra were collected using FT-IR microspectroscopy with a focal plane (FPA) detector regarding the absorbance obtained by rationing the single beam spectrum against the slide background (Santos *et al.*, 2013).

Infrared Spectroscopy Measurements and Pre-processing of Data

The FT-IR spectra were acquired with a Bruker Tensor 27 spectrometer (Global source) coupled with a Bruker Hyperion 3,000 microscope (Bruker Optics Inc., Billerica, MA, USA). The microscope was equipped with 64×64 element mercury-cadmium-telluride FPA detector, which allowed simultaneous acquisition of spectral data with a 15x objective. The FT-IR samples were recorded in reflection mode using 4 cm⁻¹ spectral resolution and 64 scans. Images at an aperture 8×8 μm² were selected for FT-IR analysis. Absorbance spectra were acquired in the spectral range of 4,000-700 cm⁻¹. OPUS 7.5 software (Bruker Optics Inc., Billerica, MA, USA) was used to obtain the FT-IR spectral data and control the instrument system. The FT-IR spectra from milk spoilage were inspected and checked for outliers by PCA using variability of the Unscrambler X 10.1 software (Camo Analytics AS, Oslo, Norway). The spectra were processed using the second derivative and vector normalised by the Savitzky-Golay method (9 smoothing points) and then normalised with extended multiplicative signal correction (EMSC) in the spectral regions from 3,000 to 2,800 cm⁻¹ and 1,800-900 cm⁻¹. Score plots (2 dimensions) and loading plots were used to represent the different classes of data and relations among variables of the data set, respectively.

Partial Least Squares

The relationship between the FT-IR spectral and the total viable counts using the multivariate statistical methods were analysed using the PLS regression model (Nicolaou and Goodacre, 2008). The PLS was employed for the 3 different spoilage experiments (the *B. cereus* and *P. aeruginosa* in mono-cultures,

and the co-culture of *B. cereus* with *P. aeruginosa*) to predict the bacterial quantification of the TVC from the FT-IR spectra by determining the coefficient quality of the calibration model (R²), root mean square error of cross-validation (RMSECV), residual prediction deviation (RPD), and a bias value. The external validation was summarized as % accuracy and standard deviation (SD). The following equations were calculated for the validation step (Junhom *et al.*, 2017):

$$\% \text{ accuracy} = 100 - \% \text{ error}$$

$$\% \text{ error} = \text{relative error} \times 100$$

$$\text{Relative error} = \left| \frac{X_{\text{mean}} - X_a}{X_a} \right|$$

when X_{mean} = the FT-IR predicted value and X_a = the actual TVC value.

Results and Discussion

Total Viable Counts of Bacterial Spoilage in Milk During Incubation

The TVC for the *B. cereus* and *P. aeruginosa* in the mono-cultures and the co-culture in milk after incubation at 37°C were expressed as the mean log of colony-forming units per ml (cfu/ml). The results are shown in Table 1. The co-culture grown on the TSA medium was undoubtedly different in morphology, size, shape, colour, and texture (Nicolaou *et al.*, 2011). The colonies of *B. cereus* grown on the TSA were irregular, approximately 3-7 mm in diameter, opaque in colour, and smooth; the colonies of *P. aeruginosa* were irregular, approximately 2 mm in diameter, transparent to light yellow, shiny in colour, and smooth, as depicted in Figure 1. The results obtained from conventional TVC methods showed that the initial inocula of bacteria were relatively low in the milk samples. The initial inoculation numbers of all experiments were similar to the mean log the TVC of 3.74 and 3.39 for the pure cultures of *B. cereus* and *P. aeruginosa*, respectively. Likewise, the co-culture also showed the average log TVC of

Table 1. Mean log TVC for *B. cereus* and *P. aeruginosa* growths at 37°C in UHT milk, both in the mono-cultures and the co-culture

Storage Time (minutes)	Microbial analysis (log cfu/ml)			
	Pure culture		Mixed culture	
	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>
0	3.74 ± 0.06 ^a	3.39 ± 0.12 ^a	3.60 ± 0.00 ^a	3.00 ± 0.00 ^a
60	4.13 ± 0.07 ^b	3.59 ± 0.16 ^b	3.96 ± 0.26 ^a	3.35 ± 0.49 ^a
120	5.07 ± 0.04 ^c	3.80 ± 0.45 ^c	4.90 ± 0.01 ^a	4.00 ± 0.00 ^b
150	5.00 ± 0.06 ^c	4.06 ± 0.03 ^d	6.02 ± 0.03 ^b	3.95 ± 0.36 ^b
180	6.14 ± 0.20 ^d	4.15 ± 0.21 ^e	6.27 ± 0.02 ^b	4.15 ± 0.21 ^b
210	6.36 ± 0.08 ^d	4.35 ± 0.49 ^f	7.20 ± 0.00 ^b	5.00 ± 0.00 ^c
240	6.19 ± 0.02 ^d	4.65 ± 0.07 ^f	7.24 ± 0.09 ^c	5.15 ± 0.21 ^c
300	6.45 ± 0.21 ^d	5.65 ± 0.07 ^f	7.30 ± 0.06 ^d	5.45 ± 0.21 ^c
360	7.45 ± 0.02 ^e	6.47 ± 0.26 ^g	7.52 ± 0.06 ^e	5.74 ± 0.06 ^d
420	7.79 ± 0.05 ^f	7.40 ± 0.00 ^h	7.78 ± 0.03 ^f	6.69 ± 0.30 ^e
480	7.85 ± 0.02 ^f	8.06 ± 0.01 ^h	7.97 ± 0.04 ^g	6.85 ± 0.21 ^e
720	7.97 ± 0.01 ^f	8.89 ± 0.14 ^h	8.17 ± 0.12 ^h	7.65 ± 0.05 ^f
960	7.90 ± 0.08 ^f	9.01 ± 0.09 ^h	8.02 ± 0.09 ^h	8.00 ± 0.06 ^f
1440	7.65 ± 0.07 ^f	9.02 ± 0.03 ^h	7.99 ± 0.12 ^h	8.19 ± 0.34 ^g

Mean values ± standard error.

A significant difference between each group was compared using ANOVA and Fisher's least significant difference (LSD) *post hoc* test at a $p < 0.05$. abcdefgh, Mean log TVC within a column with the same superscripts are not statistically significant difference ($p < 0.05$).

Supplemental Table 1. Milk components attributed to different regions of FT-IR spectra (Grewal *et al.*, 2017)

Wave number (cm ⁻¹)	Milk component
3000-2800	Milk lipids
1800-1700	Ester bond of milk fatty acids
1700-1600	Milk proteins-amide I
1600-1500	Milk proteins-amide II
1500-1200	Milk proteins-amide III, interactions between different milk components (Fingerprint region)
1200-900	Milk minerals; milk fat, lactose (Fingerprint region)

3.60 and 3.00 for *B. cereus* and *P. aeruginosa*, respectively. These findings indicated that the presence of *B. cereus* in the co-culture interfered with the growth of *P. aeruginosa* resulting in a lower final mean log TVC of 8.19 at 24 h when compared to 9.02 in the mono-cultures.

FT-IR Microspectroscopic Analysis for Milk Spoilage

FT-IR Reflectance Spectra

Vibrational micro-spectroscopic techniques are rapidly emerging as effective tools and have become more popular in studying biological samples. These techniques are used to investigate the vibrational modes of the molecules and provide spectra that are unique to the molecular properties and chemical

nature of the species (Singh *et al.*, 2012). Grewal *et al.* (2017) demonstrated the FT-IR spectra of the functional group of the biochemical compositions of milk, as shown in supplemental Table 1. Figure 2 displays the representative FT-IR microspectroscopy at 480 min (8 h) of incubation. The FT-IR spectra of the 2 distinct bacterial species in the mono-cultures and co-culture exhibited only some differences between the spectra. The representative FT-IR spectra revealed similar characteristics and were located in 4 regions: lipids (3,000-2,800 cm⁻¹), ester bond of fatty acids (1,800- 1,700 cm⁻¹), proteins (1,700-1,500 cm⁻¹), and carbohydrates (1,200-900 cm⁻¹) in all the milk samples. Accordingly, for the FT-IR spectra measurement, the weak absorbance <0.35 units or high absorbance >1.2 units were

ignored for spectral pre-processing because they are of poor spectral quality due to the absorbance <0.35 units which might show a very flat peak and the intensity was too low for interpretation, while the absorbance >1.2 units exhibited saturation of the amide I band and may result in misinterpretation.



Figure 1. *B. cereus* and *P. aeruginosa* showed the differences in morphology of the colonies from the co-culture on tryptic soy agar at 37°C after 20 h incubation

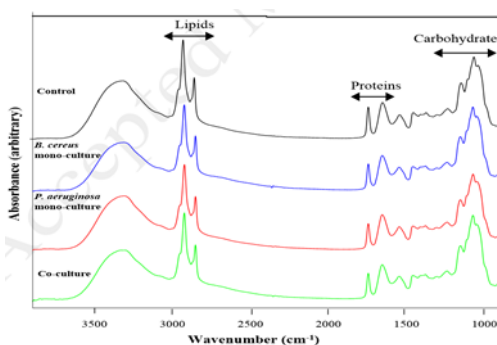


Figure 2. FT-IR spectra of bacterial-free milk control and milk samples inoculated with *B. cereus* and *P. aeruginosa* in mono-cultures and co-culture of *B. cereus* and *P. aeruginosa* at 480 min (8h). Spectra features could be observed

Principal Component Analysis

FT-IR microspectroscopy was used to analyse a significant change in the metabolic fingerprints of the milk compositions after inoculating with mono-cultures of *B. cereus* or *P. aeruginosa* and the co-culture of these particular bacteria. Graphic inspection of the spectra obtained from PCA analysis of the *B. cereus* in mono-culture revealed the differences between the FT-IR spectra cluster analysis and the PCA results. The results of the PCA analysis of the spectra for the bacterial-free control group in the region between positive and negative are illustrated in Figure 3(a). The spectra of samples incubated at the 360 min time point were different from the control group, the 480 min group tended to spread towards the negative left-hand side, while then the 960 min group were upward rising.

PCA analysis was constructed by using the second derivative, normalising spectra with EMSC, over the spectral regions between 3,000-2,800 cm^{-1} and 1,800-900 cm^{-1} . The PCA separated from each group corresponded to the results of the loading (Figure 3(b)). The typical second loading plot separated the clusters along PC1 which can be explained by distinct PC1 loadings in the region of 3,000-2,800 cm^{-1} . These regions were assigned to the stretching mode of CH_2 and CH_3 (2,957, 2,923, and 2,852 cm^{-1}) of milk lipid (Figure 3(c)). These results indicated the dramatic increase proportionally in milk lipid together with the time of incubation increasing when compared to the no bacteria control. These results were similar to the wavelength number of 1,745 cm^{-1} which represented the significant increase in milk fatty acids (Figure 3(d)). In addition, the present study also observed a slight change in the amide I band (wavelength number 1,652 cm^{-1}) of the α -helix structure of the protein at 960 min (16 h) of incubation.

Figure 4(a) reveals representatives of the PCA results retrieved from the second derivative analysis at different time points in *P. aeruginosa* grown singly at 360, 420, and 720 min. The findings showed that the no bacteria control was well-separated from the *P. aeruginosa* mono-culture with PC1 and PC2

values of 49% and 19%, respectively. Figure 4(b) depicts that the loading plot to classify a group of treatment corresponded to the PCA. The typical second loading plot separation of clusters along PC1 can be explained by distinct PC1 loadings in the region of 3,000-2,800 cm^{-1} and 1,745 cm^{-1} . These regions are represented by the stretching mode of CH_2 and CH_3 (2,960, 2,923, and 2,852 cm^{-1}) of milk lipid (Figure 4(c)) and the ester bond of fatty acids (1,745 cm^{-1}) (Figure 4(d)).

The metabolic fingerprints of *P. aeruginosa* in the mono-culture illustrates dominant peaks at the lipid and ester bond of fatty acid regions at 360, 420, and 720 min of incubation. The results showed that there was significant change in the lipid compositions (wavelength numbers of 2,960, 2,923, and 2,852 cm^{-1}) when compared to the bacterial free control (Figure 4(c)). A dramatic change was also observed in the ester bond of fatty acid compositions at wavelength number of 1,745 cm^{-1} . These

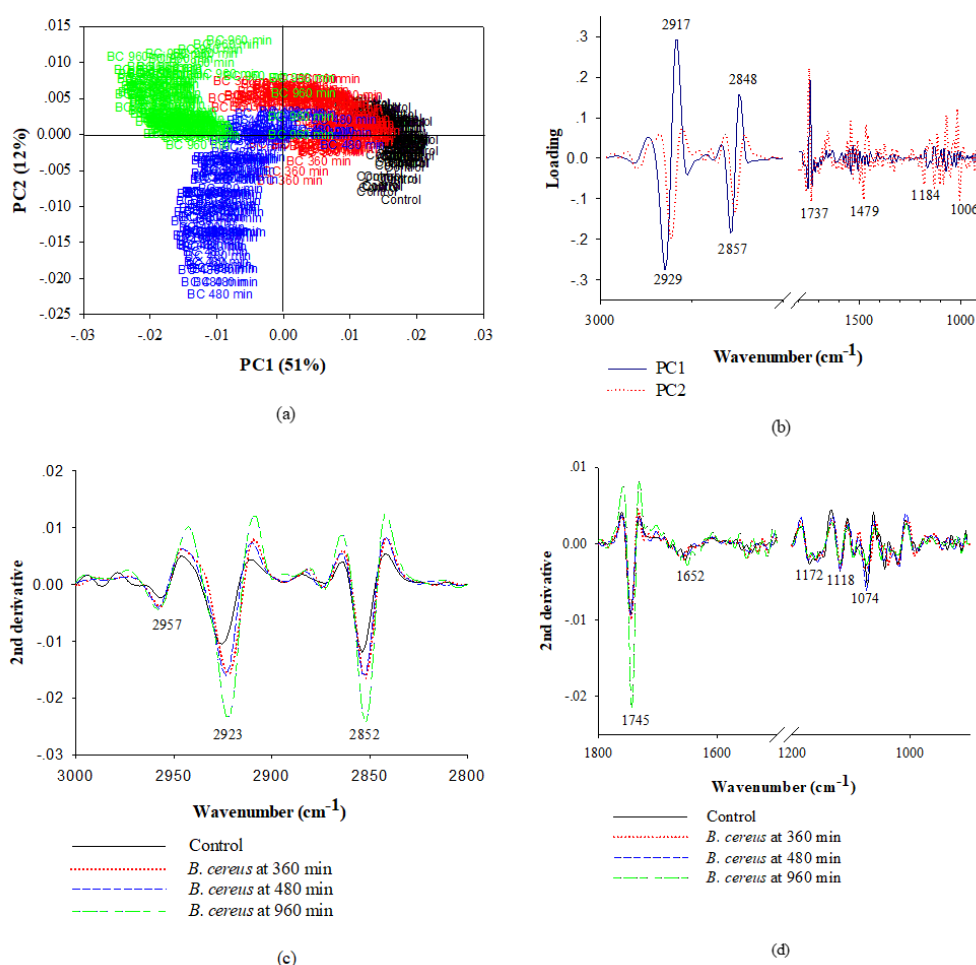


Figure 3. PCA results of bacterial-free milk control and *B. cereus* in mono-culture at 360, 480, and 960 min of the 1st (PC1) and 2nd (PC2). (a) score scatter plot, (b) the loading plot, (c) the representative 2nd derivative transformation spectra (3,000-2,800 cm^{-1}), (d) the representative 2nd derivative transformation spectra (1,800-900 cm^{-1})

findings are similar to the *B. cereus* grown singly in the UHT milk.

Figure 5(a) demonstrates representatives of the PCA results retrieved from the second derivative analysis at different time points in bacterial free control and *P. aeruginosa* grown together with *B. cereus* at 150, 360, and 1,440 min of incubation. The results showed that the no bacterial control was separated from the co-culture with PC1 and PC2 values of 35% and 28%, respectively. The distinct separations

by PC1 and PC2 were observed in the regions of 3,000–2,800 cm^{-1} (Figure 5(b)). In a difference from the mono-cultures, gradual increases in milk lipid at wavelength numbers of 2,922 and 2,852 cm^{-1} were observed at 150 and 360 min of incubation, whilst there was no significant change in these wavelength numbers at 1,440 min (24 h) of incubation (Figure 5(c)). Furthermore, at the wavelength number representing the ester bond of fatty acids (1,752 cm^{-1}), the significant increase in fatty acids was observed

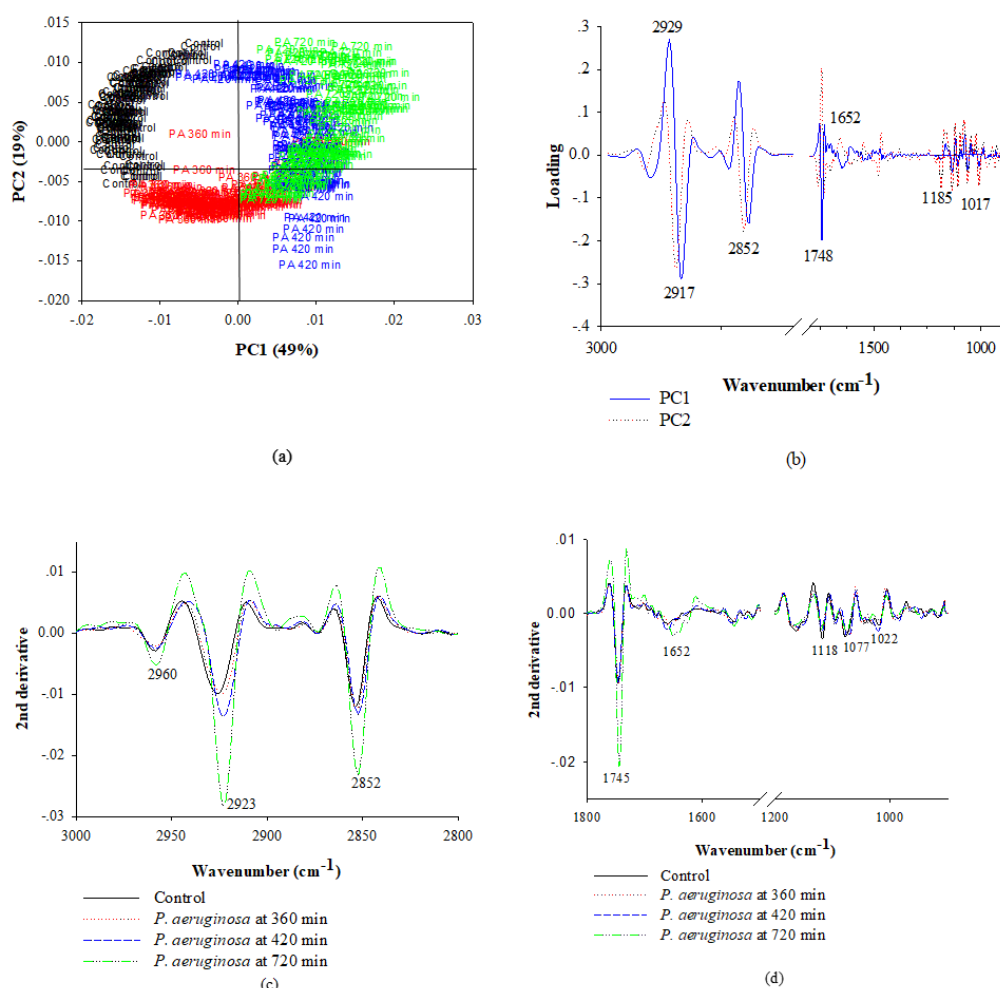


Figure 4. PCA results of bacterial-free control and *P. aeruginosa* in mono-culture at 360, 420 and 720 min of the 1st (PC1) and 2nd (PC2). (a) score scatter plot, (b) the loading plot, (c) the representative 2nd derivative transformation spectra (3,000–2,800 cm^{-1}), (d) the representative 2nd derivative transformation spectra (1,800–900 cm^{-1})

at 150 min of incubation and the reductions were then seen after the incubation time increased (360 min) and were similar to the control after 24 h of incubation (Figure 5(c)).

Quantification Model of Bacteria in Milk Spoilage using the PLS Analysis

The PLS analysis was used to predict the feasibility of FT-IR for quantification of the TVC of *B. cereus* and *P. aeruginosa* either grown singly or together in milk. A total of 15,250 FT-IR spectra was selected for model

calibration and calculated for a coefficient of determination (R^2) (a representation of accuracy of the model fit) and root mean squares error for the cross-validation (RMSECV) (an indication of the modelling error), respectively. The present study discovered that the PLS analysis for the mono-cultures and co-culture gave a good TVC prediction. The TVC prediction model of *B. cereus* in mono-culture provided R^2 of 0.972 and RMSECV of 0.24 (Figure 6(a)), and in the co-culture it was 0.982 and 0.16 for R^2

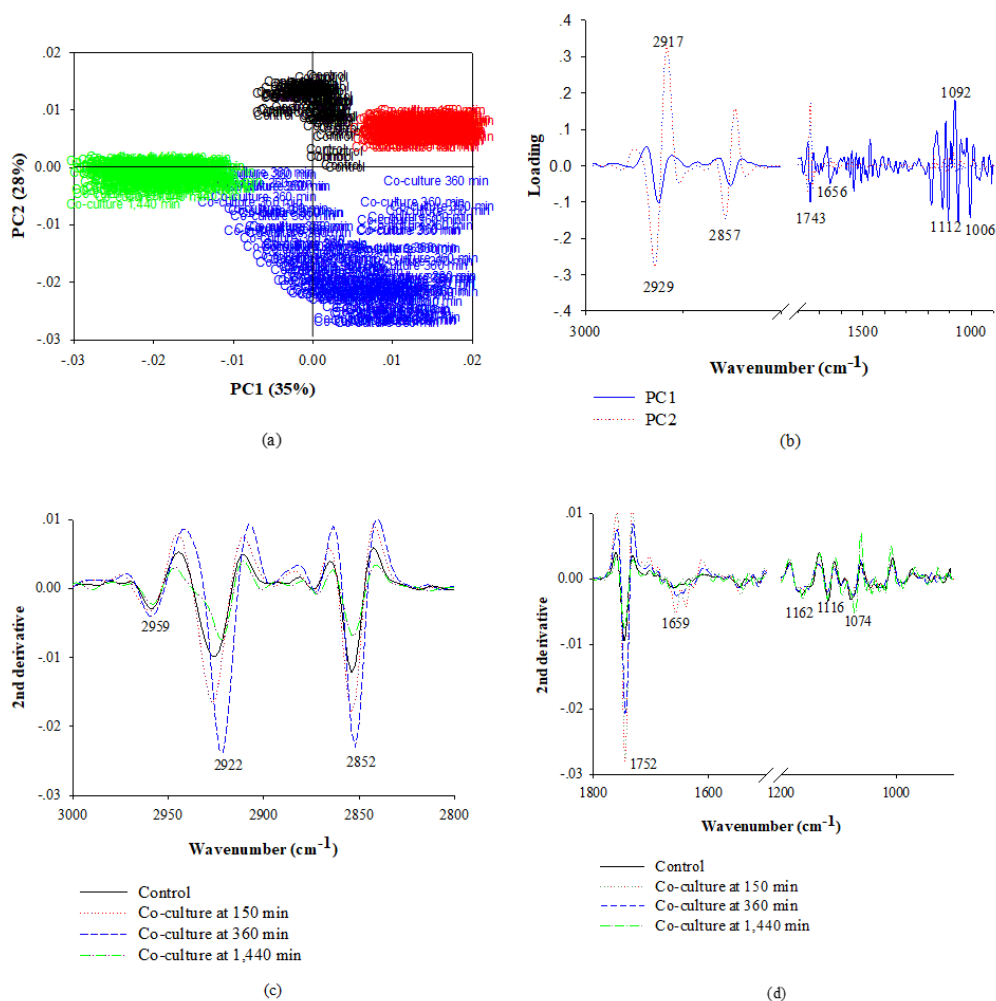


Figure 5. PCA results of bacterial-free control, *B. cereus* and *P. aeruginosa* in co-culture at 150, 360, and 1,440 min of the 1st (PC1) and 2nd (PC2). (a) score scatter plot, (b) the loading plot, (c) the representative 2nd derivative transformation spectra (3,000-2,800 cm^{-1}), (d) the representative 2nd derivative transformation spectra (1,800-900 cm^{-1})

and RMSECV, respectively (Figure 6(b)). Likewise, the model for *P. aeruginosa* in mono-culture gave R^2 of 0.971 and RMSECV of 0.35 (Figure 7(a)), while in the co-culture it provided R^2 of 0.981 and RMSECV of 0.22 (Figure 7(b)). These results are also summarised in Table 2. The predicted TVC by FT-IR through the PLS analysis provided very high accuracy of 94-99% for the mono-cultures and 95-99% for the co-culture, compared to the standard TVC (Table 3). Therefore, FT-IR can be exploitable for the detection of the bacterial load in milk and quantification of the *B. cereus* and *P. aeruginosa* mediated spoilage of milk.

Discussion

FT-IR microspectroscopy has been extensively used to analyse a wide range of metabolites present in food and produced by microorganisms. This technique was able to detect a significant amount of biochemical fingerprint data. Nicolaou and Goodacre (2008), the first researchers who used FT-IR techniques and chemometrics in pasteurized milk; they allowed it to perish naturally during storage at 15°C, and showed that metabolic fingerprinting using FT-IR spectroscopy had a very good application potential in dairy production as a rapid and non-invasive

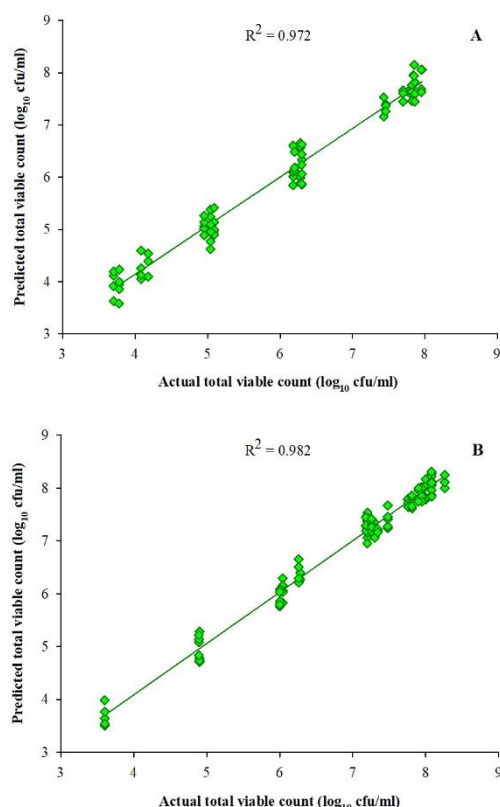


Figure 6. PLS calibration models for the relationship between the actual and predicted total viable count for *B. cereus*, (A) mono-culture and (B) co-culture. The FT-IR was predicted using the second derivative

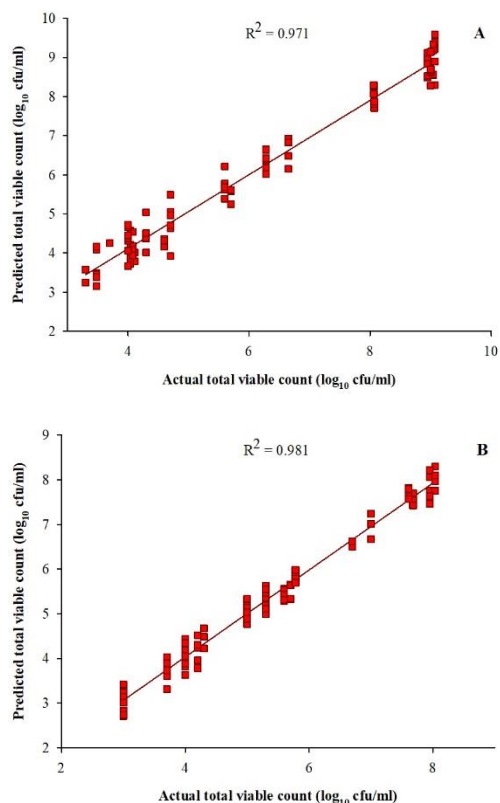


Figure 7. PLS calibration models for the relationship between the actual and predicted total viable count for *P. aeruginosa*, (A) mono-culture and (B) co-culture. The FT-IR was predicted using the second derivative

technique of bacterial spoilage detection. Furthermore, the assessment of the growth interactions between *Staphylococcus aureus* and *Lactococcus lactis* subsp. *cremoris* in milk has been also used successfully in the analysis of the rapid discrimination and enumeration (Nicolaou *et al.*, 2011). So, this study utilized FI-IR for rapid detection of the bacterial load of *B. cereus* and *P. aeruginosa* and for quantitative estimations of the TVC of these particular bacteria in milk.

PLS was previously used for prediction of fat, crude protein, lactose, and urea after pre-processing IR data. PLS provided excellent results for the prediction of crude

protein, lactose, and urea content ($R^2 > 0.99$, 0.98, and 0.86, respectively) in raw and homogenized milk using micro-attenuated total reflection IR spectroscopy (Aernouts *et al.*, 2011). In our study, implementation of PLS provided very high correlations between the actual TVC and predicted TVC with R^2 of 0.972 and 0.982 for the mono-culture and co-culture of *B. cereus*, respectively, whereas, a high correlation was also seen in *P. aeruginosa* in the mono-culture and co-culture with R^2 of 0.971 and 0.981, respectively. Interestingly, the PLS analysis provided a high accuracy of 94-95 % for the prediction of the TVC in milk.

Table 2. Results of PLS carried out on FT-IR spectra ($\sim 3,000$ - $2,800\text{ cm}^{-1}$ and $1,800$ - 900 cm^{-1}) and microbial data obtained by total viable counts

Model	Calibration samples	RMSECV	R^2	RPD	Bias
<i>B. cereus</i> (mono-culture)	78	0.24	0.972	5.98	-0.00457
<i>P. aeruginosa</i> (mono-culture)	90	0.35	0.971	5.84	-0.0137
<i>B. cereus</i> (co-culture)	113	0.16	0.982	7.35	-0.000646
<i>P. aeruginosa</i> (co-culture)	97	0.22	0.981	7.2	0.000309

R^2 = Coefficient of determination.

RPD = Residual prediction deviation.

RMSECV = Root mean square error of cross-validation.

Bias = mean value of deviation or systematic error.

Table 3. The PLS model obtained from 2 data pre-processes were used to compare the actual TVC and the predicted (FT-IR predicted value) for *B. cereus* and *P. aeruginosa* concentrations in the mono and co-cultures

Model	Mean actual Total viable counts	Mean of predicted Total viable counts $\pm$ SD	% Accuracy
<i>B. cereus</i> (mono-culture)	3.74	3.939 ± 0.228	94.68
	4.13	4.290 ± 0.218	96.12
	5.0	5.021 ± 0.152	99.58
	6.19	6.190 ± 0.231	99.99
	7.79	7.630 ± 0.109	97.95
<i>B. cereus</i> (co-culture)	3.60	3.687 ± 0.194	97.59
	4.90	4.970 ± 0.236	98.57
	6.02	5.989 ± 0.173	99.49
	6.27	6.352 ± 0.156	98.69
	7.20	7.253 ± 0.167	99.26
<i>P. aeruginosa</i> (mono-culture)	3.39	3.506 ± 0.326	96.59
	4.06	4.121 ± 0.289	98.49
	5.65	5.643 ± 0.286	99.88
	6.47	6.480 ± 0.337	99.83
<i>P. aeruginosa</i> (co-culture)	8.06	7.970 ± 0.237	98.89
	3.35	3.193 ± 0.304	95.32
	4.00	4.014 ± 0.224	99.65
	5.45	5.446 ± 0.162	99.92
	6.85	6.780 ± 0.288	98.98
	7.65	7.627 ± 0.133	99.70

Conclusions

FI-IR microspectroscopy is a physico-chemical analytical technique for analysis of the functional group changes in biological specimens. The present study applied this technique to detect *B. cereus* and *P. aeruginosa* in the spoilage of milk and utilised FT-IR spectra coupled with PLS analysis to construct a model to predict the TVC in milk spoilage. The FT-IR technique is able to detect the changes in milk lipids and fatty acids in milk inoculated with *B. cereus* and *P. aeruginosa*. PLS analysis is capable of predicting the TVCs of *B. cereus* and *P. aeruginosa* with an accuracy rate as high as 94-99%. The findings from this study support the exploitation of FT-IR and PLS analysis for rapid detection and enumeration of *B. cereus* and *P. aeruginosa* spoilage in milk.

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References

- Aernouts, B., Polshin, E., Saeys, W., and Lammertyn, J. (2011). Mid-infrared spectrometry of milk for dairy metabolomics: A comparison of two sampling techniques and effect of homogenization. *Anal. Chim. Acta.*, 705(1-2):88-97.
- Ellis, D.I., Broadhurst, D., Kell, D.B., Rowland, J.J., and Goodacre, R. (2002). Rapid and quantitative detection of the microbial spoilage of meat by Fourier transform infrared spectroscopy and machine learning. *Appl. Environ. Microb.*, 68(6):2,822-2,828.
- Eumkeb, G., Sakdarat, S., and Siri Wong, S. (2010). Reversing β -lactam antibiotic resistance of *Staphylococcus aureus* with galangin from *Alpinia officinarum* Hance and synergism with ceftazidime. *Phytomedicine*, 18(1):40-45.
- Grewal, M.K., Chandrapala, J., Donkor, O., Apostolopoulos, V., Stojanovska, L., and Vasiljevic, T. (2017). Fourier transform infrared spectroscopy analysis of physicochemical changes in UHT milk during accelerated storage. *Int. Dairy J.*, 66:99-107.
- Junhom, C., Weerapreeyakul, N., Tanthanuch, W., and Thumanu, K. (2017). Partial least squares regression and Fourier transform infrared (FTIR) microspectroscopy for prediction of resistance in hepatocellular carcinoma HepG2 cells. *Exp. Cell Res.*, 351(1):82-90.
- Ledenbach, L.H. and Marshall, R.T. (2009). Microbiological spoilage of dairy products. In: *Compendium of the Microbiological Spoilage of Foods and Beverages*. Sperber, W.H. and Doyle, M.P., (eds). Springer, New York, NY, USA, p. 41-67.
- Lianou, A., Malavazos, C., Triantafyllou, I., Nychas, G.-J.E., and Panagou, E.Z. (2017). Rapid assessment of the microbiological quality of pasteurized vanilla cream by means of Fourier transform infrared spectroscopy in tandem with support vector machine analysis. *Food Anal. Method.*, 11(3):840-847
- Lin, M., Al-Holy, M., Chang, S.S., Huang, Y., Cavinato, A.G., Kang, D.H., and Rasco, B.A. (2005). Rapid discrimination of *Alicyclobacillus* strains in apple juice by Fourier transform infrared spectroscopy. *Int. J. Food Microbiol.*, 105(3):369-376.
- Liu, I.X., Durham, D.G., and Richards, R.M.E. (2000). Baicalin synergy with β -lactam antibiotics against methicillin-resistant *Staphylococcus aureus* and other β -lactam-resistant strains of *S. aureus*. *J. Pharm. Pharmacol.*, 52(3):361-366.
- Martínez, N., Martín, M.C., Herrero, A., Fernández, M., Alvarez, M.A., and Ladero, V. (2011). qPCR as a powerful tool for microbial food spoilage quantification: Significance for food quality. *Trends Food Sci. Tech.*, 22(7):367-376.
- Nicolaou, N. and Goodacre, R. (2008). Rapid and quantitative detection of the microbial spoilage in milk using Fourier transform infrared spectroscopy and chemometrics. *Analyst*, 133(10):1,424-1,431.
- Nicolaou, N., Xu, Y., and Goodacre, R. (2011). Fourier transform infrared and Raman spectroscopies for the rapid detection, enumeration, and growth interaction of the bacteria *Staphylococcus aureus* and *Lactococcus lactis* ssp. *cremoris* in milk. *Anal. Chem.*, 83(14):5,681-5,687.
- Nollet, L. and Toldra, F. (2010). *Handbook of Dairy Foods Analysis*. CRC Press, Boca Raton, FL, USA, 918p.
- Oliver, S.P., Jayarao, B.M., and Almeida, R.A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathog. Dis.*, 2(2):115-129.
- Osawa, M., Chalmers, J., and Griffiths, P. (2002). *Handbook of Vibrational Spectroscopy*. Wiley, Chichester, UK, 785p.
- Panagou, E.Z., Mohareb, F.R., Argyri, A.A., Bessant, C.M., and Nychas, G.J. (2011). A comparison of artificial neural networks and partial least squares modelling for the rapid detection of the microbial spoilage of beef fillets based on Fourier transform infrared spectral fingerprints. *Food Microbiol.*, 28(4):782-790.

- Papadopoulou, O., Panagou, E.Z., Tassou, C.C., and Nychas, G.J.E. (2011). Contribution of Fourier transform infrared (FTIR) spectroscopy data on the quantitative determination of minced pork meat spoilage. *Food Res. Int.*, 44(10):3,264-3,271.
- Robinson, R. (2002). *Dairy microbiology handbook: the microbiology of milk and milk products*. 3rd ed. Wiley, Hoboken, NJ, USA, 784p.
- Rysstad, G. and Kolstad, J. (2006). Extended shelf life milk - advances in technology. *Int. J. Dairy Technol.*, 59(2):85-96.
- Santos, P.M., Pereira-Filho, E.R., and Rodriguez-Saona, L.E. (2013). Rapid detection and quantification of milk adulteration using infrared microspectroscopy and chemometrics analysis. *Food Chem.*, 138(1):19-24.
- Schmidt, V.S.J., Kaufmann, V., Kulozik, U., Scherer, S., and Wenning, M. (2012). Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. *Int. J. Food Microbiol.*, 154(1-2):1-9.
- Singh, B., Gautam, R., Kumar, S., Kumar, V.B., Nongthomba, U., Nandi, D., Mukherjee, G., Santosh, V., Somasundaram, K., and Umapathy, S. (2012). Application of vibrational microspectroscopy to biology and medicine. *Curr. Sci. India*, 102(2):232-244.
- Wang, J., Yue, T., Yuan, Y., Lu, X., Shin, J.H., and Rasco, B. (2011). Discrimination of *Alicyclobacillus* strains using nitrocellulose membrane filter and attenuated total reflectance Fourier transform infrared spectroscopy. *J. Food Sci.*, 76(2):M137-142.