Rapid and quantitative detection of the microbial spoilage in milk using Fourier transform infrared spectroscopy and chemometrics

Nicoletta Nicolaou and Royston Goodacre*

Received 14th March 2008, Accepted 21st May 2008
First published as an Advance Article on the web 11th July 2008
DOI: 10.1039/b804439b

Microbiological safety plays a very significant part in the quality control of milk and dairy products worldwide. Current methods used in the detection and enumeration of spoilage bacteria in pasteurized milk in the dairy industry, although accurate and sensitive, are time-consuming. FT-IR spectroscopy is a metabolic fingerprinting technique that can potentially be used to deliver results with the same accuracy and sensitivity, within minutes after minimal sample preparation. We tested this hypothesis using attenuated total reflectance (ATR), and high throughput (HT) FT-IR techniques. Three main types of pasteurized milk – whole, semi-skimmed and skimmed – were used and milk was allowed to spoil naturally by incubation at 15 °C. Samples for FT-IR were obtained at frequent, fixed time intervals and pH and total viable counts were also recorded. Multivariate statistical methods, including principal components-discriminant function analysis and partial least squares regression (PLSR), were then used to investigate the relationship between metabolic fingerprints and the total viable counts. FT-IR ATR data for all milks showed reasonable results for bacterial loads above 10^5 cfu ml^{-1}. By contrast, FT-IR HT provided more accurate results for lower viable bacterial counts down to 10^3 cfu ml^{-1} for whole milk and, 4×10^2 cfu ml^{-1} for semi-skimmed and skimmed milk. Using FT-IR with PLSR we were able to acquire a metabolic fingerprint rapidly and quantify the microbial load of milk samples accurately, with very little sample preparation. We believe that metabolic fingerprinting using FT-IR has very good potential for future use in the dairy industry as a rapid method of detection and enumeration.

Introduction

Milk is an important constituent of our diet containing a variety of nutrients, and it is essential for good bone development in infants and children. It is therefore not surprising that a big proportion of the world's population uses milk on a daily basis. Quality control of milk and milk products is therefore of paramount importance. Recent outbreaks of foodborne illnesses associated with milk and dairy product consumption have been found to be contaminated with pathogenic microorganisms such as *Listeria* spp., *Salmonella* spp., *Campylobacter jejuni* and *Yersinia enterolytica*.[1] Microbial analysis of milk and dairy products therefore has a critical role to play in the quality evaluation of these products, promoting public health safety.

A dairy product is described as spoiled when organoleptic changes within it make it unacceptable to the consumer. These organoleptic taints, among others, cause defects in appearance and unpleasant odours and flavours. These are characteristics which make food unacceptable for human consumption.[2–5] It is generally acceptable that organoleptic changes are a result of microbiological spoilage and that the compounds responsible for these changes are the various metabolites produced by the metabolic activity of the microorganisms.[3] The type of change produced varies according to the species of the microorganisms present in milk, the chemical composition of milk and the physical environment under which it is stored.

Milk is an ideal medium for microbial growth because of its high water content and the large variety of available nutrients which can be used by microorganisms as an energy source.[6] The main components of whole milk are 87.3% water, 4.8% carbohydrates (mainly lactose), 3.7% fat, 3.2% proteins, and 1% non-protein nitrogenous compounds, minerals and vitamins.[6] Furthermore, its pH is almost neutral ranging from 6.5 to 6.7 making it an ideal growth environment for bacteria.[7] Temperature also has a part to play as spoilage microorganisms can become active at temperatures between 2 and 30 °C. Within this temperature range the growth of one particular bacterial species, the Gram negative *Pseudomonas*, is dramatic. The other major members of the spoilage flora on pasteurized milk include the endospore-forming bacteria of the *Bacillus* genera, and other Gram positive rods and cocci such as *Lactobacillus*, *Corynebacterium* and *Lactococcus* species.[8,9]

The application of the Hazard Analysis and Critical Control Point (HACCP) system in the dairy industry, in order to maintain high quality levels during manufacturing and production of foods for safe consumption, has increased the requirements for rapid and more automated microbiological techniques.[10] In the recent past, several methods and instruments have been developed for the identification, detection, enumeration and the characterisation of microorganisms in milk and dairy

School of Chemistry and Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester, UK, M1 7DN. E-mail: roy.goodacre@manchester.ac.uk

products.[7,11–13] However, none of these methods have so far been ideal. In the absence of a simultaneously rapid, accurate and sensitive method, the dairy industry currently relies on classical microbiological plate counting techniques and other methods for the detection and enumeration of spoilage bacteria in pasteurized milk which typically take 1–2 days; but may take longer depending on the growth of the organism. The dairy industry therefore requires a method for the detection and enumeration of bacteria in dairy samples which will be accurate and sensitive, must be able to measure viable bacterial numbers and provide results within 2–3 h so that appropriate measures within HACCP can be made.[5,14]

Fourier transform infrared (FT-IR) spectroscopy is a metabolic fingerprinting technique that can potentially be used to reduce this time significantly, by measuring the biochemical fingerprints produced through the metabolic activity of the viable microorganisms in milk and delivering results within minutes after very minimal sample preparation. Its validity has also recently shown in the detection of spoilage in meat.[5,14] FT-IR spectroscopy is based on the principle of detecting the specific frequencies of energy absorbed by a molecule within a functional group once it has been irradiated with IR radiation (usually in the mid IR range; 4000–600 cm^{-1}) and has been excited to a higher energy level, usually reaching its first vibrational excited state. As each molecule only absorbs energy when the frequency of the infrared energy directed on the molecule is equal to the frequency of one of the fundamental vibration modes of that molecule, the end result of this absorbance is a highly specific infrared spectrum.[15–19] The main advantage of this technique is that it is very fast as a spectrum can be obtained within a few seconds after minimum sample preparation. Furthermore, it is a simple technique to use, it has high sensitivity, and it is inexpensive to operate. This technique in combination with appropriate multivariate statistical methods, including partial least square regression (PLSR), may therefore be an attractive solution for the detection and enumeration of bacteria in dairy samples.

Therefore, the aim of this study was to investigate the ability of FT-IR spectroscopy to quantify the bacterial contamination of the three types (*viz.* whole, semi-skimmed and skimmed) of pasteurized cow's milk accurately, sampling using attenuated total reflectance (ATR), and a high throughput (HT) transmission-based technique with PLSR analysis.

Materials and methods

Sample preparation

Three cartons of milk with the same use-by-date, each of a different type of milk (whole, semi-skimmed and skimmed milk) were purchased from a national retail outlet. The milk was then separately poured into sterilized flasks (1 L) and was placed in a rotation incubator at 15 °C and 200 revolutions per minute (rpm). Samples were then taken at eight-hourly intervals for 104 h. Once samples were obtained they were mixed for 1 min and then the organoleptic changes and pH of each type of milk were recorded and the total viable bacterial counts were also determined using a classical microbiological plating method (see below). Fifteen millilitres of the milk samples were divided to 1 ml volumes and preserved at −80 °C. Six of these aliquots were used for ATR FT-IR and HT FT-IR analysis.

Total viable counts (TVCs)

TVCs were measured according to the national standard method.[20] Using peptone saline diluent (containing 1.0 g peptone, 8.5 g sodium chloride in 1 L distilled water) serial dilutions were undertaken. Each dilution was mixed for 1 min and 1 ml was inoculated into three Petri dishes. Subsequently, milk plate count agar (containing 2.5 g yeast extract, 5.0 g tryptone, 1.0 g glucose, 1.0 g skimmed milk powder, 15.0 g agar in 1 L distilled water) was added, mixed with the inoculum, and incubated aerobically at 30 °C for 72 h. The plate colonies were then counted and the total viable count per millilitre was calculated. The number of colonies per plate was only taken into account when it was between 30 and 300. The number of viable microorganisms per millilitre of sample was calculated using the equation:

$$N = \frac{\sum c}{(n_1 + 0.1n_2)d}$$

were $\sum c$ is the sum of the colonies counted from all plates (between 30 and 300 colonies), n_1 is the number of plates counted at the first dilution, n_2 is the number of plates at the second dilution and d is the dilution from which the first counts were obtained (*i.e.* least dilute).

Attenuated total reflectance (ATR) FT-IR spectroscopy

FT-IR analysis was undertaken using a ZnSe Gateway ATR Horizontal 6 Reflection accessory (Specac Ltd, London) on a Bruker Equinox 55 infrared spectrometer equipped with a DTGS (deuterated triglycine sulfate) detector (Bruker Ltd, Coventry, UK). For ATR the evanescent wave allows penetration into the surface above the crystal and this was calculated to be 0.98 μm at 1500 cm^{-1} (arising from the N–H vibration of the Amide II band) and 0.45 μm at 2900 cm^{-1} (from the centre of fatty acid CH_x stretches).[15]

Samples were defrosted on ice, one sample at a time, and were then mixed for 1 min. Aliquots of 800 μl were then taken from the sample and placed in intimate contact with the ZnSe crystal and the sample's spectrum was obtained. In total, six replicates were taken from each time point. Between samples the crystal surface was first cleaned with distilled water, then with analytical grade acetone and again with distilled water and dried with a soft cloth and left for approx. 5 min to air dry. Prior to making any sample measurements, reference spectra were acquired from the clean blank crystal. All the spectra were collected within the wavenumber range of 4000–600 cm^{-1} with a resolution of 8 cm^{-1} and in order to improve the signal-to-noise ratio 64 scans were co-added and averaged. In total, 252 spectra were collected for every type of milk in the series of three experiments and the collection time for each spectrum was approximately 30 s.[5,14] Spectral acquisition was achieved using an IBM compatible computer which controlled the spectrometer.

High throughput transmission (HT) FT-IR spectroscopy

FT-IR analysis was undertaken using a ZnSe plate on the same Bruker Equinox 55 infrared spectrometer equipped with a motorised microplate module HTS-XT™ [21] utilising a DTGS detector (Bruker Ltd). Samples were defrosted on ice one sample at a time, then mixed for 1 min, and then 5 µl from each sample was placed onto a ZnSe plate (which can hold 96 samples). The ZnSe plates were then oven dried at 50 °C for 30 min. In total, six replicates were taken from each sample and were placed randomly onto the ZnSe plates. Again the wavelength range collected was 4000–600 cm^{-1}, with a resolution of 8 cm^{-1}, and 64 scans were co-added and averaged. As also detailed above, a total of 252 spectra were collected and each spectrum took 30 s to acquire.

Data analysis

Pre-processing. For FT-IR, ASCII data were exported from the Opus software used to control the FT-IR instrument and imported into Matlab version 7 (The Mathworks, Inc Matick, MA). To minimize problems arising from baseline shifts Matlab was used to correct the CO_2 vibrations seen in the HT FT-IR measurements by removing the CO_2 peaks at 2403–2272 and 683–656 cm^{-1} and filling them with a trend. Both HT and ATR FT-IR spectra then were scaled by using extended multiplicative scatter correction (EMSC);[22] we also tried the first and second Savitzky–Golay derivatives with five-point smoothing but this was no better than EMSC. Prior to PCA and PLSR, as is normal practice, the data were mean-centred. At this stage we visually inspected the spectra and checked for outliers using principal components analysis (see below) and these were subsequently removed from further analyses. These samples included a single biological replicate from the 24 h time point from the HT spectra collected from whole milk, all the biological replicates from the 104 h time point from the HT spectra collected from skimmed milk and all three biological replicates from the 64 h time point from the HT spectra collected from whole, semi-skimmed and skimmed milk; this suggested that this later time point was due to a sampling error, as the sample was collected in the middle of the night!

To investigate the relationship between the FT-IR spectra and the total viable count, multivariate statistical methods were used including cluster analysis and partial least squares regression (PLSR). These multivariate analysis methods were performed in PyChem version 3, details of which are available from Jarvis *et al.*[23] and the programme is also available on the web (http://pychem.sf.net/).

Cluster analysis. Cluster analysis was carried out in two steps. Firstly, principal component analysis (PCA) was used.[24,25] PCA is a well established analysis technique which works by finding the correlation between a set of variables and then creating a new set of uncorrelated variables named principal components (PCs). Subsequently, discriminant function analysis (DFA; also known as canonical variate analysis) was used.[26] DFA was programmed to discriminate data based on the first few PCs with the prior knowledge of which spectra are biological replicates.

Validation of the PC-DFA model was performed for the HT FT-IR data and for the ATR FT-IR data, for every type of milk (whole, semi-skimmed and skimmed) as detailed in ref. 27. In brief, the data were divided into two subsets: the training set and the test set. The training set consisted of the first two biological replicates (two groups at each time point), which were used to construct a PC-DFA model. After the PC-DFA model was constructed the test data of the third biological replicate was projected into PCA space and then the projected PCs were projected into DFA space. The model was considered valid if the test set data were projected coincident with the training data.

Finally, in order to inspect the tightness of the clusters 95% tolerance regions were constructed around the PC-DFA group means using the χ^2 confidence intervals using two degrees of freedom.[28]

Partial least squares regression (PLSR). For quantitative prediction of TVCs from the FT-IR spectra the multivariate supervised learning method of partial least squares regression was used[29] as detailed in refs 2, 30 and 31. The aim of supervised learning is to construct a model which correctly associates inputs with targets, where in the calibration phase both input and targets are already known. PLSR was calibrated with FT-IR data from the first two spoilage experiments (biological replicates) to predict the known $\log_{10}$ TVC values. During calibration these data were divided randomly into training data and cross-validation data, and the number of latent variables used in the model was the point at which the lowest RMS error in the validation data was seen. Once this model was constructed it was challenged with independent test set data from the third unseen spoilage experiment (biological replicate).

Results and discussion

pH, TVC and organoleptic changes

The pH levels during the 104 h of the three spoilage experiments are shown in Fig. 1. The initial mean pH was 6.72. After 104 h incubation at 15 °C the final mean pH was 7.07. In general, the pH showed mild fluctuation prior to 80 h and then increased significantly when the bacterial levels reached 2×10^7 cfu ml^{-1} (Table 1). Similar results were shown for semi-skimmed and skimmed milk were the initial mean pH was 6.72 and 6.74 and then increased to 7.11 and 7.10 respectively (data not shown). The results from these experiments suggest that the use of pH as

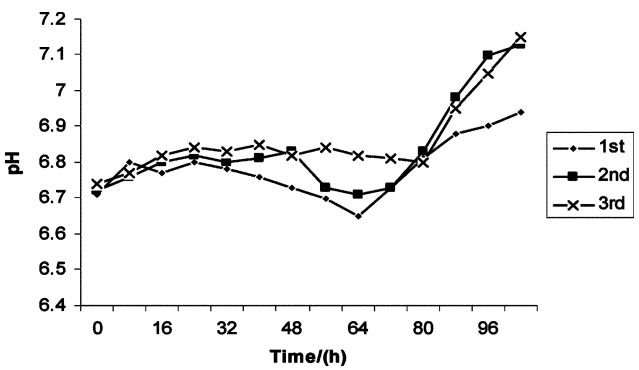

Fig. 1 Plot of the pH levels for whole milk observed through the series of three experiments.

Table 1 Mean $\log_{10}$ TVC of bacteria acquired from milk spoilage samples from three experiments for the three different types of milk

	Whole			Semi-skimmed			Skimmed		
Time/h	Mean $\log_{10}$ TVC	Negative SD	Positive SD	Mean $\log_{10}$ TVC	Negative SD	Positive SD	Mean $\log_{10}$ TVC	Negative SD	Positive SD
0	3.22	0.51	0.23	2.62	0.38	0.20	2.64	1.67	0.30
8	3.07	0.55	0.23	2.66	0.21	0.14	2.62	1.38	0.29
16	3.11	0.65	0.25	2.75	0.28	0.17	2.56	1.16	0.32
24	3.06	0.32	0.18	2.65	0.28	0.17	2.69	2.29	0.30
32	3.37	1.21	0.31	2.66	0.21	0.14	2.61	0.63	0.25
40	5.35	0.14	0.44	2.93	1.02	0.28	3.12	0.17	0.12
48	4.99	0.59	0.24	4.07	0.91	0.27	4.21	0.43	0.37
56	6.11	1.73	0.31	5.47	0.14	0.39	4.96	0.60	0.35
64	6.77	0.14	0.11	6.39	0.72	0.34	5.69	0.79	0.26
72	7.02	0.09	0.08	7.01	0.84	0.27	6.39	0.44	0.37
80	7.36	1.46	0.29	7.06	0.80	0.27	6.71	0.66	0.25
88	7.32	1.17	0.32	7.08	0.52	0.23	6.74	0.21	0.14
96	7.54	0.79	0.26	6.89	0.73	0.34	6.92	0.62	0.25
104	8.08	0.50	0.37	7.12	0.41	0.38	7.41	0.22	0.15

an indicator of remaining shelf life in the different types of milk would be insufficient.

The results of the spoilage experiment for whole milk are shown in Table 1. The initial mean $\log_{10}$ TVC was 3.22 which is a usual finding for fresh pasteurized whole milk. After 104 h of incubation at 15 °C the final $\log_{10}$ TVC increased to 8.08. An example of the increase in the growth of bacteria in the whole milk for the three replicate experiments is shown on Fig. 2. For semi-skimmed and skimmed milk the initial mean $\log_{10}$ TVC was between 2.62 and 2.64 and the final $\log_{10}$ TVC increased to 7.12 for semi-skimmed and 7.41 for skimmed milk. Both the initial and the final $\log_{10}$ TVC were lower for the semi-skimmed and skimmed milk than whole milk. This finding is different from previous research which found that the TVC for whole and skimmed milk during the spoilage did not have significant differences when they were stored at 5–8 °C,[32] and may be a consequence of the incubation temperature for our studies being 15 °C.

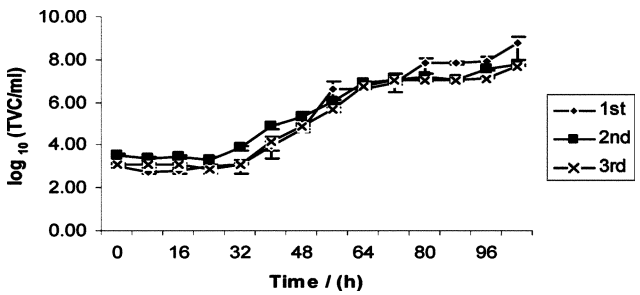

Fig. 2 Plot showing the $\log_{10}$ (total viable count per ml) against time for whole milk samples spoiled at 15 °C for 104 h. Averages of three replicate measurements for the three biological repeats are shown and error bars show the standard deviations.

In our experiments general organoleptic changes indicative of spoilage became apparent when the number of bacteria reached approximately 10^7 cfu ml^{-1} in all types of milk. Whilst of course these observations are personal and therefore subjective, we found that at this stage the milk started to smell bitter and cheesy. These organoleptic changes result from bacterial metabolism which produces a complex mixture of volatile esters, ketones, aldehydes, fatty acids, ammonia and amines, collectively comprising the off-odours detected. The different off-odours are most probably due to protease and lipase enzyme activities,[33–35] some well known spoilage effects caused by *Pseudomonas* species, which is one of the most common species in the bacterial population at 15 °C.[8,9] It has been shown that these enzymes are detectable when the bacterial counts reach a level of 10^6 cfu ml^{-1} or higher and are stable at high temperatures, surviving pasteurization (72 °C for 15 s).[33–35] Proteases can act directly on micellar casein resulting in its degradation and the liberation of bitter peptides.[6,34] When proteolysis is continued the degradation of lower molecular weight products such as ammonia and amines produces the putrid aroma and flavour.[6,33,34,36]

Lipolysis occurs when fatty acids are released from milk triglycerides. High molecular weight fatty acids produce a soapy flavour and unsaturated fatty acids are oxidised to ketones and aldehydes producing a different odour and flavour.[6,34,37] Fruity flavours and aroma mainly occur in pasteurized milk from the action of *P. fragi* and *P. fluorescens*, esterifying free fatty acids with ethanol, as a result of post-pasteurization contamination. Ethyl esters such as ethyl acetate, ethyl butanoate and ethyl lexanoate are responsible for the fruity aroma.[4]

FT-IR ATR spectroscopy

Representative FT-IR ATR spectra collected from whole milk and the identities of the main absorption bands relating to milk are shown in Fig. 3. Using simple visual inspection no obvious quantitative differences were observed between fresh milk at 0 h, and spoiled milk at 48 h and 104 h. Similar spectra were also obtained from semi-skimmed and skimmed milk (data not shown).

In order to detect any differences between the FT-IR ATR spectra, cluster analysis was employed, and the PC-DFA results for spoilage of whole-fat milk from FT-IR ATR are shown in Fig. 4. The spectra for the first 48 h group together in the same region on the bottom left of this figure, as evident from their overlapping 95% tolerance regions; the TVC during this period was between 10^3 and 9×10^4 cfu ml^{-1}. Spectra of samples incubated after the 48 h time point are clearly different from the earlier samples and tended to spread in both axes towards the right hand side of the figure and then upwards followed by

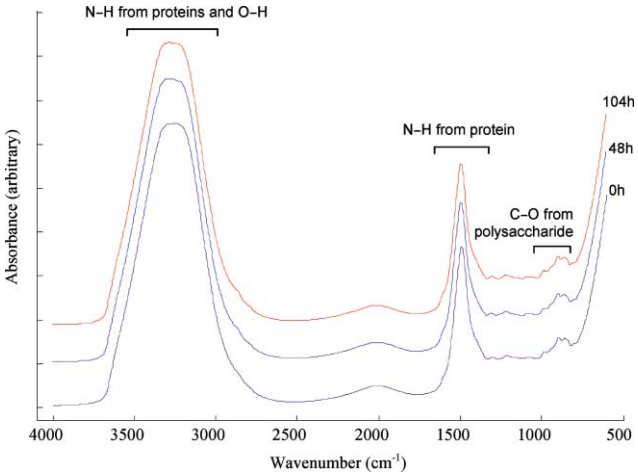

Fig. 3 FT-IR ATR spectra for whole milk at 0 h (purple), 48 h (blue), and 104 h (red).

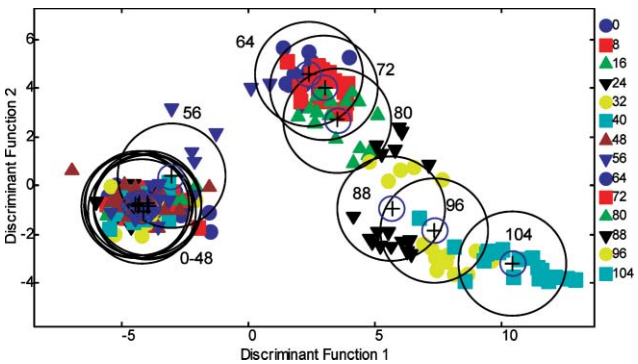

Fig. 4 PC-DFA plot of the ATR FT-IR spectra for the three repeat experiments of whole milk. PCs 1–20 (accounting for 96.54% of the total variance) were used by the DFA algorithm with *a priori* knowledge of machine replicates (*i.e.* one class per time point, giving 14 classes in total). The different symbols represent the different time points of spoilage. The circles represent the 95% tolerance region constructed around the mean by the χ^2 confidence intervals using two degrees of freedom.

a downward dip. The latter trend appears to occur when the viable bacterial numbers are between 10^6 and 10^8 cfu ml^{-1}, and for some of these the 95% tolerance regions are seen to overlap sequentially.

In semi-skimmed milk (data not shown) similar results in PC-DFA were observed where the spectra for the first 56 h appeared in the same region, with TVCs between 4×10^2 and 2×10^5 cfu ml^{-1}. After this time point the spectra again followed a trend correlated to the number of bacteria. For skimmed milk (data also not shown) the spectra for the first 56 h also appeared clustered together (TVCs from 4×10^2 to 9×10^4 cfu ml^{-1}), after which a trend relating to the TVCs from 4×10^5 to 2×10^7 cfu ml^{-1} was also observed.

Since trends were observed in the PC-DFA of all milk types undergoing spoilage, we sought to correlate the known TVC with its representative FT-IR spectra. Therefore supervised learning analysis using PLSR was used to quantify the bacteria in spoilage milk. As described above, the PLSR algorithm was first calibrated and cross-validated with FT-IR spectra and the known TVC; after calibration these models were challenged with FT-IR spectra collected from an independent experiment set (*i.e.* data that were unseen/new to the model). Preliminary modeling for all milk types was performed on the FT-IR ATR spectra and it was found (data not shown) that PLSR could not give accurate estimates of TVC for very low bacterial numbers. Therefore, depending on the milk type, either 48 or 56 h to 104 h were used in PLS modeling and this also corresponds to when PC-DFA could not differentiate between the early sampling points.

PLS analysis on FT-IR ATR spectra from whole milk (between 56 and 104 h) found that the best model occurred when five PLS factors (latent variables) were used. The PLSR result for this model is shown in Fig. 5, where it can be clearly observed that the plot of the estimated TVC *versus* the known TVC values shows very reasonable prediction (*i.e.* the estimates lie close to the $y = x$ line shown on this plot). Overall, PLS gave accurate results at bacterial levels higher than 1×10^6 cfu ml^{-1} (Table 2), and this level is in agreement with the findings of previous research performed for the detection of microbial spoilage in chicken by using ATR FT-IR and PLSR.[5]

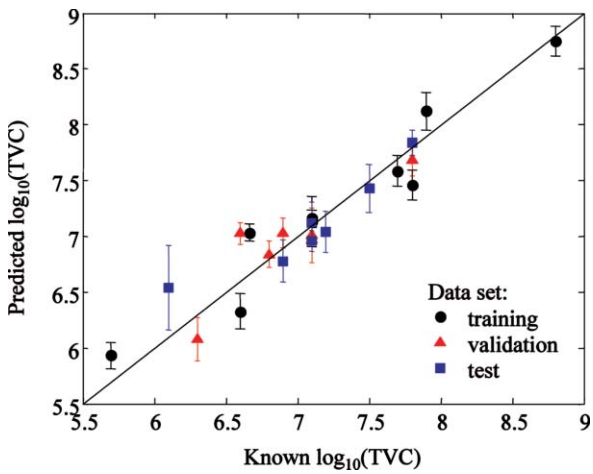

Fig. 5 Plot showing the predicted $\log_{10}$ TVC from PLS *versus* the actual $\log_{10}$ TVC for whole milk measured using ATR FT-IR spectroscopy. The RMS error ($\log_{10}$) in these measurements is 0.25 for the calibration, cross-validation and independent tests.

In semi-skimmed and skimmed milk, FT-IR ATR spectra from samples between 56 and 104 h, and from between 48 and 104 h, respectively, were analyzed. Table 2 gives the overall performance of the PLS models and shows that PLSR for semi-skimmed milk showed a good predictive value, when the total viable counts above 2×10^5 cfu ml^{-1} could be assessed. By contrast, for skimmed milk reasonable predictions were observed when the total viable counts were above 1×10^4 cfu ml^{-1}, which is an order of magnitude lower than those obtained for whole and semi-skimmed milk; remodeling of the whole-fat and semi-skimmed milk including the 48 h time point could not predict $<1 \times 10^4$ cfu ml^{-1}.

FT-IR HT spectroscopy

The same chemometric strategy to that discussed above was also used to analyse the FT-IR spectra obtained from the high throughput screening approach from dried milk. This novel

Table 2 Comparison between HT FT-IR and ATR FT-IR showing the root mean square errors for calibration, validation and test for each type of milk

Type of milk	FT-IR technique	TVC prediction range with PLSR	PLS factors	Error calibration (log_{10})	Error cross-validation (log_{10})	Error test (log_{10})
Whole	HT	10^3 to 10^8	18	0.33	0.84	0.84
Semi-skimmed	HT	4×10^2 to 10^7	13	0.45	1.20	0.66
Skimmed	HT	4×10^2 to 2×10^7	4	1.10	1.28	0.79
Whole	ATR	10^6 to 10^8	5	0.25	0.25	0.25
Semi-skimmed	ATR	2×10^5 to 10^7	4	0.20	0.35	0.41
Skimmed	ATR	10^4 to 2×10^7	4	0.7	0.82	0.87

screening approach has not previously been used to investigate food spoilage.

Representative FT-IR HT spectra collected from whole milk with the main absorption bands identified are shown in Fig. 6. Minimal qualitative differences were observed between the fresh milk at 0 h, and spoiled milk at 48 and 104 h; however, on close inspection quantitative differences were visible, especially in the spectral region from 900 to 1600 cm^{-1} which arise from carbohydrates, proteins and lipids.

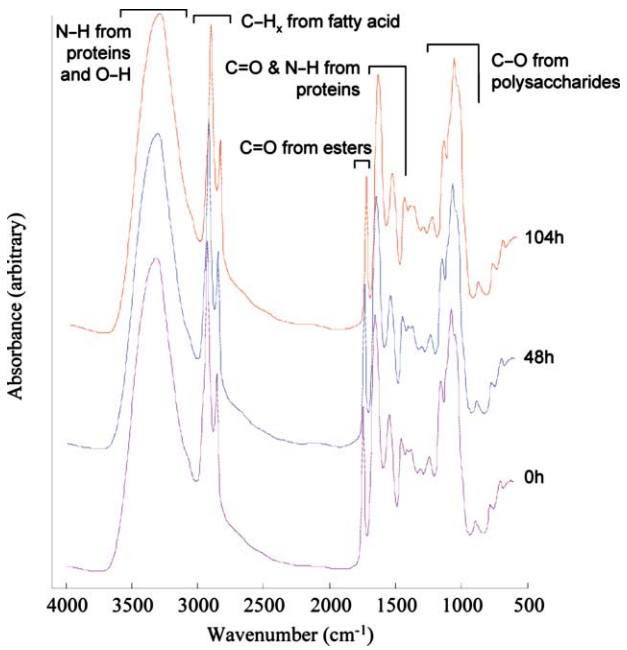

Fig. 6 FT-IR HT spectra for full milk at 0 h (purple), 48 h (blue), and 104 h (red).

Similar spectra were observed for semi-skimmed and skimmed milk (data not shown) with two main differences. The first difference involved the CH_x absorption band related to fatty acids at 2800 cm^{-1}. Since this involved the absorption of lipids, as expected this was found to be weaker in skimmed milk compared to whole-fat milk, as a result of the different fat quantities in the different types of milk. The other major difference appeared on the absorption band related to the C=O group of esters at 1750 cm^{-1}. This absorption was again found to be very strong in whole milk, less strong in semi-skimmed milk and did not appear in skimmed milk. The explanation behind this finding is most likely related to the esterification of fatty acids from lipids, which occurs in higher levels in whole and semi-skimmed milk and in very low levels in skimmed milk.

The PC-DFA results for spoilage of whole milk from FT-IR HT are shown in Fig. 7. It can be seen that the spectra for the first 48 h appear in the same region on the left of the pane. After that time point, subsequent time points tend to spread towards the right and upwards. The latter trend occurred when the viable bacterial numbers were between 10^6 and 10^8 cfu ml^{-1}, and the sample points were more discrete compared to the same analysis on the ATR accessory (Fig. 4) as evident from the 95% tolerance regions not overlapping, suggesting that the FT-IR HT spectra were more information-rich. The spectra of semi-skimmed milk during the first 56 h appeared within the same region, after which the time points spread again in a trend that corresponded to the bacterial load. For skimmed milk the trend in PC-DFA space was again very similar to this.

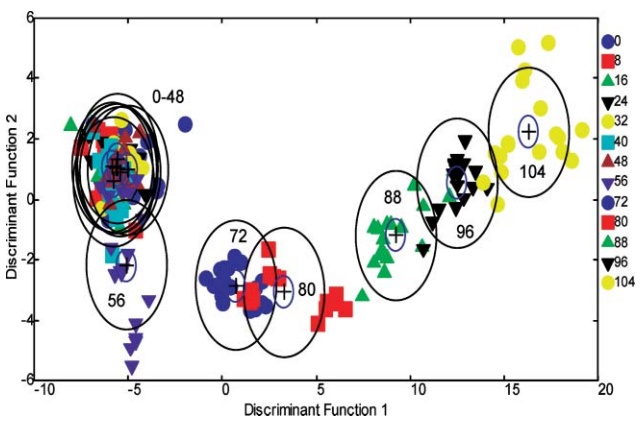

Fig. 7 PC-DFA plot on HT FT-IR spectra for the three repeat experiments of full milk. PCs 1–20 (accounting for 99.45% of the total variance) were used by the DFA algorithm with *a priori* knowledge of machine replicates (*i.e.* one class per time point, giving 13 classes in total). The different symbols represent the different time points of spoilage. The circles represent the 95% tolerance region constructed around the mean by the χ^2 confidence intervals using two degrees of freedom.

In contrast to the FT-IR ATR PLSR modeling it was possible to use the full time course of the FT-IR HT results for analysis. The PLSR results for whole milk are shown in Fig. 8, where the plot of the estimated TVC *versus* the known TVC values for whole milk showed good predictive values and gave relatively accurate results even at very low number viable counts (1×10^3 cfu ml^{-1}). The results for all three milk types are summarized in Table 2. When semi-skimmed milk was tested, PLSR again gave good predictions. However, the results

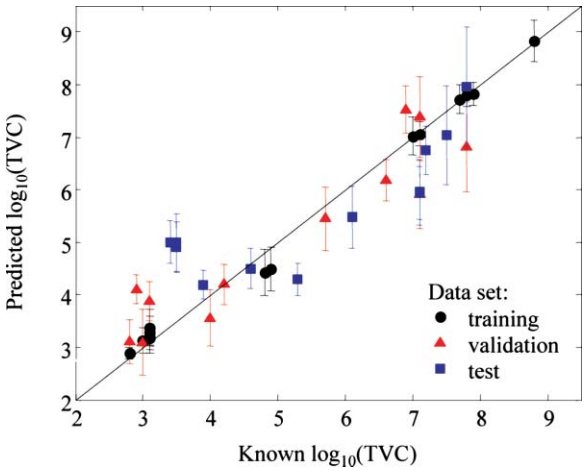

Fig. 8 Plot showing the predicted $\log_{10}$ TVC from PLS *versus* the actual $\log_{10}$ TVC for whole milk measured using HT FT-IR spectroscopy. The RMS error ($\log_{10}$) in these measurements is 0.33, 0.84, and 0.84, for the calibration, cross-validation and independent tests respectively.

from skimmed milk were not quite as good as the results for whole and semi-skimmed milk but were never-the-less still very encouraging.

Comparison of the two techniques

The most noticeable difference between FT-IR HT (Fig. 3) and FT-IR ATR (Fig. 6) spectra was the absence of CH_x vibrations at 2900 and 2800 cm^{-1} in the FT-IR ATR spectra, the peaks related to the acyl chain of fatty acids. Obviously these chemical species will not have disappeared during analysis and this is likely to have occurred because of the nature of the FT-IR ATR technique, which detects only the surface chemistry of cells or substances. For ATR the evanescent wave allows penetration into the surface above the crystal, and as reported in the Materials and Methods section for the CH_x stretches this is *ca.* 0.45 μm. As lipids in milk exist in the form of micellular globules surrounded by a protective membrane which is composed of glycoproteins, lipoproteins and phospholipids, the acyl chains will be internal to these globules with the polar head group exposed to the aqueous environment of the milk, and this may be why the CH_x stretches are missing from the ATR spectra. By contrast, the FT-IR HT technique employed uses dried milk and is a transmission-based measurement in which infrared light penetrates the whole of the sample and provides a spectrum characteristic of the total components of the milk. Furthermore, the peak shape of the spectra between 1700 and 900 cm^{-1} collected with ATR are different from those observed with FT-IR HT spectroscopy.

An overall comparison of the PLS modeling between HT FT-IR and ATR FT-IR for the root mean square errors of calibration, validation and test sets for each type of milk can be found in Table 2. It can be seen that the accuracy of ATR FT-IR spectroscopy for all whole and semi-skimmed types is better than the HT FT-IR approach, with both having similar predictive ability for skimmed milk. However, HT FT-IR does have significantly lower detection limits compared to ATR FT-IR and generally produces acceptable models with two lower orders of magnitude.

Conclusions

The use of FT-IR ATR and HT techniques in combination with multivariate statistical methods, including PC-DFA and PLSR, was able to acquire a metabolic snapshot and quantify the microbial load of milk samples accurately and rapidly, within 30 s, with little sample preparation. We believe that metabolic fingerprinting using FT-IR has a very good potential for future use in the dairy industry as a rapid method of viable bacterial detection and enumeration. As such it could therefore be incorporated in the HACCP system increasing consumer safety and lowering product-related risks and hazards.

Acknowledgements

This research was partially funded through the European Commission's BIOTRACER Integrated Project (www.biotracer.org).

References

1 R. R. Zall, in *Dairy microbiology: The microbiology of milk*, ed. R. K. Robinson, Elsevier Science Publishers, New York, 2nd edn, 1990, vol. 1, pp. 115–162.
2 D. I. Ellis and R. Goodacre, *Trends Food Sci. Technol.*, 2001, **12**, 414–424.
3 L. Gram, L. Ravn, M. Rasch, J. B. Bruhn, A. B. Christensen and M. Givskov, *Int. J. Food Microbiol.*, 2002, **78**, 79–97.
4 F. B. Whitfield, *Int. J. Food Sci. Technol. D*, **33**, 31–51.
5 D. I. Ellis, D. Broadhurst, D. B. Kell, J. J. Rowland and R. Goodacre, *Appl. Environ. Microbiol.*, 2002, **68**, 2822–2828.
6 J. F. Frank, in *Food Microbiology – Fundamentals and Frontiers*, ed. M. P. Doyle, L. R. Beuchat and T. J. Montville, ASM Press, Washington, 2nd edn, 2001, pp. 101–116.
7 M. R. Adams and M. O. Moss, *Food Microbiology*, Royal Society of Chemistry, Cambridge, UK, 2nd edn, 2000.
8 R. E. Chandler and T. A. McMeekin, *Aust. J. Dairy Technol.*, 1985, **40**, 37–42.
9 M. W. Griffiths and J. D. Phillips, *J. Soc. Dairy Technol.*, 1988, **41**, 96–102.
10 D. Bernard, in *Food Microbiology – Fundamentals and Frontiers*, ed. M. P. Doyle, L. R. Beuchat and T. J. Montville, ASM Press, Washington, 2nd edn, 2001, pp. 833–846.
11 D. Y. C. Fung, *Food Rev. Int.*, 1994, **10**, 357–375.
12 M. Karwoski, *Food Rev. Int.*, 1996, **12**, 155–174.
13 P. C. Vasavada, *J. Dairy Sci.*, 1993, **76**, 3101–3113.
14 D. I. Ellis, D. Broadhurst and R. Goodacre, *Anal. Chim. Acta*, 2004, **514**, 193–201.
15 B. Stuart, *Biological Applications of Infrared Spectroscopy*, John Wiley & Sons Inc., Chichester, UK, 1st edn, 1997.
16 H. Yang and J. Irudayaraj, *J. Mol. Struct.*, 2003, **646**, 35–43.
17 J. Schmitt and H.-C. Flemming, *Int. Biodeterior. Biodegrad.*, 1998, **41**, 1–11.
18 C. N. Banwell and E. M. McCash, *Fundamentals of Molecular Spectroscopy*, McGraw-Hill, London, 4th edn, 1994.
19 N. P. Cothup, L. H. Daly and S. E. Wiberly, *Introduction to Infrared and Raman spectroscopy*, Academic Press, New York, 1990.
20 *Plate count test at 30 °C*, National Standard method D 2 Issue 3, Health Protection Agency, 2004, www.hpa-standardmethods.org.uk/pdf_sops.asp.
21 C. L. Winder, S. V. Gordon, J. Dale, R. G. Hewinson and R. Goodacre, *Microbiology*, 2006, **152**, 2757–2765.
22 H. Martens, P. J. Nielsen and S. B. Engelsen, *Anal. Chem.*, 2003, **75**, 394–404.
23 R. M. Jarvis, D. Broadhurst, H. E. Johnson, N. M. O'Boyle and R. Goodacre, *Bioinformatics*, 2006, **22**, 2565–2566.
24 I. T. Jolliffe, *Principal Component Analysis*, Springer-Verlag, New York, 1st edn, 1986.
25 L. Mariey, J. P. Signolle, C. Amiel and J. Travent, *Vib. Spectrosc.*, 2001, **26**, 151–159.

26 B. F. J. Manly, *Multivariate Statistical methods: a primer*, Chapman and Hall, London, 1994.
27 R. M. Jarvis and R. Goodacre, *Anal. Chem.*, 2004, **76**, 40–47.
28 W. J. Krzanowski, *Principles of Multivariate Analysis – A User's Prespective*, Oxford University Press, New York, revised edn, 2000.
29 H. Martens and T. Naes, in *Multivariate Calibration*, John Wiley & Sons, Chichester, UK, 1st edn, 1989, pp. 73–236.
30 R. Goodacre, *Vib. Spectrosc.*, 2003, **32**, 33–45.
31 R. Goodacre and D. B. Kell, in *Metabolic Profiling – Its Role in Biomarker Discovery and Gene Function Analysis*, ed. G. H. Harrigan and R. Goodacre, Kluwer Academic Publishers, London, 2003, pp. 239–256.
32 H. C. Deeth, T. Khusniati, N. Datta and R. B. Wallace, *J. Dairy Res.*, 2002, **69**, 227–241.
33 C. P. Champagne, R. R. Laing, D. Roy, A. A. Mafu and M. W. Griffihs, *Crit. Rev. Food Sci. Nutrit.*, 1994, **34**, 1–30.
34 N. P. Shah, *Milchwissenchaft*, 1994, **49**, 432–437.
35 T. Sorhaug and L. Stepaniak, *Trends Food Sci. Technol.*, 1997, **8**, 35–41.
36 L. A. Mabbitt, *Kieler Milchwirtschaftliche*, 1981, 273–280.
37 H. J. Jos and H. in'tVeld, *Int. J. Food Microbiol.*, 1996, **33**, 1–18.