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Microbially induced changes in the volatile constituents of fresh chilled pasteurised milk during storage

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ABSTRACT

Off-odours caused by volatile organic compounds (VOCs) are often the first indicators consumers have of milk spoilage. In this study the VOCs associated with three types (trim, 0.25–0.40% fat; lite, 1.40–1.50% fat; and full-cream, 3.18–3.28% fat) of fresh chilled pasteurised milk (FCPM), held for up to 17 days at 4.5 ± 0.5 °C, were measured using proton-transfer-reaction mass spectrometry (PTR-MS). The chemical identification of VOCs in the headspace of the milk was supported by SPME–GC–MS analysis. Bacterial numbers (aerobic plate count at 25 °C) in the milk were also estimated. Replicate sets of milk types treated with sodium azide (NaN_3) to inhibit microbial activity were investigated. The relationship between microbial numbers and VOCs was not linear; rather the concentrations of VOCs only started to change after a threshold number of bacteria ranging from 10^6–10^8 CFU mL^{-1} was reached. This combined approach provided new insights on the effect of microbial growth on FCPM shelf-life.

1. Introduction

Fresh chilled pasteurised milk (FCPM) packaged in high-density polyethylene (HDPE) containers is generally given a shelf-life of 14 days if stored under appropriate temperature and light conditions. Milk producers frequently use total bacterial numbers to determine the end of shelf-life, while consumers usually rely on a sensory assessment, mainly odour, to determine if the milk is suitable to consume.

Regardless of the assessment method, it is generally accepted that the growth of micro-organisms limits the shelf-life of FCPM (Fromm & Boor, 2004; Sørhaug & Stepaniak, 1997). If post-pasteurisation contamination is low, shelf-life is limited by Gram-positive psychrotolerant, endospore-forming bacteria, such as *Paenibacillus* and *Bacillus* spp. that survive

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pasteurisation and have the ability to grow at typical refrigeration temperatures (Fromm & Boor, 2004; Huck, Hammond, Murphy, Woodcock, & Boor, 2007; Huck, Sonnen, & Boor, 2008). If post-pasteurisation contamination occurs due to the design, age or operations of the milk packaging plant, milk spoilage has been found to be associated with the growth of psychrotrophic Gram-negative bacteria such as *Pseudomonas* spp. (Craven & Macauley, 1992; Dogan & Boor, 2003; Sørhaug & Stepaniak, 1997). Despite efforts to reduce post-pasteurisation contamination, it is still believed to be a major cause of spoilage of FCPM (Martin et al., 2011).

Many bacterial species, including *Pseudomonads* spp., produce enzymes such as proteinases and lipases (Champagne et al., 1994; Sørhaug & Stepaniak, 1997) that break down proteins and fat in the milk, altering its physicochemical, functional and sensory properties and eventually resulting in consumers rejecting the milk. Such enzymes are only believed to occur at a significant concentration in milk when the bacterial counts reach around 10^6–10^7 colony forming units (CFU) per millilitre (Champagne et al., 1994). Bacterial numbers of 10^7 CFU mL^{-1} have also been defined as marking the end of shelf-life for milk (Griffiths & Phillips, 1986).

When consumers sniff milk to determine if it is suitable to drink they are actually assessing the odour-active volatile organic compounds (VOCs) present in the headspace of the milk container. These VOCs comprise compounds from the fresh milk and compounds arising due to a combination of chemical, microbial, enzymatic or light-induced reactions (Shipe et al., 1978).

For milk stored in light-exposed containers a relatively good correlation has been reported between sensory quality and the formation of VOCs, including light-induced and auto-oxidation compounds such as carbonyls (hexanal, pentanal, heptanal) and dimethyl disulphide. In contrast, in light-protected containers there was no correlation between VOCs produced and the milk's sensory attributes. Under all storage conditions there was a poor relationship between microbiological data and either VOC or sensory data (Karatapanis, Badeka, Riganakos, Savvaidis, & Kontominas, 2006). A poor correlation between the sensory properties and microbial numbers was also reported from studies on the shelf-life of both raw or pasteurised milk (Duyvesteyn, Shimoni, & Labuza, 2001). Gas chromatography–mass spectrometry (GC–MS) has been widely used in the analysis of milk for the early detection of microbial spoilage metabolites such as dimethyl sulphide, dimethyl disulphide, methanethiol, methional (3-(methylthio)propionaldehyde), skatole (3-methylindole), 3-methylbutanol, acetaldehyde, acetic acid, 1-octen-3-one, and 1,5-cis-octadien-3-one (Belitz, Grosch, & Schieberle, 2001; Cormier, Raymond, Champagne, & Morin, 1991; Marsili & Miller, 1998; Töpel, 2007; Toso, Procida, & Stefanon, 2002). Although GC–MS is the reference analytical method for the analysis of food VOCs (Snow & Slack, 2002), it is prone to artefacts and losses and it is a relatively laborious and costly test (Andersen, Hansen, Feilberg, 2012; López-Feria, Cárdenas, & Valcárcel, 2008). In general, GC–MS analysis of milk is made by prior extraction of the volatile fraction of milk using various methods like solid phase micro-extraction (SPME), vacuum distillation or simultaneous steam distillation and extraction (Contarini & Povolo, 2002). However, these methods are time-consuming and some require a large sampling volume, making them not entirely suitable for routine monitoring of a large number of samples (Contarini, Povolo, Leardi, & Toppino, 1997). Electronic nose instruments have been trialled as rapid methods to assess milk quality (Magan, Pavlou, & Chrysanthakis, 2001) and while they have had some success, they do not provide the detailed chemical information offered by GC–MS methods and there is concern that the technique lacks sensitivity (Marsili, 1999).

Proton-transfer-reaction mass spectrometry (PTR-MS) is a chemical ionisation-based technique for the gas-phase analysis of VOCs (Lindinger, Hansel, & Jordan, 1998). The technique allows rapid, direct, and accurate determination of the concentration of VOCs at trace levels (down to low part per trillion by volume, pptv, levels) (Jordan et al., 2009). PTR-MS has distinct advantages over GC–MS, as sample pre-treatment is not required, the technique is insensitive to inorganic air constituents and sample gas humidity variations, and it is a fast, sensitive and non-destructive method (Aprea et al., 2006). A drawback of PTR-MS is that the chemical identity of a measured VOC often cannot be determined unequivocally, especially in the complex gas matrix of food headspace, owing to the low mass resolution ($m/\Delta m$ of 1 Da) of the quadrupole mass spectrometer; hence multiple compounds, clusters and fragments from other VOCs can potentially contribute to the integral signal of a detected ion. Therefore, for confirmation of sample identification a secondary technique such as SPME–GC–MS should be used. PTR-MS has been successfully applied for the headspace analysis of several foods (Biasioli, Gasperi, Yeretzian, & Tilmann, 2011), including dairy products (Fabris et al., 2010; Soukoulis et al., 2010; van Ruth et al., 2008). Operational details of the PTR-MS technique can be found in the literature (Blake, Monks, & Ellis, 2009; de Gouw & Warneke, 2007).

The objectives of the present study were to investigate changes in VOCs in the headspace of refrigerated (4.5 ± 0.5 °C) bovine milk of different fat content (trim at 0.25–0.40% fat, lite at 1.40–1.50% fat, and full-cream at 3.18–3.28% fat) as a result of microbial activity over a storage period of up to 17 days. To control against changes arising from light-induced oxidation, the samples were kept in brown bottles in the dark throughout the storage period. In order to determine the relative impact of growth of the psychrotrophic Gram-negative bacteria such as *Pseudomonas* spp. and chemical or enzymatic processes on VOC production in the milk, a duplicate series of the analysed milk samples that were treated with sodium azide (NaN_3), a bacteriostatic agent that works by inhibiting respiration in Gram-negative bacteria (Lichstein & Soule, 1943), were also analysed. Bacterial numbers were determined using standard techniques used in the dairy industry. PTR-MS was used as the principal tool for monitoring the temporal development of the VOCs in the milk headspace over storage time. Off-line SPME–GC–MS analysis of the headspace of a subset of the frozen milk samples was made in order to aid chemical identification of the VOCs detected by PTR-MS.

2. Materials and methods

2.1. Milk samples

Fresh chilled pasteurised milk (FCPM) was purchased at a local supermarket (Dunedin, Otago, NZ) in 2 L high-density

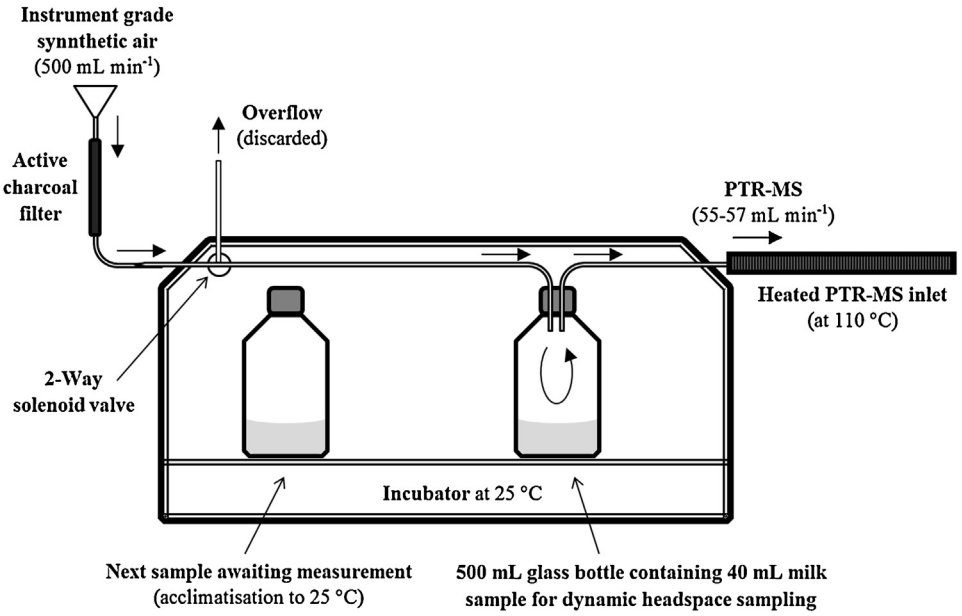

Fig. 1 – Experimental set-up for PTR-MS headspace analysis of milk samples.

polyethylene (HDPE) bottles. Three pasteurised milk types were used: *trim* (skim; fat 0.25–0.40%, protein 3.5–3.6%), *lite* (semi-skimmed; fat 1.40–1.50%, protein 3.40–3.50%), and *full-cream* (whole milk; fat 3.18–3.28%, protein 2.98–3.10%). The stated fat and protein concentrations were those provided by the milk producer on the packaging and were not additionally determined experimentally. The bottles used for each type of milk were all from the same production batch. Sodium azide (NaN_3) was added to half of the milk to give a final concentration of 10 ppmv NaN_3. 50 aliquots of each type of milk, both with and without NaN_3, were aseptically dispensed into sterile brown glass bottles (50 mL), ensuring that the bottle was completely filled before being closed with a sterile lid and held at 4.5 ± 0.5 °C in the dark for up to 15 days (17 days in total since bottling). Triplicate bottles of FCPM were analysed at regular intervals by the following protocol. 10 mL of each 50 mL sample was used to estimate bacterial numbers. The remaining 40 mL was used for headspace analysis by PTR-MS (see details in Sections 2.4 and 2.2, respectively). The milk, which was two days old when purchased, was distributed into sub-samples on the day of purchase, and was first sampled the following day, i.e. three days after it was bottled at the factory. Microbial and PTR-MS analyses were carried out on days 3, 6, 9, 10, 13, 15 and 17 after bottling. The pH of a subset of samples was measured directly after PTR-MS analysis to determine potential effects of pH on VOC profiles.

2.2. *Headspace analysis of milk samples using PTR-MS*

The VOC composition of the milk headspace was determined using a high sensitivity proton-transfer-reaction mass spectrometer (PTR-MS; IONICON Analytik GmbH, Innsbruck, Austria). The PTR-MS inlet consisted of a ~1 m long 1/16″ outer diameter Silcosteel™ capillary (Restek Co., Bellefonte, PA) heated to 110 °C and with a continuous flowrate of 55–57 mL min^{-1}.

The experimental set-up comprised a 500 mL sampling bottle (Schott Duran, Mitterteich, Germany) fitted with a lid containing two polytetrafluoroethylene (PTFE) tube connection ports for gas inlet and outlet (see Fig. 1). The sampling bottle and all downstream sampling tubes were placed in an incubator at 25 ± 2 °C to maintain a constant sample temperature throughout the PTR-MS analysis. Instrument grade synthetic air was flushed at a flow rate of 500 mL min^{-1} through an active charcoal filter (Supelcarb®, Supelco, Bellefonte, PA) to remove potential contaminant VOCs. This VOC-free air (termed zero-air from hereon) was subsequently connected via a 2-way solenoid valve (Parker, Cleveland, OH) to the inlet of the glass sampling bottle. Placing the solenoid valve upstream of the sample bottle inlet allowed the zero-air flow through the bottle to be switched on and off for flushing and static sampling, respectively. Operation of the valve was made by direct electrical connection with the PTR-MS and via the PTR-MS Control software (IONICON Analytik GmbH).

For the measurements, the PTR-MS was operated in mass scan mode over a mass range of *m*/*z* 20–200 with a dwell time of 100 ms per *m*/*z*, which resulted in a cycle time of 18 s. The PTR-MS drift tube was held at 600 V, 2.2 mbar and 70 °C, which corresponded to an electric field strength (*E*) to buffer gas number density (*N*) ratio (*E*/*N*) of 136 Td (1 Td (Townsend) = 10^{-17} V cm^2). Prior to measurement, each milk sample was placed in a water bath for 15 min for pre-warming to 25 °C and subsequently placed inside the incubator for a further 12 min before connection to the PTR-MS for sampling.

Headspace measurements of each sample proceeded as follows. A clean sample bottle was connected to the zero-air gas flow line (outlet line open) and flushed at a gas flow rate of 500 mL min^{-1} for 3 min in order to exchange the laboratory air present in the bottle (as a result of opening) with VOC-free air.

The PTR-MS inlet line was then connected via a piece of 1/8″ outer diameter PTFE tubing – by direct insertion of the inlet capillary into this tube – to the bottle outlet. PTR-MS analysis (over five measurement cycles, i.e. ~90 s) of this empty bottle under dynamic conditions provided a level of VOC contamination within the set-up (i.e. background noise), which was used during data post-processing (see Section 2.5). The PTR-MS inlet was then disconnected from the sample bottle outlet and the pre-warmed milk was poured into the bottle. The bottle was then flushed with zero-air (at 500 mL min^{-1}) for 3 min to again remove laboratory air from the bottle. Disconnection of the PTR-MS inlet from the bottle outlet enabled this higher flow rate to be achieved and thereby provided a faster gas exchange within the bottle. The PTR-MS inlet was subsequently reconnected and the milk headspace was flushed for a further ~90 s at the reduced rate of the PTR-MS inlet flow (55–57 mL min^{-1}) to enable a build-up of VOCs from the milk in the headspace. The gas flow at the bottle inlet was then stopped (by switching the solenoid valve; thereby closing the bottle inlet flow) and static headspace measurements were made for 8–10 cycles (up to 180 s). The measurements were stopped once the pressure in the PTR-MS drift tube started to decrease. This pressure drop was additionally used as a quality control to check that the sampling lines and bottle were gas tight; the data from samples where this criterion was not fulfilled were discarded. This procedure resulted in a headspace measurement time of 12 min per sample. Analysis of all milk types were carried out in triplicate using a different 50 mL milk sample aliquot poured into a different 500 mL sample bottle for each replicate, resulting in the measurement of 18 samples per sampling day (three replicates of three milk types, both with and without NaN_3).

2.3. GC–MS analysis of milk volatiles

Gas chromatography–mass spectrometry (GC–MS) analyses of a subset of samples were made using solid-phase microextraction (SPME) to aid identification of the PTR-MS *m/z* signals. Milk samples from days 3 and 17 were frozen immediately after PTR-MS analysis at −18 °C. Prior to GC–MS analysis, frozen samples were defrosted and stored at 4 °C. Subsequently, 4.00 ± 0.05 g of the milk samples were weighed (KERN ABT 220-4M, Kern & Sohn GmbH, Balingen, Germany) into 20 mL screw-capped vials (Wheaton Industries Inc., Millville, NJ). Two SPME fibres (Supelco, Sigma–Aldrich Co., St. Louis, MO) of different polarities were used for analysing the headspace volatiles: a carboxen/polydimethylsiloxane fibre (CAR/PDMS, 75 μm film thickness, bonded) and a polydimethylsiloxane/divinylbenzene fibre (PDMS/DVB, 65 μm film thickness, bonded). Before analyses, the fibre was cleaned and preconditioned by thermal desorption in the injector port of the GC at a temperature of 240 °C (Agilent 6890N, Agilent Technologies, Santa Clara, CA). The capped vials, filled with the defrosted milk samples, were equilibrated to 40 °C for 10 min in a dry block heater (DBH20D, Ratek Instruments Pty. Ltd., Boronia, Australia) before the fibre was exposed to the milk headspace, following the methodology reported by Soukolis and coworkers (2010). After an exposure time of 30 min the fibre was immediately inserted into the injector port of the GC where the VOCs were thermally desorbed at 240 °C for 2 min in splitless mode. The fibre was held in the injector port for another 3 min in split mode (purge flow: 50 mL min^{-1}) to clean the fibre for the next measurement. The volatiles were separated on a ZB-1701 ZebronTM capillary GC column (60 m, 0.25 mm i.d., 0.25 μm film thickness, Phenomenex Inc., Torrance, CA). The following GC oven programme was executed: 40 °C for 10 min, 40–180 °C at 5 °C min^{-1}, 180–220 °C at 10 °C min^{-1}, 220 °C for 5 min. The method was run in constant flow mode at 1 mL min^{-1} with helium as carrier gas (initial pressure: ca. 110 kPa). The GC was coupled to a mass spectrometer (Agilent 5975B VL MSD, Agilent Technologies, Santa Clara, CA) and the MS was operated in electron ionisation (EI) mode at an ionisation energy of 70 eV and a scan range of *m/z* 35–200. The compounds were tentatively identified based on mass spectra matching with the standard NIST-08 MS library (National Institute of Standards, Gaithersburg, MD).

2.4. Determination of the number of bacteria

The number of bacteria in three replicated samples of each milk type incubated in the presence or absence of NaN_3 was determined on each sampling day by the spread plate method. Immediately prior to PTR-MS headspace analysis, a 10 mL aliquot of the milk was aseptically removed from each bottle. A number of milk dilutions (1:9) were prepared in 0.1% peptone water (DIFCO, Fort Richard, New Zealand) in universal bottles. Each dilution was plated (100 triplicate $n = 3$) on plate count agar (PCA, DIFCO) using the standard spread plate method. Plates were incubated at 25 °C for 72 h. The number of microbial cells associated with the milk samples was expressed in colony-forming units as CFU mL^{-1} milk. The detection limit for the microbial counts was 30 CFU mL^{-1}.

2.5. PTR-MS data evaluation

The raw PTR-MS signal data in counts per second (cps) for each *m/z* in the range *m/z* 20–200 were normalised to the sum of the primary ions signal (the isotopologue at *m/z* 21 and the water cluster at *m/z* 37), as well as to the drift tube pressure using factors of 10^6 and 2.2, respectively, which resulted in a normalised cps (n-cps) dataset. Mean values of both the background and sample measurement cycles were calculated using the last three cycles (first two discarded) for the former and cycles 3–9 (first two and last cycles discarded) for the latter to rule out potential carry-over contamination after switching of the sampling valve. The background data were used to calculate the respective system limit of detection for each sample measurement, which was taken as three times the standard deviation of the background noise. Signals of samples that were below this threshold were discarded and not used for further analysis. The background mean was then subtracted from the sample mean to produce a net signal. An overall mean and standard deviation of these net signals was then calculated from the sample triplicates. A conversion from n-cps to gas phase concentration was not carried out as only relative differences over time were compared and because calibration of the PTR-MS was not possible for all of the VOCs detected in the milk headspace as some were unknown.

2.6. *Statistical analyses*

The mean normalised PTR-MS (n-cps) data of each sample triplicate were initially visually scrutinised to determine which signals changed noticeably over the storage period.

The data matrix consisted of as many rows as samples (n = 108, 54 each with and without NaN_3 consisting of two milk types and nine time points) and as many columns as variables (180 for the m/z matrix). Since bacterial numbers for the full-cream (3.5% fat) milk were less than10^6 CFU mL^{-1} and the generally accepted number of counts for spoiled milk is in the range of 10^6 to 10^7 CFU mL^{-1} (Urbach & Milne, 1987) the full-cream milk was considered not spoiled and thus excluded from the statistical analysis comparing the NaN_3 treated and untreated samples.

A *U*-Mann–Whitney test was conducted to determine the effect of microbial spoilage on the VOC composition of milk. The distributions of m/z measured by PTR-MS were compared between milk samples with and without NaN_3 (significance level $p \leq 0.05$). To determine what changes in the milk could be attributed to non-microbial mechanisms a Kruskal–Wallis test ($p \leq 0.05$) was carried out comparing the distributions of the n-cps data of the NaN_3-treated milk over the storage period. The software used for non-parametric tests was SPSS v20.0 (IBM Statistics Inc, Chicago, IL).

3. Results and discussion

In the absence of sodium azide (NaN_3) the number of bacteria detected in the milk increased with increasing storage time (Fig. 2). The different milk sample types showed different profiles of microbial growth. The number of bacteria detected in the milk samples on day 3 ranged from being below the detection limit (of 30 CFU mL^{-1}) to 4.8 $\log_{10}$ CFU mL^{-1}. In both the trim (Fig. 2a) and lite (Fig. 2b) milk samples in the absence of NaN_3 bacterial numbers in at least one of the replicates tested had exceeded 10^6 CFU mL^{-1} by day 6 of their shelf-life with numbers continuing to increase until reaching a plateau on around day 13. In the full-cream milk (Fig. 2c) microbial numbers started off lower than in the trim and lite milk samples with bacteria not being detectable (less than 30 CFU mL^{-1}) in some of the samples until day 10, after which time in the absence of NaN_3 they steadily started to increase. It is likely that the lower number of bacteria detected in the full-cream milk over the trial reflects a lower initial level of post pasteurisation contamination. For each milk type and sampling time, the number of bacteria within the triplicate measurements also varied, which is also believed to be due to differences in the bacterial population (numbers, species, strains) that contaminated the milk during packaging (Salustiano et al., 2009).

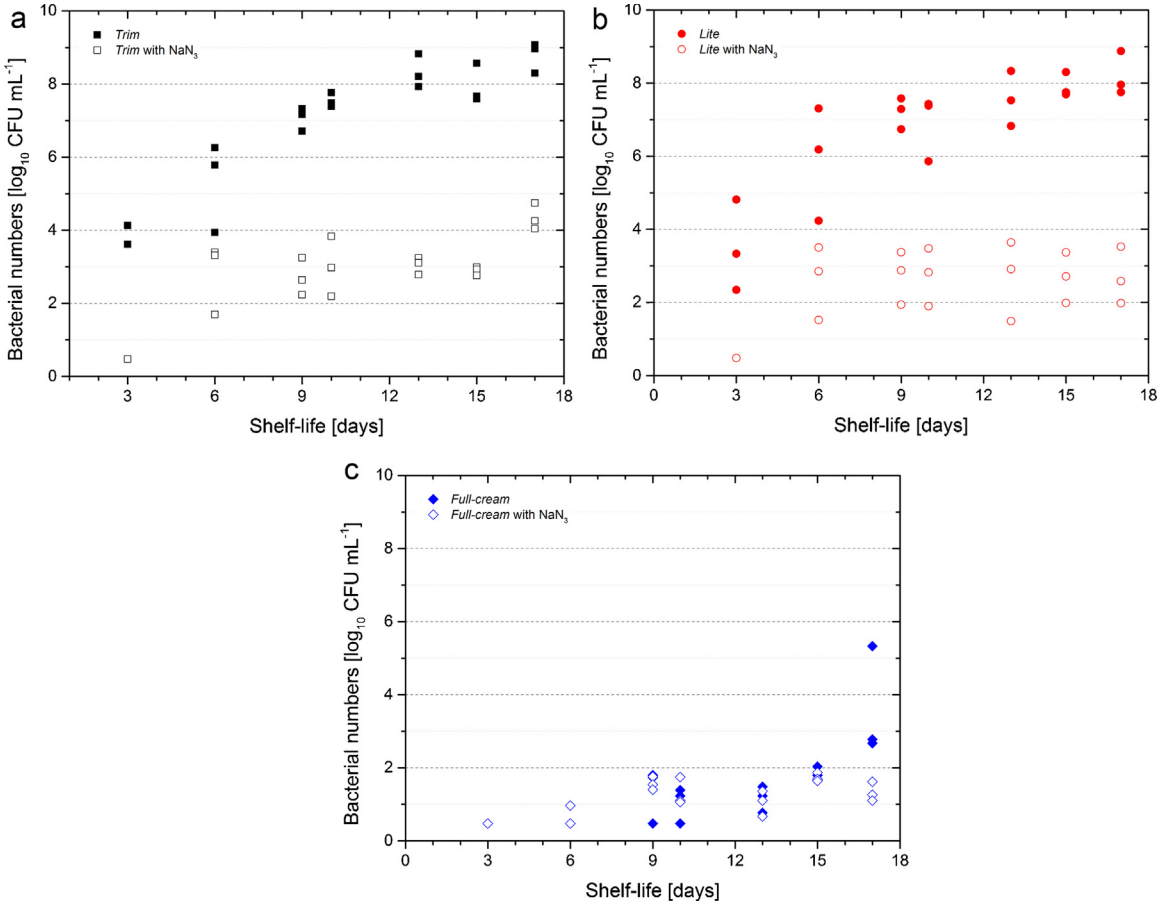

Fig. 2 – Total bacterial numbers in (a) *trim*, (b) *lite* and (c) *full-cream* fresh chilled pasteurised milk (FCPM) samples over time. Open symbols represent control samples (treated with 10 ppmv NaN_3) and solid symbols represent untreated samples. Each data point is the mean value of three counts from one bottle (n = 3).

Table 1 – Mean PTR-MS signal intensity across days 3–17 after packaging (in normalised counts per second; n-cps) (±standard error) for the VOCs (*m/z*) that significantly discriminated between skim and lite milk with microbial growth (untreated) from milk with no microbial growth (treated with 10 ppmv NaN_3).

m/z	Tentative identity	Untreated milk	Milk treated with NaN_3
41	Fragments of alcohols (e.g. 1-propanol, 2-propanol[a]). In NaN_3-treated milk it correlated with *m/z* 69, tentatively identified as isoprene.	305.5 ± 56.4	23.7 ± 0.9
43	Fragment of alcohols, esters and acids	1151.5 ± 287.8	89.0 ± 2.6
44	Azide product (HN_3) (+^{13}C isotope of *m/z* 43, ca. 2.2%)	28.8 ± 7.2	624.6 ± 11.9
45	Acetaldehyde ethanal[b]	889.6 ± 212.5	32.6 ± 1
47	Ethanol[a,b], formic acid[b]	601.5 ± 192	2.3 ± 0.6
49	Methanethiol[b]	2.7 ± 0.6	0.3 ± 0
57	Fragments of carboxylic acids and alcohols	21.7 ± 4.7	2.7 ± 0.4
59	Acetone[a,b]	2117.5 ± 229.8	4236.9 ± 142.7
61	Acetic acid[a,b], ethyl acetate (fragment)[b]	318.7 ± 86.9	20.8 ± 0.9
63	Dimethyl sulphide (DMS)[a,b]	67.3 ± 10.6	23.2 ± 1.1
65	Ethanol hydrate (cluster)	11.9 ± 3.3	0.7 ± 0.1
71	Fragment related to *m/z* 89	198.2 ± 73.3	2.7 ± 0.3
73	2-Butanone[a,b]	73.4 ± 14.9	64.6 ± 2.9
75	Methyl acetate[a], ethyl ether [a]	7.2 ± 1.5	1.9 ± 0.2
77	Acetone hydrate (cluster)	2.0 ± 0.3	3.2 ± 0.3
87	Butane-2,3-dione (diacetyl), 1,2-pentanone[a,b], 3-methylbutanal[a], pentanal[a]	18.8 ± 3.7	2.2 ± 0.2
89	Acetoin (3-hydroxybutanone)[a,b], ethyl acetate[a], butanoic acid[a], 1-pentanol[a,b], 3-methyl-1-butanol[a]	210.4 ± 72.2	2.2 ± 0.3
103	1-Hexanol[a,b], 2-hydroxy-3-pentanone[b], 1-methylethyl acetate[a], ethyl propanoate[a], methyl butanoate[a]	2.7 ± 0.9	0.6 ± 0.1
115	2-Heptanone[a,b], ethyl 2-butenoate[a], heptanal[a]	7.2 ± 1.5	0.5 ± 0.1
117	Hexanoic acid[a,b], 2-heptanol[a], ethyl butanoate[a], 1-methylethyl propanoate[a]	8.5 ± 2.5	1.5 ± 0.2
129	1-Methylethyl 2-butenoate[a], 2-methylethyl 2-butenoate[a]	0.9 ± 0.2	0.3 ± 0
131	Unidentified	1.7 ± 0.6	0.4 ± 0.1
143	2-Nonanone[a,b], nonanal[a], ethyl-2-hexenoate[a]	2.3 ± 0.5	0.4 ± 0
145	Octanoic acid[a,b], 3-methyl, 1-methylethyl butanoate[a], 4-methyl, ethyl pentanoate[a]	4.1 ± 1.6	0.6 ± 0.1

[a] Detected by SPME–GC–MS in this study.
[b] Reported by Soukoulis et al. (2010).

In the samples treated with NaN_3 microbial numbers did not increase generally over time (Fig. 2). Previous trials have shown that the majority of the bacteria detected within the first few days post production are due to the outgrowth of spore-forming bacteria that have survived pasteurisation and that their numbers do not subsequently increase in milk held at 4 °C (data not shown). As NaN_3 does not kill the spore formers, it was expected that the number of these organisms in the milk would remain constant over time.

It was found that 46 mass ions (*m/z*) detected in the headspace above the FCPM samples significantly differentiated ($p \leq 0.05$) between milks with or without NaN_3. The number of *m/z* differing significantly between the two treatments was reduced to 38 when *m/z* signals relating to ^{13}C isotopes were removed. The key significant *m/z* detected are summarised in Table 1 along with tentative identification from SPME–GC–MS analysis and literature reports.

In the presence of NaN_3 six *m/z* significantly ($p \leq 0.05$) changed during storage, namely *m/z* 33, 43, 44, 59, 73 and 124 (data not shown). In the 3.5% full-cream milk three additional *m/z*, 41, 69 and 87, significantly ($p \leq 0.05$) decreased during storage, although all three *m/z* are potentially from the same VOC, i.e. fragments and/or water clusters (data not shown). The signal at *m/z* 44 (Fig. 3b) was attributed to protonated HN_3, a volatile product of NaN_3, which decreased in its concentration over time, which in turn may explain why microbial numbers increased slightly on day 17 in some NaN_3-treated samples (Fig. 2). Note that the increase in the *m/z* 44 peak intensity as bacterial numbers increased in samples that did not contain NaN_3 is believed to be due to the increase in *m/z*m/z 43 (i.e. the ^{13}C isotope), rather than related to NaN_3.

In the absence of NaN_3 the production of alcohols, esters, and volatile acids in the milk indicates bacterial activity with the relative production of these metabolites being dependent on the composition of the bacterial population. For example, CO_2, acetoin, ethanol, and acetate have been reported to be abundant in the headspace of milk inoculated with pure and mixed cultures of *Serratia marcescens* and *Serratia proteamaculans*, whereas CO_2 and acetate were the major volatiles (90–98% of the GC–MS peak areas) in *Pseudomonas putida* cultures (Haugen, Rudi, Langsrud, & Bredholt, 2006). The growth of coliform bacteria in milk has been associated with high levels of ethanol while the production of compounds like acetaldehyde and 2,3-butanedione (diacetyl) is considered to be related to the growth of lactic acid bacteria (e.g., *Lactococcus lactis*) (Bassette, Bawdon, & Claydon, 1967). In addition to specific bacterial metabolites, the release of enzymes during the growth of bacteria in food can result in the production of VOCs like alcohols, acids, aldehydes, ketones, and esters (Sabatini, Mucciarella, & Marsilio, 2008).

In the current study both univariate and multivariate data analysis methods failed to find a meaningful relationship

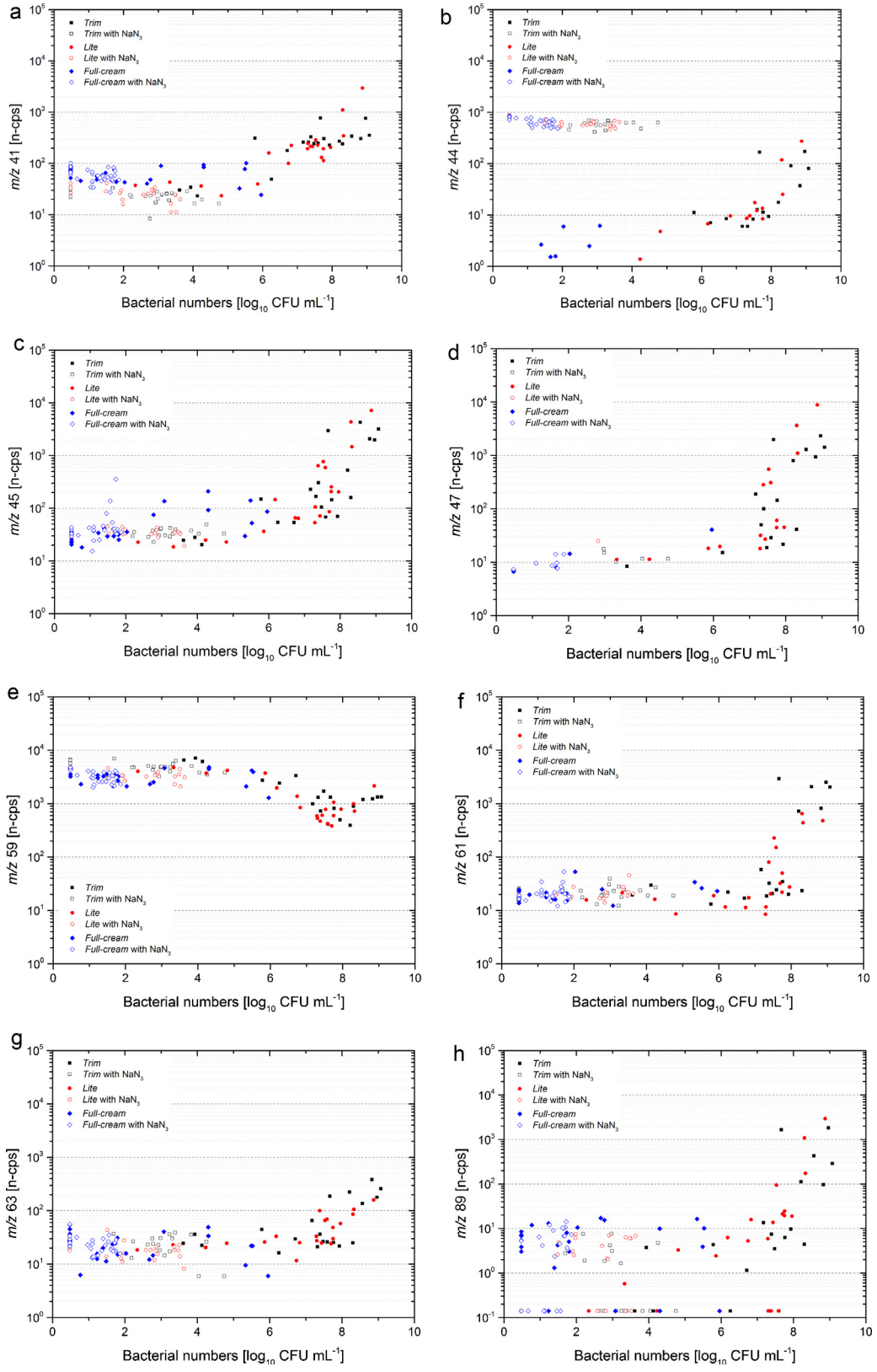

Fig. 3 – Milk headspace concentration (PTR-MS signal intensity) of selected VOCs and corresponding bacterial numbers in all fresh chilled pasteurised milk (FCPM) samples (*trim*, *lite* and *full-cream*, either in the presence or absence of 10 ppmv NaN_3). Tentative identifications are (see text and Table 1): *m/z* 41, fragments of alcohols; *m/z* 44, HN_3; *m/z* 45, acetaldehyde; *m/z* 47, ethanol; *m/z* 59, acetone; *m/z* 61, acetic acid; *m/z* 63, dimethyl sulphide; *m/z* 89, acetoin, ethyl acetate, butanoic acid, 1-pentanol, 3-methyl-1-butanol. Open symbols represent control samples (treated with 10 ppmv NaN_3) and solid symbols represent untreated samples.

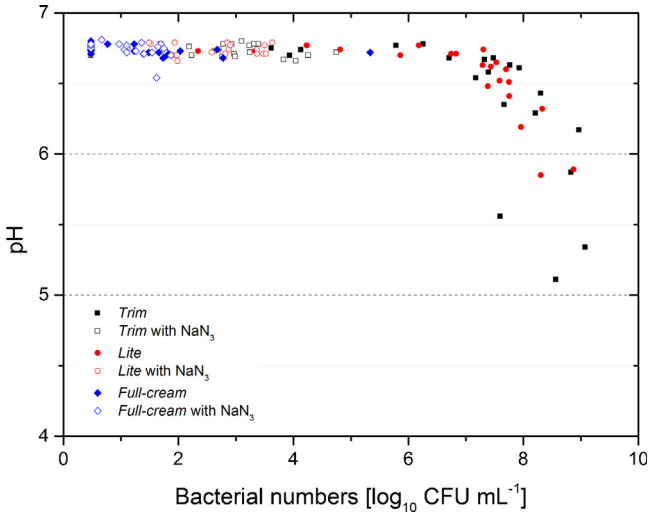

Fig. 4 – Total bacterial numbers in trim, lite and full cream fresh chilled pasteurised milk (FCPM) samples versus pH. Open symbols represent control samples (treated with 10 ppmv NaN_3) and solid symbols represent untreated samples. Each data point is the mean value of three counts from one bottle ($n = 3$).

between VOC concentrations and bacterial numbers. However, when the VOC signal intensities versus bacterial numbers were plotted (Fig. 3) it became apparent that the reason for the lack of a correlation between total bacterial numbers and VOC concentrations was because VOC concentrations remain largely unchanged until the number of bacteria reached a threshold number. Interestingly, however, not all VOCs started to change in concentration at the same threshold number of bacteria and the rate of change at the inflexion point varied from compound to compound. For example, when bacterial numbers reached about 10^6 to 10^7 CFU mL^{-1}, *m/z* 41 (fragments of alcohols, Fig. 3a) started to increase and *m/z* 59 (acetone, Fig. 3e) started decreasing. In general the rate of evolution of VOCs as the number of bacteria increased showed a similar pattern for all three milk types (Fig. 3).

The intensities of *m/z* 45 (acetaldehyde), *m/z* 47 (ethanol), *m/z* 61 (acetic acid, ethyl acetate), *m/z* 63 (dimethyl sulphide) and *m/z* 89 (acetoin, ethyl acetate, butanoic acid, 1-pentanol, 3-methyl-1-butanol) started to increase at varying rates once the number of bacteria reached between 10^7 to 10^8 CFU mL^{-1} (Fig. 3). When bacterial numbers were plotted against the pH of the milk samples a similar trend was evident, with a threshold number of bacteria being required prior to a change being detected (Fig. 4). The relationship between log bacterial numbers versus log VOC concentration has previously been described by Mayr et al. (2003) for bacteria growing on air-packaged beef or pork. In this instance, while the data appeared to show a similar threshold effect as seen in the current study, whereby the VOC concentrations remained relatively unchanged until microbial numbers exceeded 10^7 CFU g^{-1}, the authors chose to fit a linear equation to their data. In another study with pasteurised milk, ethanol and acetaldehyde levels in the headspace of the milk at concentrations corresponding to the onset of off-flavour development, were reported to occur when bacterial numbers reach around 10^6–10^7 CFU mL^{-1} (Urbach & Milne, 1987). These authors also reported an increase in propan-2-ol with a decrease in acetone levels, but such results were dependent on the contaminating bacterial strain. Similar studies on a mixed lettuce medium (Ragaert et al., 2006) or cooked peeled grey shrimp (Noseda et al., 2012) have also reported that bacterial numbers in excess of 10^7–10^8 CFU cm^{-2} or g^{-1} are required before VOC production occurs at rates sufficient to cause spoilage. On the shredded mixed lettuce it was reported that VOC generation was linked to sugar consumption, which was low until microbial numbers exceeded approximately 10^7 CFU cm^{-2} (Ragaert et al., 2006). In the studies reported above and in the current study the slight difference in the total number of bacteria required to be present before variations in VOC concentrations become significant is likely to depend on the bacteria species present, their rate of VOC generation, and the substrates available.

4. Conclusions

The present data show that the relationship between microbial numbers and volatile organic compound (VOC) concentration in fresh chilled pasteurised milk (FCPM) held at 4.5 °C is not linear, rather the concentrations of spoilage-related VOCs detected only start to change once a threshold number of bacteria has been reached. This data suggests that the estimation of bacteria numbers in milk at a single time point will not necessary accurately reflect consumer (odour) perception of the milk at that time. For example, microbial numbers in excess of 10^6–10^7 CFU mL^{-1} will not necessarily result in the milk being rejected by consumers. However, within a short period of time additional bacterial growth and further VOC production will result in the development of off-odours and consumer rejection. The approach of combining non-destructive, on-line VOC analysis by PTR-MS in conjunction with microbial testing has provided additional information on the relationship between microbial growth and the shelf-life of milk as assessed by its VOC concentration.

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