International Dairy Journal 97 (2019) 86–91

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

16S rDNA high-throughput sequencing and MALDI-TOF MS are complementary when studying psychrotrophic bacterial diversity of raw cows' milk

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ARTICLE INFO

Article history:
Received 26 March 2019
Received in revised form
1 June 2019
Accepted 2 June 2019
Available online 8 June 2019

ABSTRACT

Refrigeration of raw milk favours the growth of psychrotrophic bacteria (psychrotrophs) that can produce spoilage enzymes, which may deteriorate final dairy products. The psychrotrophs from raw milk across four seasons were identified using culture-dependent and culture-independent approaches. Both 16S rDNA high-throughput sequencing (HTS) and MALDI-TOF MS showed the *Pseudomonas* genus to predominate; however, predominant species differed between the two identification methods. There was no geographical trend in microbiota, but a seasonal variation was evident. The use of HTS and MALDI-TOF MS was complementary in describing the psychrotrophic bacterial diversity of raw milk and provides an understanding of the raw milk microflora that may influence milk quality. This is the first report to compare data obtained from 16S rDNA high-throughput sequencing and MALDI-TOF analysis to assess the psychrotrophic microbial quality of refrigerated raw milk in New Zealand.

1. Introduction

Milk provides an excellent environment for the growth of a variety of microorganisms due to its neutral pH, high water activity and richness in nutrients (Champagne et al., 1994). The microbiological quality of raw milk is closely linked to the health of the cow, the teat surfaces, the feed, bedding material, milking equipment, bulk tank storage, and transportation conditions (Mangia, Fancello, & Deiana, 2016; Vithanage et al., 2016). Refrigeration of raw milk immediately after milking and maintaining chilled temperatures (<7 °C) before processing is a common international practice to delay the growth of bacteria and maintain quality (Law, 1979). However, it is well known that prolonged refrigerated storage of raw milk can favour the growth of psychrotrophic bacteria, which can produce heat-resistant proteolytic and lipolytic enzymes. These heat-resistant enzymes are able to remain active following heat treatment (including ultra-high temperature processing) and then hydrolyse the milk proteins and fats during storage, leading to the spoilage and reduced shelf-life of commercial dairy products (Samaržija, Zamberlin, & Pogačić, 2012).

The majority of the bacteria found in fresh raw milk directly isolated from the udder are Gram-positive mesophilic aerobic bacteria with only 10% being psychrotrophic. The populations of psychrotrophic bacteria in raw milk can then increase to 50% of the whole microbial load after one-day cold storage and can finally increase to more than 90% of the whole microbial load after 2 days cold storage (Lafarge et al., 2004). They include the following genera *Acinetobacter*, *Achromobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Flavobacterium*, *Pseudomonas* and *Serratia*, with *Pseudomonas* being the predominant genera (Cousin, 1982; Ercolini, Russo, Ferrocino, & Villani, 2009).

Different psychrotrophic bacteria can secrete different types of enzymes, such as proteases, lipases and phospholipases with different molecular sizes. For example, *Pseudomonas fluorescens* are able to produce proteases, lipases and phospholipases, whereas *Ancinetobacter guillouiae* were reported to be only lipolytic (Vithanage et al., 2016). Most *Pseudomonas* strains can produce only one type of proteinase, a neutral zinc metallo-proteinase with molecular masses ranging from 39.2 ± 0.7 to 45.3 ± 1.3 kDa (Marchand et al., 2009). *Bacillus* species can produce many diverse types of proteinases with different molecular masses (Fairbairn &

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https://doi.org/10.1016/j.idairyj.2019.06.001
0958-6946/

Law, 1986). Stoeckel et al. (2016) reported that the proteinases produced by different *Pseudomonas* species have various effects on protein destabilisation and flavour defects of UHT milk. Since psychrotrophic bacteria are capable of producing variety of enzymes that can cause defects in dairy products, it is important to determine the diversity of psychrotrophic bacteria in raw milk to ensure effective storage practices for milk before processing to minimise the growth of these bacteria.

Studies investigating the microbial diversity in raw milk have used culture-based methods, which may only detect a proportion of total microbiota. Molecular based methods, such as 16S rDNA high-throughput sequencing (HTS), are useful new technologies to study raw milk microbial communities that capture both the culturable and non-culturable populations. Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) is a relatively new method for the rapid identification of culturable microorganisms that is simpler and easier than API, Biolog, and culture-based 16S rDNA gene sequencing (Weber, Geiβert, Kruse, & Lipski, 2014). In this study, both culture-independent (HTS) and culture-dependent (MALDI-TOF) approaches were used to determine the diversity of psychrotrophic bacteria in raw milk collected from New Zealand during different seasons.

2. Materials and methods

2.1. Raw milk sampling

Raw milk samples were obtained during each season (autumn, winter, spring and summer) and represented the main raw milk collection regions of New Zealand (Northland, Waikato, Bay of Plenty, Hawkes Bay, Clandeboye, and Southland). Raw milk samples (35 mL) were randomly obtained from 10 different farms in each region ($n = 60$ per season, total $n = 240$) and transferred to Massey University within 12 h. The 10 samples from within each region were commingled and then stored at 7 °C for 5 days before analysis to select for the predominant psychrotrophic populations.

2.2. DNA extraction and 16S rDNA high-throughput sequencing

The total bacterial gDNA of raw milk was extracted using the Presto™ Mini gDNA Bacteria kit following the manufacturer's instructions. The kit was directly applied to raw milk without any treatment beforehand. The quality and concentration of extracted DNA was assessed by 2% agarose gel electrophoresis and Colibri spectrophotometry (Berthold Detection Systems, Germany) (optical density at 260/280 nm ratio). The DNA samples were then sent to New Zealand Genomics Ltd (NZGL; Massey Genome Service at Massey University, Palmerston North) for sequencing on the Illumina MiSeq Sequencing Platform. Sterile distilled water was used as the negative control.

16S rRNA gene libraries were constructed using PCR to amplify the variable regions V3 and V4 using the forward 16Sf (5′-CCTACGGGAGGCAGCAG-3′) and the reverse 16Sr (5′-GGACTACHVGGGTWTCTAAT-3′) primers. Amplicons were generated using a high-fidelity polymerase (AccuPrime; Invitrogen) and then were purified using a magnetic bead capture kit (Ampure; Agencourt) and quantified using a fluorometric kit (QuantIT PicoGreen; Invitrogen). The purified amplicons were then pooled in equimolar concentrations using a SequalPrep plate normalization kit (Invitrogen), and the final concentration of the library was determined using a SYBR green quantitative PCR (qPCR) assay with primers specific to the Illumina adapters (Kappa). The 24 libraries were pooled by equal molarity and run on 25% of one 2 × 250 base PE run.

The sequence reads from the 24 samples were analysed inside the QIIME2 environment (version 2018.2) (Caporaso et al., 2010). The paired sequences were imported as the type ‘SampleData [PairedEndSequencesWithQuality]’ using a manifest file. The sequences had quality trimming and analysis performed on them using the incorporated dada2 method (Callahan et al., 2016). No samples with low reads were discarded, as the full set of 24 samples were needed for the analysis. Within the QIIME2 environment, the quality plots were manually inspected using the qiime2view viewer (www.view.qiime2.org/) after running “qiime tools import” and “qiime demux summarize”. The trimming was then performed in the dada2 denoise-paired command as “qiime dada2 denoise-paired –i-demultiplexed-seqs demux-paired-end.qza –o-table table_tf10_tr10_truf250_trur240 –o-representative-sequences rep-seqs_tf10_tr10_truf250_trur240 –p-trim-left-f 10 –p-trim-left-r 10 –p-trunc-len-f 250 –p-trunc-len-r 240”. In other words, after visual inspection, both read sequence files had the first 10 bases removed, and only read2 sequence files had the last 10 bases removed.

The data were summarised using a metadata file and also tabulated. Alpha (Shannon, chao1 and observed_otus) and beta (Euclidean and Jaccard) diversity metrics were calculated on the individual samples, and also when grouped based on the metadata factor “Season and region”. The samples were classified taxonomically using the QIMME2 feature-classifier with a modified version of the NCBI 16S rRNA BLAST database (“16SMicrobial.tar.gz”; downloaded and processed in August 2018) that had been programmatically modified to remove some of the intervening taxonomic levels from the classification.

2.3. Bacterial isolation and procedure for MALDI-TOF identification

Each of the milk samples was serially diluted using 10-fold volumes of 0.1% (w/v) sterile peptone water and cultured on milk plate count agar (MPCA) (Oxoid, Basingstoke, UK), using the spread plate technique, in triplicate. The plates were then incubated at 7 °C for 7 days to enumerate the populations of psychrotrophic bacteria (Hantsis-Zacharov & Halpern, 2007). Bacterial colonies with unique morphologies were selected from a readable plate (10–100 colonies) and streaked on MPCA to obtain pure cultures. The isolated bacteria were grown in nutrient broth (Becton Dickson, USA) overnight and then were kept on Cryopreserved beads at −80 °C for future work.

The MALDI-TOF MS method was used to rapidly identify the selected bacteria (Bruker Daltonik GmbH, Bremen, Germany). An overnight colony on tryptic soya agar (TSA) (Becton Dickinson, Cockeysville, MD, USA) was transferred into a 1.5 mL Eppendorf tube with 300 μL of sterile water and 900 μL of 100% ethanol (Sigma–Aldrich) and vortex mixed for at least 1 min, followed by centrifugation for 2 min at 13,000× *g*. The suspension was mixed with 30 μL of 70% formic acid (Fluka) and acetonitrile (Merck, Darmstadt, Germany) by vortex mixing. The supernatant was again centrifuged for 2 min at 13,000× *g*. 1 μL of mixture was dipped onto the MALDI stainless steel target plate. One microlitre of the matrix solution (HCCA, cyano-4-hydroxycinnamic acid, Bruker Daltonik) was then overlaid on the dried samples. The HCCA was made by the mixture of 50% acetonitrile and 2.5% trifluoroacetic acid (Fluka by Sigma–Aldrich, St. Louis, MO, USA). The dried target steel was loaded in the microflex LT mass spectrometer. The resulting spectra were analysed using the Bruker Daltonik MALDI Biotyper 3.0 Real Time Classification (RTC) program (Lindsay, Hill, & Venter, 2014). Each sample was tested in triplicate.

3. Results

3.1. Comparison between HTS and MALDI-TOF MS

The average psychrotrophic bacteria count from unenriched milk was in the range of 2–3 log cfu mL^{-1}. The population of psychrotrophic bacteria from the milk after 5-day enrichment at 7 °C reached to 6–7 log cfu mL^{-1}. From Milk Test New Zealand, the origin of the milk samples before enrichment, the numbers of somatic cells counts for all milk samples were less than 100,000. To compare the differences in microbial composition of the enriched milk using HTS and MALDI-TOF MS, a taxonomic analysis of 24 milk samples at the genus and species level are provided in Table 1. HTS generated a total of 4,334,304 raw reads from 24 raw milk samples, with a minimum of 66,095 and a maximum of 296,169 reads. HTS identified more than 11 genera, namely *Acinetobacter*, *Carnobacterium*, *Chryseobacterium*, *Erwinia*, *Flavobacterium*, *Hafnia*, *Kluyvera*, *Lactococcus*, *Leuconostoc*, *Pseudomonas* and *Serratia*. *Pseudomonas* appeared to be the predominant genus among all 24 samples. *Serratia*, *Lactococcus* and *Acinetobacter* were the second most prevalent genera in all other samples, whereas the remaining 7 genera together only represented a small proportion of the population. At the species level, 9 species showed higher than 1% prevalence, with *Pseudomonas psychrophila* (39.02%) and *P. fluorescens* (37.53%) dominant.

MALDI-TOF was used as a rapid method to identity culturable bacteria based on the spectra produced from protein extracts. A total of 127 psychrotrophs were cultured in this study and identified by MALDI-TOF MS, representing 6 families, 9 genera, and 20 species. MALDI-TOF analysis showed similarities in all the microbial communities and agreed with the HTS results with *Pseudomonas* dominating. The dominant genus was *Pseudomonas* (93.68%), with other genera making up 6.32% of the isolates comprising *Acinetobacter*, *Bacillus*, *Buttiauxella*, *Carnobacterium*, *Lactococcus*, *Hafnia*, *Rahnella* and *Serratia*. Among 21 the species belonging to *Pseudomonas*, *Pseudomonas lundensis* was the predominant species (24.41%), followed by *Pseudomonas fragi* (20.47%) and *P. fluorescens* (5.51%). A total of 28.33% (36/127) of *Pseudomonas* were not identifiable to the species level (1.7 < score < 2.0).

3.2. Seasonal variation

To evaluate the differences in the psychrotrophic bacterial community in raw milk across the seasons, the results from HTS are shown in Table 2. *Pseudomonas* spp. were the most abundant detected across all 4 seasons. HTS showed that *P. psychrophila* and *P. fluorescens* were the two most prevalent *Pseudomonas* species in all samples. The abundance of *P. psychrophila* was higher than *P. fluorescens* in spring, whereas the opposite was observed across the other three seasons. The abundance of the second dominant species varied in summer (*A. guillouiae*), autumn (*Serratia proteamaculans*), winter (*Lactococcus raffinolactis*) and spring (*Lactococcus lactis*). However, the percentages were all very low compared with the dominant species.

In terms of regional variation, there was no variation in predominant species that could be attributed to different locations.

Table 1
Bacteria identified by HTS and MALDI-TOF at genus level and species level.

Genus - HTS	Abundance (%)	Genus - MALDI	Abundance (%)	Species - HTS	Abundance (%)	Species - MALDI	Abundance (%)
Acinetobacter	5.37	*Acinetobacter*	0.79	*Acinetobacter guillouiae*	2.03	*Acinetobacter guillouiae*	0.79
Carnobacterium	0.23	*Bacillus*	0.79	*Acinetobacter johnsonii*	0.91	*Bacillus cereus*	0.79
Chryseobacterium	0.12	*Buttiauxella*	0.79	*Acinetobacter radioresistens*	0.73	*Buttiauxella brennerae*	0.79
Erwinia	0.50	*Carnobacterium*	0.79	*Carnobacterium maltaromaticum*	0.55	*Carnobacterium maltaromaticum*	0.79
Flavobacterium	0.22	*Lactococcus*	0.79	*Chryseobacterium carnipullorum*	0.10	*Lactococcus raffinolactis*	0.79
Hafnia	0.25	*Hafnia*	0.79	*Erwinia aphidicola*	0.44	*Hafnia alvei*	0.79
Kluyvera	1.59	*Rahnella*	0.79	*Flavobacterium hibernum*	0.15	*Rahnella aquatillis*	0.79
Lactococcus	5.33	*Pseudomonas*	93.68	*Hafnia psychrotolerans*	1.21	*Pseudomonas antarctica*	0.79
Leuconostoc	0.34	*Serratia*	0.79	*Kluyvera cryocrescens*	1.01	*Pseudomonas extremorientalis*	1.57
Pseudomonas	78.67			*Lactococcus lactis*	1.52	*Pseudomonas fluorescens*	5.51
Serratia	4.18			*Lactococcus raffinolactis*	3.03	*Pseudomonas fragi*	20.47
Others	3.20			*Pseudomonas abietaniphila*	0.01	*Pseudomonas gessardii*	2.36
				Pseudomonas chlororaphis	0.05	*Pseudomonas koreensis*	2.36
				Pseudomonas fluorescens	37.53	*Pseudomonas libanensis*	0.79
				Pseudomonas graminis	0.77	*Pseudomonas lundensis*	24.41
				Pseudomonas koreensis	1.21	*Pseudomonas protegens*	0.79
				Pseudomonas psychrophila	39.02	*Pseudomonas rhodesiae*	3.15
				Serratia proteamaculans	3.43	*Pseudomonas synxantha*	0.79
				Others	6.30	*Pseudomonas taetrolens*	2.36

Table 2
Seasonal variation in the predominant isolates detected by HTS at species level.

Isolate	Abundance (%)			
	Autumn	Winter	Spring	Summer
Pseudomonas psychrophila	38.44	37.50	49.26	30.86
Pseudomonas fluorescens	37.15	50.49	28.92	33.54
Serratia proteamaculans	9.51			
Lactococcus raffinolactis		3.04		
Lactococcus lactis			5.13	
Acinetobacter guillouiae				6.60

4. Discussion

The microbiological quality of raw milk before processing is essential to ensure the quality of processed dairy products. Although the combination of refrigeration and pasteurisation of raw milk extends the shelf life of dairy products, psychrotrophic bacterial growth before pasteurisation and the resultant spoilage of milk before or after pasteurisation has the potential to have an adverse impact on the quality of dairy products. The objective of this present study was to investigate the psychrotrophic bacterial communities of raw milk across seasons, based on culture-independent and culture-dependent approaches.

In the present work, the milk samples differed in their microbial ecology; however, both culture-independent and culture-dependent approaches showed that *Pseudomonas* were the predominant microbiota in all raw milk samples after a few days refrigeration, which is in accordance with many other studies (Champagne et al., 1994; Xin et al., 2017; Yuan et al., 2017). The reason for the high prevalence of *Pseudomonas* spp. may be due to the relatively short generation time (<4 h) at 0–7 °C of these bacteria in comparison with other psychrotrophic bacteria, which implies that one single cell can reach 10^6 cfu mL^{-1} after 8 days storage (Dogan & Boor, 2003). Yuan et al. (2017) stated that *Pseudomonas* require very limited nutrients to survive and grow even in unfavourable conditions. This might also account for the dominance of *Pseudomonas* in refrigerated raw milk. *Pseudomonas* spp. are naturally present in raw milk having their origin in the cows' feed, drinking water, bulk milk storage tank, milking machine and the farm environment (Carloni et al., 2016). Poor farm hygiene and contamination of cows' feed and water utensils may be the origins for the *Pseudomonas* in the raw milk.

Pseudomonas spp. present in raw milk are responsible for the defects in final products, such as UHT milks, due to the production of heat-resistant spoilage proteases and lipases (Stoeckel et al., 2016). The secretion of these two enzymes is commonly at a maximum in the late exponential or early stationary phase of bacteria growth (Chen, Daniel, & Coolbear, 2003).

Proteases can be defined as the enzymes that hydrolyse peptide bonds, and lipases are the carboxylesterases that hydrolyse acylglycerols. The bacterial protease present in milk preferentially degrades casein micelles, and then releases the β-lactoglobulin- κ-casein complex. The complex subsequently attaches to whey proteins and as κ-casein and forms a three-dimensional network of cross-linked proteins. These mixed proteins can accelerate the off-flavours and early gelation of UHT milk during storage (Datta & Deeth, 2003). Lipases can hydrolyse milk triacylglycerol into short-chain fatty acid, such as butyric acid, caproic acid, and caprylic acid, resulting in a soapy taste of final products (Chen et al., 2003).

The enzymes produced by *Pseudomonas* spp. can be very heat-resistant. For example, a protease from *Pseudomonas* strains retained 55–65% of the initial activity after a heat treatment at 72 °C, 15s and 20–40% activity after heat treatment at 140 °C, 5s (Haryani, Datta, Elliott, & Deeth, 2003). According to Adams, Barach, and Speck (1975), proteases from 10 different *Pseudomonas* strains all remained active after 149 °C, 10s heat treatment. The heat-stable protease is a major concern in UHT milk since it can survive the heat treatment used to produce commercially sterile milk. Even a small amount of active proteases can lead to a shortened shelf life of dairy products. Normally, the shelf life of UHT is about 6–9 months. Richardson and Newstead (1979) pointed out that UHT milk containing as little as 1 ng bacterial protease per mL had only a 3 month shelf life. Therefore, to ensure the quality of final products, raw milk should be maintained in the appropriate condition (chilled in clean vessels) before processing and processed as soon as possible to minimise the outgrowth and selection of *Pseudomonas* in raw milk.

The subdominant bacterial genera found in raw milk by both HTS and MALDI-TOF were *Acinetobacter*, *Carnobacterium*, *Hafnia*, *Lactococcus*, and *Serratia,* which agrees with previous studies using both culture-independent and culture-dependent approaches to observe the psychrotrophic microbiota in raw milk. Other genera detected in this study, *Bacillus*, *Buttiauxella*, *Chryseobacterium*, *Erwinia*, *Kluyvera* and *Rahnella* have been isolated from raw cows' milk in many other studies (Quigley et al., 2013; Vithanage et al., 2016; Yuan et al., 2017). *Acinetobacter* was the second most prevalent genus detected by both HTS and MALDI-TOF MS methods. *Acinetobacter* is frequently found in refrigerated milk samples. *A. guillouiae* is a strong lipolytic enzyme producer but less of a protease producer However, the heat stability of the enzymes produced by *Acinetobacter* is not well studied (Ercolini et al., 2009; Hantsis-Zacharov & Halpern, 2007; Vithanage et al., 2016; Xin et al., 2017; Yuan et al., 2017). *Bacillus*, *Hafnia* and *Serratia* have also been reported to produce spoilage enzymes, which influence the quality of dairy products (Vithanage et al., 2016; Xin et al., 2017; Yuan et al., 2017). *Bacillus* spp., especially *Bacillus cereus*, have been reported to be the most common Gram-positive psychrotrophic bacteria in raw milk. 40–84% of *Bacillus* spp. can produce both proteases and lipases (Samaržija et al., 2012). *Chryseobacterium* occurs frequently in raw milk, and is associated with bovine mastitis in previous studies (Hagi, Sasaki, Aso, & Nomura, 2013; Kuang et al., 2009). The *Buttiauxella* genus has been found to remain active after pasteurization (Kuang et al., 2009). The *Rahnella* genus is able to grow rapidly in milk, cream and cheese (Baruzzi, Lagonigro, Quintieri, Morea, & Caputo, 2012). Other genera have not been reported for their spoilage enzyme-producing ability, but they also have potential for limiting dairy product quality, through high cell numbers, acid-production and biofilm formation (Kives et al., 2005; Millière, Michel, Mathieu, & Lefebvre, 1994; Xin et al., 2017).

HTS revealed only 6 *Pseudomonas* spp. whereas MALDI-TOF revealed 13 different *Pseudomonas* spp. including 28.33% only identified of genus level. This is due to the selection of the isolates based on their different morphologies, which can lead to a bias. Furthermore, a relatively large proportion of *Acinetobacter*, *Lactococcus* and *Serratia* were revealed by HTS compared with MALDI-TOF analysis. One of the limitations of the culture-dependent method is the reliance on growth on agar, which is not able to support the growth requirements for all the microorganisms. Less than 2% of microorganisms on this planet can be cultivated *in vitro* (Wade, 2002). Some bacteria may not be able to grow on an agar plate due to their specialised growth requirements but they still can exist and grow in milk. *P. psychrophila* was the predominant species detected by HTS whereas it was not detected by MALDI-TOF. One possible reason is that it may not grow optimally on MPCA. The reason for the lack of detection of 28.33% culturable *Pseudomonas* spp. is most likely due to the species not included in the MALDI-TOF database (Weber et al., 2014).

No identification method is ideal. The use of HTS will detect both live and dead bacteria so may not be relevant to some studies that rely on the viable cell population. In addition, milk is a complex matrix of fats, protein, carbohydrates and minerals, which can interfere with the distribution of PCR products with overloading with bacterial biomass, and so the total DNA might overburden the PCR reaction (Jost, Lacroix, Braegger, & Chassard, 2013; Šuranská et al., 2016). Both approaches (HTS and MALDI-TOF) present unique challenges for identification and interpretation of biologically meaningful information, and for the moment, the high costs associated with high-throughput sequencing limits full exploitation. Although HTS can provide a full picture for profiling the microbial ecology of raw milk, for single bacterial isolation and study, MALDI-TOF is a cheap, reliable and efficient method for identifying culturable bacteria. As such, combined approaches may offer the best possibility for achieving an understanding of complex microbial communities. Thus, sequencing of the 16S rDNA gene and MALDI-TOF continues to offer a powerful and economic way to gain insight into the bacterial community composition in large numbers of samples.

The effects of seasonal variation on microbial quality of raw milk have been extensively explored in many other studies but there is little information on psychrotrophic bacteria (Doyle, Gleeson, O'Toole, & Cotter, 2017; Kable et al., 2016; Mallet et al., 2012). In the present study, the changes in the microbial ecology of milk due to seasonality revealed by HTS can potentially be important for milk suppliers and dairy manufacturers. There is a clear trend in the abundance of *Pseudomonas* spp. according to the season, with most being found in winter, followed by spring, autumn, and fewest in summer. According to The National Institute of Water and Atmospheric Research (1981–2010) (www.niwa.co.nz), the average temperatures in winter, spring, autumn, and summer, are 14, 17, 19 and 22 °C, respectively. The rainfall is 114.5, 118.0, 117.7, and 105.2 mm, respectively. In New Zealand, dairy cows are mainly fed year-round by grazing pastures, which comprises 96% of the total diet (De Klein, Smith, & Monaghan, 2006). As *Pseudomonas* thrive in wet and cold environments, the seasonal climate changes may influence microbial composition in the farm environment. The increased growth of *Pseudomonas* in the farm environment will increase the chance of raw milk contamination.

HTS show the bacterial species dominance differed across the seasons of the year with *P. psychrophila* predominating in spring and *P. fluorescens* in the other three seasons. The prevalence of *P. fluorescens* and *P. psychrophila* in raw milk samples has been widely reported (Munsch-Alatossava & Alatossava, 2006; Samaržija et al., 2012). *P. fluorescens* is believed to be a stronger proteolytic producer than *P. psychrophila* due to the presence of the apex gene encoding heat-resistant protease (Malmgren et al., 2017; Matéos et al., 2015; Xin et al., 2017). *P. fluorescens* also produces lipases. Xin et al. (2017) reported that *P. psychrophila* was able to produce protease, but did not mention lipases.

Previous studies suggested that the fluctuation of microbiota may be linked to the optimum growth temperature of these bacteria (Griffiths, Phillips, & Muir, 1987). The optimum growth conditions for *P. psychrophila* are mild temperatures and wet conditions, which can favour the growth of *P. psychrophila* on farm even before milking. With the high numbers of *P. psychrophila* on farm, the milk will have higher chance of contamination. As New Zealand dairy cows are pasture fed, the feed during spring is relatively wet, which creates a niche for them to grow. In addition, contamination of drinking water and uncleaned utensils used on the farm will provide additional opportunity for milk contamination.

This result suggests that samples of milk taken in spring appear to favour a different predominant *Pseudomonas* spp. than the other three seasons. HTS showed an increase in the relative proportion of bacteria belonging to *A. guillouiae* in summer. Kable et al. (2016) reported that the environment in which the herd was kept was the primary driver of the milk microbiota. The variations in microbial populations observed in this study are likely to be due to warmer temperatures and less precipitation in summer, which favours the growth of *Acinetobacter*. Yuan et al. (2017) stated that *Acinetobacter* spp. is characterised by its tendency to tolerate drying. Another possible reason is the temperature abuse of milk during transportation and storage at the processing plant.

According to the Ministry for Primary Industries New Zealand Code of Practice for the design and operation of farm dairies (www.foodsafety.govt.nz), raw milk must enter the bulk tank at 6 °C or below. However, in New Zealand, raw milk is transported from the dairy farm to the dairy processing plant by milk tanker. Most of the milk tankers in New Zealand are single-skinned and without refrigeration systems. There is concern that milk is collected from the farm at temperatures below 6 °C but may be transported and stored at temperatures higher than 6 °C before pasteurisation. Failure to keep raw milk under appropriate refrigeration may lead to a shift in the raw milk microbiota community.

S. proteamaculans was the third predominant species in autumn followed by two *Pseudomonas* spp. (Machado et al., 2016). They have been isolated from previous studies and able to produce proteases but their growth characterisation and effect on dairy product has not been well reported. A relatively small abundance of *L. raffinolactis* and *L. lactis* were found in winter and spring, respectively. They are mesophilic lactic acid bacteria that naturally thrive in the environment and mostly found on cow teats. They are not able to produce spoilage enzymes and can be totally eliminated through pasteurisation.

There is some evidence on how seasonal variation affects milk quality (Hantsis-Zacharov & Halpern, 2007; Kable et al., 2016; Vithanage et al., 2016). However, seasonal variation cannot be controlled and therefore controlling farm hygiene and avoiding raw milk temperature abuse continue to be important management tools in controlling raw milk quality on the farm. In this study, milk samples were combined farm samples from each region, not individual, to make some attempt at providing a representative sample. On farm problems at each farm may also contribute to the changes of the microbial composition of raw milk.

5. Conclusions

This study demonstrated the complementarity of culture-independent (HTS) and culture based (MALDI-TOF) methods for evaluating the psychrotrophic populations that may be present in raw milk. Although MALDI-TOF can be used as a rapid and accurate method to identify cultured isolates, combining this technique with HTS might provide a more comprehensive insight into the microbial composition of raw milk. Studies profiling the bacterial communities of raw milk can demonstrate changes in the microbial populations through a dairy season. This understanding may assist in selecting milk supplies that are best suited to the manufacture of specific products with the highest microbial quality requirements.

Acknowledgement

This work was financially supported by Tetra Pak, New Zealand.

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