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Culture-independent bacterial community profiling of carbon dioxide treated raw milk

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ABSTRACT

Due to technical simplicity and strong inhibition against the growth of psychrotrophic bacteria in milk, CO_2 treatment has emerged as an attractive processing aid to increase the storage time of raw milk before downstream processing. However, it is yet to be adopted by the industry. In order to further explore the suitability of CO_2 treatment for raw milk processing, the bacterial populations of carbonated raw milk collected locally from five different sources in Australia were analysed with next-generation sequencing. Growth inhibition by CO_2 was confirmed, with spoilage delayed by at least 7 days compared with non-carbonated controls. All non-carbonated controls were spoiled by Gammaproteobacteria, namely *Pseudomonas fluorescens* group bacteria, *Serratia* and *Erwinia*. Two out of the five carbonated samples shared the same spoilage bacteria as their corresponding controls. The rest of the three carbonated samples were spoiled by the lactic acid bacterium (LAB) *Leuconostoc*. This is consistent with higher tolerance of LAB towards CO_2 and selection of LAB in meat products stored in CO_2-enriched modified atmosphere packaging. No harmful bacteria were found to be selected by CO_2. LAB are generally regarded as safe (GRAS), thus the selection for *Leuconostoc* by CO_2 in some of the samples poses no safety concern. In addition, we have confirmed previous findings that 454 pyrosequencing and Illumina sequencing of 16S rRNA gene amplicons from the same sample yield highly similar results. This supports comparison of results obtained with the two different sequencing platforms, which may be necessary considering the imminent discontinuation of 454 pyrosequencing.

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1. Introduction

The establishment of the cold chain has extended the storage time of raw milk before processing due to inhibition of mesophilic bacteria. However, low temperature favours the growth of psychrotrophic organisms, among which those belonging to the *Pseudomonas fluorescens* group are predominant spoilage bacteria of raw milk (Boor and Fromm, 2009; Heyndrickx et al., 2010; Sørhaug and Stepaniak, 1997; Ternström et al., 1993). Although the majority of psychrotrophic bacteria can be effectively killed by pasteurisation, many species, particularly those of the *P. fluorescens* group, produce heat-stable lipases and proteinases which remain active post-pasteurisation (Fairbairn and Law, 1986; Sørhaug and Stepaniak, 1991, 1997; Stead, 1986). Therefore, controlling the proliferation of psychrotrophic bacteria during refrigerated storage of raw milk is crucial to maintaining the shelf-lives of the derived dairy products.

Carbon dioxide has emerged as an attractive preservative of raw milk as it is inhibitory against the growth of psychrotrophic bacteria including *P. fluorescens* (Hotchkiss et al., 2006; Loss and Hotchkiss, 2003; Martin et al., 2003), it is generally regarded as safe (GRAS), its treatment method is non-thermal, simple and economical, and it does not have adverse effects on the nutritional content of milk (Loss and Hotchkiss, 2003; Ruas-Madiedo et al., 1996; Sierra et al., 1996). The effectiveness of CO_2 in extending the storage life of raw milk and cottage cheese has been well established (Amigo et al., 1995; Chen and Hotchkiss, 1991, 1993; Espie and Madden, 1997; King and Mabbitt, 1982; Kosikowski and Brown, 1973; Maniar et al., 1994; Moir et al., 1993; Roberts and Torrey, 1988; Ruas-Madiedo et al., 1996; Ruas-Madiedo et al., 1998b). CO_2 treatment is already routinely used in commercial cottage cheese production in the US (Loss and Hotchkiss, 2003), and an unpublished scaled-up field trial of CO_2 treatment of raw milk showed a 4 day increase in storage life of the treated sample (Hotchkiss et al., 2006). However, CO_2 remains to be adopted by the industry for raw milk processing.

Some reasons for the hesitation of introducing CO_2 into raw milk processing could be the possible selection of pathogens and sporeformers and increased risk of toxin production during the

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extended storage time before downstream processing. Studies to date on the effect of CO_2 on the growth of *Listeria monocytogenes*, *Bacillus cereus* and *Clostridium sporogenes* in milk or cottage cheese and toxingenesis of *Clostridium botulinum* in milk have shown that CO_2 treatment does not pose increased risk with regard to these factors (Chen and Hotchkiss, 1993; Glass et al., 1999; Werner and Hotchkiss, 2002). In addition, culture-independent studies on the native bacterial composition of carbonated raw milk during cold storage have found no evidence of selection of harmful bacteria (Rasolofo et al., 2011; Rasolofo et al., 2010). The current study aims to further explore the bacterial profiles of carbonated raw milk using culture-independent next-generation sequencing of 16S rRNA gene amplicons. Compared to the 16S rRNA gene-based techniques used in previous studies, namely gene clone libraries, terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) (Rasolofo et al., 2011; Rasolofo et al., 2010), next-generation amplicon sequencing offers much greater sampling depth and requires less manual handling and subjective judgement, making results more representative and reproducible. Thus the current study will be a meaningful addition to the literature of CO_2 treatment of dairy products.

Pyrosequencing has already been used in a large number of recent studies in which the microbial communities of dairy products were characterised, including raw and pasteurised milk and various types of cheese (Aldrete-Tapia et al., 2014; de Filippis et al., 2014; de Pasquale et al., 2014; Ercolini et al., 2012; Guidone et al., 2016; Masoud et al., 2011; Masoud et al., 2012; Quigley et al., 2013a; Quigley et al., 2012; Riquelme et al., 2015). We have recently analysed the bacterial populations of fresh and spoiled carbonated raw sweet whey using pyrosequencing of 16S rRNA gene amplicons (Lo et al., 2016). The carbonated sample was spoiled by the same bacteria as the non-carbonated control, namely *Pseudomonas*, *Serratia* and other *Enterobacteriaceae*, which are common milk spoilage organisms. It would be of interest to determine whether similar organisms would be found in spoiled carbonated raw milk.

This study aims to confirm the effectiveness of CO_2 in inhibiting bacterial growth in raw milk samples obtained from geographically distinct sites and to characterise the microbial populations of fresh and spoiled raw milk using culture-independent 16S rRNA community profiling. The findings will help determine the suitability of incorporating CO_2 treatment into raw milk processing.

2. Materials and methods

2.1. Sources of raw milk

Raw milk was collected from five different sources located in two different states of Australia (Table 1) and transported to the University of Queensland (UQ), where carbonation and analyses were performed. Milk samples were transported in an insulated container with ice bricks. Samples collected in Queensland arrived at UQ within an hour. SF2, which was collected in Victoria, was first transported to Dairy Innovation Australia Limited (DIAL) where it was stored in a 4 °C cold room before being packaged with ice bricks and transported by plane to Queensland. The SF2 sample arrived at UQ within the same day as sample collection. Upon arrival at UQ, all samples were held in a 4 °C cold room before carbonation. The day of sample collection was designated as day 0. All samples were carbonated on day 0. Henceforth, the samples are abbreviated by their source and treatment. For example, DP1-control and DP1-CO_2 stand for the non-carbonated control and carbonated sample from DP1 respectively.

2.2. Carbonation, measurement of CO_2 concentration and milk storage conditions

Carbonation and measurement of CO_2 concentration were carried out as previously described (Lo et al., 2016). Samples were carbonated to achieve CO_2 saturation (2146–3540 ppm). The carbonated milk was divided into 30 mL aliquots in sterile sealed plastic containers and stored at 4 °C. One aliquot was used for analysis at each time point. A non-carbonated control was included in the analysis of each sample.

2.3. Bacterial growth studies

Bacterial growth in the raw milk samples was monitored by a plate count method. One millilitre of milk was taken from a well mixed 30 mL sample aliquot and used to prepare serial tenfold dilutions in 0.1% peptone water. One hundred microlitres were spread plated onto nutrient agar (Oxoid, Basingstoke, UK) and incubated at 30 °C for 3 days. Undiluted samples were also plated on day 0 to allow for low bacterial counts. Analysis of a sample was terminated when it was spoiled. Spoilage was defined by bacterial counts on nutrient agar reaching a threshold of 10^6 CFU/mL. CO_2-treated samples were also plated onto MRS agar (Oxoid, Basingstoke, UK) and incubated at 37 °C anaerobically to monitor the growth of *Lactobacillus* and *Leuconostoc*.

2.4. DNA extraction for pyrosequencing

One millilitre aliquots of milk were collected on the same day as plating for treatment with propidium monoazide (PMA) (Biotium, Hayward, CA, USA) and stored at −80 °C until ready for DNA extraction. The purpose of the PMA treatment was to prevent DNA from dead bacteria from being extracted and amplified in PCR (Nocker et al., 2006). All milk samples except those from DP1 were treated with PMA prior to DNA extraction. PMA treatment and DNA extraction were performed using methods described previously (Lo et al., 2016).

2.5. Pyrosequencing

PCR was performed with GoTaq® DNA Polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The primers, provided by the Australian Centre for Ecogenomics (ACE, UQ), were non-barcoded and targeted the V5–V8 region of the 16S rRNA gene (forward primer: a mixture of TTAGATACCCTGGTAGTC, TTAGATACCCSGGTAGTC, TTAGATACCCYHGTAGTC and TTAGAGACCCY GGTAGTC in a 2:1:1:1 ratio respectively; reverse primer: ACGGGC GGTGWGTRC). The resulting PCR products were then submitted to ACE for a second PCR with barcoded primers and Roche 454 pyrosequencing (Margulies et al., 2005; Rothberg and Leamon, 2008). The sequencing data were analysed with QIIME (Caporaso et al., 2010). The default parameters of the scripts were used in most cases. Reads

Table 1
Details of raw milk sample sources.

Sample source	Location[a]	Scale	Month of sample collection (season)
Dairy processing plant 1 (DP1)	Queensland	Large scale, one of major national dairy producers	February 2013 (summer)
Dairy processing plant 2 (DP2)	Queensland	Large scale, one of major national dairy producers	March 2014 (autumn)
Medium farm (MF)	Queensland	~250 cows	March 2014 (autumn)
Small farm 1 (SF1)	Queensland	~50 cows	March 2014 (autumn)
Small farm 2 (SF2)	Victoria	4 cows	May 2014 (autumn - winter)

[a] State in Australia.

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