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Inhibition of bacterial growth in sweet cheese whey by carbon dioxide as determined by culture-independent community profiling

Raquel Lo [a], Tian Xue [a], Mike Weeks [b], Mark S. Turner [a,c] and Nidhi Bansal [a,*]

[a] School of Agriculture and Food Sciences, The University of Queensland, St Lucia, QLD 4072, Australia

[b] Dairy Innovation Australia Limited, Werribee, VIC 3030, Australia

[c] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD 4072, Australia

*Corresponding author

Tel.: +61 7 33651673

E-mail address: n.bansal@uq.edu.au

Postal address: School of Agriculture and Food Sciences, Hartley Teakle Building (No. 83), The University of Queensland, St Lucia, QLD 4072, Australia

Abstract

Whey is a valuable co-product from cheese making that serves as a raw material for a wide range of products. Its rich nutritional content lends itself to rapid spoilage, thus it typically needs to be pasteurised and refrigerated promptly. Despite the extensive literature on milk spoilage bacteria, little is known about the spoilage bacteria of whey. The utility of carbon dioxide (CO_2) to extend the shelf-life of raw milk and cottage cheese has been well established, but its application in whey preservation has not yet been explored. This study aims to characterise the microbial populations of fresh and spoiled sweet whey by culture-independent community profiling using 454 pyrosequencing of 16S rRNA gene amplicons and to determine whether carbonation is effective in inhibiting bacterial growth in sweet whey. The microbiota of raw Cheddar and Mozzarella whey was dominated by cheese starter bacteria. After pasteurisation, two out of the three samples studied became dominated by diverse environmental bacteria from various phyla, with Proteobacteria being the most dominant. Diverse microbial profiles were maintained until spoilage occurred, when the entire population was dominated by just one or two genera. Whey spoilage bacteria were found to be similar to those of milk. Pasteurised Cheddar and Mozzarella whey was spoiled by *Bacillus* sp. or *Pseudomonas* sp., and raw Mozzarella whey was spoiled by *Pseudomonas* sp., *Serratia* sp., and other members of the *Enterobacteriaceae* family. CO_2 was effective in inhibiting bacterial growth of pasteurised Cheddar and Mozzarella whey stored at 15°C and raw Mozzarella whey stored at 4°C. The spoilage bacteria of the carbonated samples were similar to those of the non-carbonated controls.

Keywords

whey spoilage, whey microbiota, CO_2 treatment, pyrosequencing

1. Introduction

Historically, whey was considered as waste, or at most a by-product, from cheese and casein production. However, through advancements in science and technology over the years, whey has now become a valuable co-product (Pescuma et al., 2010). The rich lactose and protein content of whey makes it prone to spoilage by bacteria (Reddy et al., 1976; Varnam and Sutherland, 1994; Webb and Whittier, 1948). To utilise whey as a raw material for high quality whey-based ingredients, it is essential to prevent its spoilage immediately after production.

There has been considerable interest in using carbon dioxide (CO_2) to extend the shelf-life of dairy products due to its antimicrobial activity, generally regarded as safe (GRAS) status and ease of incorporation into dairy processing (Hotchkiss et al., 2006; Loss and Hotchkiss, 2003). The effectiveness of CO_2 in shelf-life extension has been well established in raw milk and cottage cheese (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, Bascarán, et al., 1998) and has already been adopted in commercial cottage cheese production in the US (Loss and Hotchkiss, 2003). However, there have been no studies on carbonation of whey, so it would be of interest to test the potential of CO_2 to act as a whey preservative.

Little is known about the specific genera of bacteria that spoil whey. Since cheese milk contains large numbers of starter bacteria, they are likely to dominate the microbial population of whey and thus may cause whey spoilage. On the other hand, whey may be spoiled by bacteria that spoil milk. Raw and pasteurised milk exposed to post-pasteurisation contamination is most often spoiled by Gram-negative bacteria, predominantly *Pseudomonas*,

while uncontaminated pasteurised milk is mainly spoiled by the Gram-positive thermoduric sporeformers, *Bacillus* and *Paenibacillus* (Boor and Fromm, 2009; Heyndrickx et al., 2010; Sørhaug and Stepaniak, 1997; Ternström et al., 1993). CO_2 is strongly inhibitory against the growth of Gram-negative bacteria, such as *Pseudomonas fluorescens* and *Escherichia coli*, but its effect on Gram-positive bacteria is less pronounced (Hotchkiss et al., 2006; Loss and Hotchkiss, 2003; Martin et al., 2003). Interestingly, CO_2 has been reported to have little effect on the growth of some cheese starter bacteria including *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus* sp. (Espie and Madden, 1997; Ruas-Madiedo, Alonso, et al., 1998; van Hekken et al., 2000; Vinderola et al., 2000).

In order to determine effective ways of controlling whey spoilage, the microbiota of fresh and spoiled whey needs to be characterised. This can be achieved by culture-independent community profiling using next generation sequencing of barcoded 16S rRNA gene amplicons (Degnan and Ochman, 2012; Rothberg and Leamon, 2008). Pyrosequencing has 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, McCarthy, et al., 2013; Quigley, O'Sullivan, et al., 2012; Riquelme et al., 2015). Many of the cheese studies cover various stages of cheese making, and fermented whey used as natural whey culture has been analysed (Aldrete-Tapia et al., 2014). However, fresh whey has not been examined. Pyrosequencing has also been used to study microbial populations of spoiled meat and seafood (Chaillou et al., 2014; Ercolini et al., 2011; Madigan et al., 2014), but spoiled dairy products has only been covered in one pyrosequencing study on high-moisture

Mozzarella (Guidone et al., 2016). Whey and other dairy products spoiled during low temperature storage has not been subjected to pyrosequencing analyses to date.

Since Gram-positive bacteria are known to be more resistant to inhibition by CO_2, there have been concerns that Gram-positive pathogens such as *Bacillus cereus* and *Listeria monocytogenes* can be enriched by CO_2. However, all reports to date have not found enhanced growth of these bacteria in CO_2-treated dairy products (Chen and Hotchkiss, 1993; Werner and Hotchkiss, 2002). In these studies, the bacteria of interest were inoculated into the products and their growth monitored by plate counts on culture media. Culture-independent community profiling using 16S rRNA gene amplicons offers another approach to determine whether undesirable native bacteria are selected by CO_2 treatment. This approach has the added benefit of a broader coverage as all bacteria are targeted using universal primers for 16S rRNA genes. CO_2-treated milk has been analysed by 16S rRNA-based culture-independent methods including 16S rRNA gene clone libraries, terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) (Rasolofo et al., 2011; Rasolofo et al., 2010). However, there has been no culture-independent community profiling of carbonated dairy products using next generation sequencing thus far.

This study aims to investigate the effectiveness of CO_2 in inhibiting bacterial growth in sweet whey and to characterise the microbial populations of fresh and spoiled sweet whey using pyrosequencing. This is the first study in which pyrosequencing is used in the analysis of fresh and spoiled sweet whey. It is also the first study in which a CO_2-treated food product is analysed with pyrosequencing.

2. Materials and methods

2.1 Sources of sweet whey

Raw sweet whey was collected from commercial cheese factories, pasteurised at 72°C for 15 s at Dairy Innovation Australia Limited (DIAL) (Werribee, VIC, Australia) and sent on ice by overnight delivery to the University of Queensland (UQ) (St Lucia, QLD, Australia). The day on which the whey was received and carbonated at UQ was defined as day 0. A total of 3 whey samples were collected from two different cheese factories in Victoria, Australia: a mixed whey sample from Cheddar and Mozzarella production from factory 1, a Cheddar whey sample from factory 2 and a Mozzarella whey sample from factory 2. The samples differ in the time elapsed between sample collection, pasteurisation and carbonation. The mixed whey was delivered 1 day after cheese production to DIAL, stored for 3 days at 4°C before pasteurisation and arrived at UQ after 2 days. The Cheddar and Mozzarella whey samples were collected 12-13 h after cheese production started, pasteurised within 4 h and arrived at UQ the next day. The samples were collected 4 months apart: the mixed whey was collected in October 2013 (spring), the Cheddar whey in February 2014 (summer), and the Mozzarella whey in June 2014 (winter).

2.2 Carbonation, measurement of CO_2 concentration and whey storage conditions

Approximately 300 mL of whey was carbonated in a sterile 500 mL PET bottle fitted with a Carbonater™ valve coupling (Liquid Bread, Orlando, FL, USA) which was connected to a CO_2 cylinder via a disconnect (ball-lock fitting) and LLDPE tubing. CO_2 at an output pressure of 0.8 bar was injected into the headspace of the whey for 20 s. Then the disconnect was removed, and the bottle shaken vigorously to aid CO_2 infusion into the whey. This injection-shaking procedure was repeated for five more times to achieve CO_2 saturation in the whey (2300-3200 ppm). Before use, the Carbonater, disconnect and LLDPE tubing were sterilised

in a 1:80 dilution of Milton solution (a commercial preparation of 2% sodium hypochlorite and 16.5% sodium chloride). The concentration of CO_2 was measured with the InPro 5000 dissolved CO_2 sensor (Mettler Toledo, Columbus, OH, USA). The whey was divided into 30 mL aliquots and stored at 15°C, and for some samples, also at 4°C. One aliquot was used for analysis at each time point. Non-carbonated pasteurised whey from the same batch of sample was studied simultaneously as a control.

2.3 Bacterial growth studies

Bacterial growth in the whey samples was monitored by plate counts. One mL of whey was taken from a well mixed 30 mL sample aliquot and used to prepare serial tenfold dilutions in 0.1% peptone water. One hundred µL of the dilutions were spread plated onto nutrient agar (NA) and M17 agar (Oxoid, Basingstoke, UK) and incubated at 30°C for 2 days. Undiluted samples were also plated out when bacterial counts were expected to be low. A whey sample was considered to be spoiled when bacterial counts increased by at least 100-fold compared to day 0, or when the dominant colony types changed drastically compared to day 0.

2.4 DNA extraction for pyrosequencing

One mL aliquots of whey were collected for pyrosequencing on the same day as plating. The samples were treated with propidium monoazide (PMA) (Biotium, Hayward, CA, USA) on the same day in order to prevent DNA from dead bacteria from being extracted and amplified in PCR (Nocker et al., 2006). The PMA treatment protocol was based on the manufacturer's instructions and a previous study (Cattani et al., 2013). The 1 mL whey sample was centrifuged and the pellet was resuspended in 500 µL of 0.1% peptone water. PMA was added to a final concentration of 30 µg/mL, followed by incubation in the dark with shaking for 7 min. After that, the samples were placed on ice and exposed to light from a 500 W

halogen lamp at a distance of 20 cm. The samples were then centrifuged, and the resulting pellets were stored at -80°C until DNA extraction with the PowerFood™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, US). DNA extraction was performed according to the manufacturer's instructions, with the following modifications: (i) the sample was incubated at 70°C for 10 min before homogenisation, based on recommendations by Quigley et al. (Quigley, O'Sullivan, et al., 2012); (ii) homogenisation was done with a Mini-Beadbeater (Biospec, Bartlesville, OK, USA) instead of vortexing.

2.5 Pyrosequencing

PCR to amplify the 16S rRNA gene 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) based at UQ, were non-barcoded and targeted the V5-V8 region of the 16S rRNA gene (forward primer: a mixture of 5'-TTAGATACCCTGGTAGTC-3', 5'-TTAGATACCCSGGTAGTC-3', 5'-TTAGATACCCYHGTAGTC-3' and 5'-TTAGAGACCCYGGTAGTC-3' in a 2:1:1:1 ratio respectively; reverse primer: 5'-ACGGGCGGTGWGTRC-3'). The resulting PCR products were then submitted to ACE for barcoding with a second PCR, multiplexing and Roche 454 pyrosequencing.

2.6 Analysis of pyrosequencing data

The sequencing data were analysed with QIIME (Caporaso et al., 2010). The default parameters of the scripts were used in most cases. Reads were excluded if their average quality score were below 25, if their length was outside 200-1000 bp, if they had more than 6 ambiguous bases, if they had a homopolymer run exceeding 6 bases or if there were any primer mismatches. Reverse primer sequences, if present, were removed from the reads.

Sequences with a similarity of at least 97% were grouped into operational taxonomic units (OTUs) by Uclust (Edgar, 2010). A representative set of sequences were then picked from the OTUs, and their taxonomy assigned by Uclust against the Greengenes 16S rRNA database (McDonald et al., 2012) with a sequence similarity threshold of 90%. OTUs with unassigned taxonomies were excluded from subsequent analyses. The most specific taxonomy assignment with this method was often to the genus level only. Representative sequences of dominant OTUs belonging to the same genus were analysed by BLAST (Altschul et al., 1990) and species assignment was given where applicable. The summarize_taxa_through_plots script in QIIME was used to combine OTUs to the genus level and for normalisation. Rarefaction curves and Good's coverage were calculated using the alpha rarefaction script in QIIME.

2.7 Accession number of pyrosequencing data

The raw pyrosequencing data of the three whey samples analysed in this study have been deposited into the European Nucleotide Archive under the study number PRJEB9517. A key listing the sample IDs in the sequence files with the corresponding sample descriptions is available in the supplemental data.

2.8 Identification of bacterial isolates

Single colonies of bacterial isolates of interest were grown in standing cultures in nutrient or M17 broth and 2 mL of stationary phase cultures were centrifuged to obtain cell pellets. The pellets were stored at -20°C until DNA extraction. DNA was extracted using chloroform-isoamyl alcohol as previously described (Prasad and Turner, 2011). The resulting DNA extract acted as template for PCR amplification of the 16S rRNA gene using primers designed against conserved regions upstream of V1 and downstream of V8 (forward primer:

5'-AGAGTTTGATCCTGGCTCA-3', reverse primer: 5'-CGGTGAATACGTTCCCG-3'). The PCR products were sent to Macrogen (Seoul, Korea) for DNA purification and Sanger sequencing. Bacteria were identified with BLAST (Altschul et al., 1990).

2.9 Reducing SDS-PAGE

Whey samples were stored at -20°C until analysis. Prior to reducing SDS-PAGE, the whey was thawed and diluted 1/6 to result in a final protein concentration of approximately 1 mg/mL. Reducing SDS-PAGE was carried out as previously described (Wijayanti et al., 2013). Briefly, 100 µL of the diluted sample was mixed with 200 µL of loading buffer (containing SDS, bromophenol blue and glycerol) and 5µL of β-mercaptoethanol and the mixture incubated at 95°C for 5 min. After cooling down, 5 µL of the sample mixture was loaded into a well of a 4-20% Mini-PROTEAN® TGX™ precast gel (Bio-Rad, Hercules, CA, USA) and electrophoresis was carried out at 200 V until the dye front ran off (approximately 50 min). The gel was stained with 0.1% Coomassie Brilliant Blue G250 overnight, destained with 10% acetic acid, and scanned with a densitometer.

3. Results and discussion

3.1 Characteristics of pyrosequencing data

Whey samples supplied by two cheese factories were analysed in this study using both culture-based and culture-independent methods. They included mixed whey (containing whey from Cheddar and Mozzarella production) from factory 1 and Cheddar whey and Mozzarella whey from factory 2. The number of pyrosequencing reads from 16S rRNA gene amplicons obtained from whey samples after quality filtering are shown in Table 1. The mean length of each sequence after quality trimming was close to 500 bp (499 bp for the mixed whey and 508 bp for the other whey samples). The shapes of the rarefaction curves plotted with the

observed number of OTUs (Fig. 1) and Good's coverage at the highest sequences per sample (ranging from 0.990 to 0.998) suggest the sequencing depths were sufficient.

3.2 Initial bacterial communities of raw whey are dominated by starter bacteria

It was hypothesised that the initial bacterial population of raw whey would be dominated by cheese starter bacteria. This was confirmed by pyrosequencing results of raw Cheddar and Mozzarella whey, in which cheese starter taxa formed 65% and 99.6% of all OTUs respectively at day 0 (Fig. 2a; pyrosequencing not done for raw mixed whey). The taxa found are also consistent with the types of starter used by the cheese factory: a mix of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Streptococcus thermophilus* and *Lactobacillus* sp. for Cheddar, and *Streptococcus thermophilus* for Mozzarella. High counts of lactic streptococci-like bacteria on M17 (10^6-10^8 CFU/mL) are consistent with the pyrosequencing results (Fig 2b; counts of raw Cheddar whey not shown).

3.3 Carbonation delays spoilage of raw Mozzarella whey and maintains the initial bacterial composition

The ability of CO_2 to inhibit spoilage bacteria growth in raw Mozzarella whey despite the high number of bacteria at day 0 was subsequently determined by carbonating raw Mozzarella whey and monitoring bacterial growth in relation to the non-carbonated control during storage at 15 and 4°C. CO_2 retarded non-starter bacteria growth (determined using NA) when the raw Mozzarella whey was stored at 15°C up to day 3, but the final numbers were similar between carbonated and non-carbonated samples at day 7 (Fig. 2b). Lowering the storage temperature to 4°C did not slow down bacterial growth in non-carbonated raw Mozzarella whey, however CO_2 addition strongly inhibited NA bacterial counts at least until day 7 (Fig. 2d). The bacterial population of the day 7 carbonated raw Mozzarella whey stored

at 4°C remained similar to that of the day 0 sample, being dominated by *S. thermophilus* (Fig. 2a).

All the spoilage taxa in raw Mozzarella whey detected by pyrosequencing are γ-proteobacteria (Fig. 2a). At day 7, both samples stored at 15°C contained *Serratia* sp. and members of the *Enterobacteriaceae* family. The spoilage *Enterobacteriaceae* OTUs could not be further resolved into specific genera. The carbonated sample contained a higher proportion of *Serratia* than the non-carbonated control (64% vs 18%, Fig. 2a). Speculative support for selection of *Serratia* by CO_2 exists as *Serratia* has been reported to be able to grow at high CO_2 concentrations (Schuerger et al., 2013).

Non-carbonated raw Mozzarella whey stored at 4°C was spoiled by members of the *Pseudomonas fluorescens* group (Fig. 2a). The spoilage microbiota of carbonated raw Mozzarella whey stored at 4°C were not analysed by pyrosequencing, but representative colony types isolated at day 34, when spoilage had already occurred (Fig. 2d), were identified by Sanger sequencing of the 16S rRNA gene. The results varied with culture media. NA contained a single colony type which was identified as a member of *Enterobacteriaceae*. On M17, the dominant colony type (88%) was *S. thermophilus*, and the second most dominant (13%) was a member of the *P. fluorescens* group. Considering the 1000-fold increase in NA counts in the carbonated sample after day 7 at 4°C (Fig. 2d), it is likely that the dominant spoilage bacteria belong to the *Enterobacteriaceae* family and *P. fluorescens* group.

Since *Pseudomonas*, *Serratia* and many *Enterobacteriaceae* are psychrotrophs, low storage temperature seems to be a dominant factor that selects for spoilage of raw Mozzarella whey by these organisms. This scenario is similar to that in raw milk which is spoiled by the same

types of psychrotrophs. It is worth considering whether the difference in the dominant spoilage genus in the non-carbonated sample stored at different temperatures (4 and 15°C) is not a random occurrence but is due to differences in bacterial growth rates. Considering that *Pseudomonas* is the leading cause of spoilage of refrigerated raw milk, it is possible that it has a competitive advantage over *Enterobacteriaceae* in the raw whey stored at 4°C, leading to its dominance at this temperature.

3.3 Initial bacterial communities of pasteurised whey consist of both starter and non-starter bacteria

Day 0 pasteurised mixed whey had a highly homogeneous bacterial population dominated by *S. thermophilus* (97%) (Fig. 3a), similar to that of raw Mozzarella whey (Fig. 2a). The type of starter used for producing the cheese from which the mixed whey originated was unknown, but the presence of *S. thermophilus* is consistent with the starter usually used for producing Mozzarella. *Thermus thermophilus* was also found as a minor component at day 0 (3%) (Fig. 3a). This species is an extreme thermophile which has been found in tube heat exchangers used in milk processing (Roberts et al., 2005).

In contrast to the mixed whey, the day 0 pasteurised Cheddar and Mozzarella whey had much more diverse bacterial profiles (Fig. 4a, 5a). The increase in diversity was particularly striking in the Mozzarella whey, as the raw whey contained almost 100% *S. thermophilus*, whereas 36% of the raw Cheddar whey microbiota was already made up of non-starter bacteria (Fig. 2a). After pasteurisation, non-starter bacteria dominated the microbiota of the Cheddar and Mozzarella whey, comprising 57-89% of all OTUs (Fig. 4a, 5a). Proteobacteria are the dominant phylum among the non-starter bacteria, constituting 57-97% of the non-starter component, but members of Firmicutes, Bacteroidetes, Actinobacteria, and

Planctomycetes were also found. The non-starter bacteria found are mostly environmental bacteria found in water or soil, and the majority of these taxa or their close relatives have been found in milk, curd and cheese (Aldrete-Tapia et al., 2014; de Filippis et al., 2014; Quigley, McCarthy, et al., 2013; Quigley, O'Sullivan, et al., 2013). One noteable prominent non-starter species that was common to the initial populations of pasteurised Cheddar and Mozzarella whey was *Petrobacter succinatimandens*, forming 14-58% of all OTUs (Fig. 4a, 5a). *P. succinatimandens* is a β-proteobacterium first isolated from an Australian oil well (Salinas et al., 2004). Pyrosequencing studies have found *Petrobacter* in oilfields and infant stool samples (Lenchi et al., 2013; Madan et al., 2012), but it has not been reported in dairy products so far. *P. succinatimandens* is a thermophilic bacterium with an optimal growth temperature of 55°C and is able to growth up to 60°C (Salinas et al., 2004). Hence the dominance of *P. succinatimandens* in the day 0 pasteurised Cheddar and Mozzarella whey samples is likely due to its high heat resistance, relative to the starter culture bacteria. This species persisted in the pasteurised samples until spoilage occurred (Fig. 4a, 5a) and was also found in raw Cheddar whey (Fig. 2a). The Cheddar and Mozzarella whey both came from the same factory, so the presence of *P. succinatimandens* in both samples which were collected four months apart is suggestive of its persistence in the factory environment.

The differences in bacterial composition of the day 0 pasteurised mixed whey sample compared to the other two pasteurised whey samples were supported by bacterial counts on M17 agar. The pasteurised mixed whey had much higher counts on M17 (5.3×10^6 CFU/mL) than the pasteurised Cheddar and Mozzarella whey (60 and 1000 CFU/mL respectively) (Fig. 3b, 4b, 5b). These discrepancies could be due to the difference in time elapsed between cheese manufacture and pasteurisation. The mixed whey was pasteurised 4 days after cheese

manufacture, whereas the other two samples were pasteurised no more than 17 hours after cheese manufacture (see section 2.1).

Day 0 samples with and without CO_2 treatment in each batch of pasteurised whey were examined, and OTU proportions and bacterial counts within the same batch were found to be broadly similar (Fig. 3a, 3b, 4a, 4b, 5a, 5b). Therefore, any subsequent differences found between the carbonated samples and non-carbonated controls would not have been due to artifactual causes such as contamination during the carbonation process or differences between sample aliquots.

3.4 Carbonation delays spoilage of pasteurised whey and maintains the initial bacterial composition

When stored at 15°C, the non-carbonated controls of all three pasteurised whey samples spoiled within 3-4 days, by which time bacterial counts reached 10^5-10^7 CFU/mL, equivalent to an increase of 400 to 200,000-fold from day 0 counts (with the exception of the M17 counts of mixed whey as it contained high initial counts of starter) (Fig. 3b, 4b, 5b). This showed that pasteurisation was not sufficient to preserve whey when stored at 15°C. In contrast, spoilage was clearly delayed in the carbonated samples. Bacterial counts remained similar to day 0 levels till at least day 6 (Fig. 3b). The most remarkable inhibition by CO_2 at 15°C was seen in the pasteurised Cheddar whey, in which bacterial counts were stable till day 28, thus bacterial growth was inhibited for at least 24 days more than the non-carbonated control (Fig. 4b).

A decrease in bacterial diversity upon spoilage has been well characterised in foods (Chaillou et al., 2014; Ercolini, 2013). Two genera were involved in spoilage of the non-carbonated

whey stored at 15°C: *Pseudomonas* in the pasteurised mixed whey (85% of population at day 3) (Fig. 3a), and *Bacillus* in the pasteurised Cheddar and Mozzarella whey (98 to 100% of population at day 7) (Fig. 4a, 5a). These genera could not be resolved to single species, but the matching species obtained in BLAST analyses indicate that they are most closely related to the *Pseudomonas fluorescens* group and the *Bacillus cereus* group. The initial microbiota of the pasteurised mixed whey was already highly homogeneous, so there was little change in diversity upon spoilage. However, there were remarkable decreases in bacterial diversity in the non-carbonated Cheddar and Mozzarella whey, from 8-17 taxa at day 0 to 1 taxon when spoiled.

High microbial diversity was maintained in the carbonated samples before spoilage, as seen in the day 7 pyrosequencing profiles of pasteurised Cheddar and Mozzarella whey treated with CO_2 (Fig. 4a, 5a). Some differences in the taxa compared to the day 0 profiles are seen, but there are no consistent patterns of enrichment of certain groups of bacteria by CO_2 across the whey samples. Carbonated Cheddar whey was spoiled by *B. cereus* group bacteria, similar to the non-carbonated control (Fig. 4a). The spoiled pasteurised carbonated Mozzarella whey was not submitted for pyrosequencing, but the dominant colony type obtained at day 23, when the whey was already spoiled (Fig. 5b), was identified as a member of the *B. cereus* group by Sanger sequencing of the 16S rRNA gene. Therefore, CO_2 did not select for different spoilage organisms in the pasteurised Cheddar and Mozzarella whey.

Pseudomonas and *Bacillus* are ubiquitous in the environment. Both *Pseudomonas* and *Bacillus* are dominant dairy spoilage genera of pasteurised milk (Boor and Fromm, 2009; Ternström et al., 1993). *Pseudomonas* is sensitive to heat but could contaminate whey post-pasteurisation, while *Bacillus* can form spores which can survive pasteurisation. It is possible

that post-pasteurisation contamination occurred in the pasteurised mixed whey but not in the pasteurised Cheddar whey and Mozzarella whey, leading to spoilage by *Pseudomonas* in the mixed whey and *Bacillus* in the Cheddar and Mozzarella whey.

3.5 Growth inhibition by carbonation is not solely due to reduced pH

Carbonation decreased the day 0 pH of whey by approximately 1 unit due to formation of carbonic acid (Fig. 2c, 3c, 4c, 5c), and the pH remained low throughout the storage period due to substantial amounts of CO_2 being retained (CO_2 concentration data not shown). Therefore, it was possible that the growth inhibition observed was due solely to reduced pH. In order to confirm that CO_2 had a specific inhibitory effect on bacterial growth, pasteurised mixed whey and Cheddar whey was acidified with lactic acid to a similar pH as that of carbonated whey (Fig. 3c, 4c) and bacterial growth was monitored during storage at 15°C. In both instances, the acidified whey spoiled much more quickly than the carbonated whey (Fig. 3b, 4b). Therefore, bacterial inhibition by CO_2 in these samples could not be attributed to pH reduction alone. These results are consistent with those previously reported in studies exploring the activity of CO_2 in milk (Hendricks and Hotchkiss, 1997; King and Mabbitt, 1982).

3.6 Protein degradation is minimal in spoiled whey

Protein is one of the key components of whey that is purified and used as a food ingredient or solely as a protein powder. We investigated if protein degradation was occurring during storage of Mozzarella whey for prolonged periods after CO_2 treatment using reducing SDS-PAGE analysis. The protein profiles from non-spoiled carbonated pasteurised Mozzarella whey stored at 15°C for 7 days were the same as those from the same whey stored for 24 days at 15°C (SDS-PAGE data not shown; see Fig. 5b for bacterial counts). The band profiles

were also similar to those of fresh whey as previously reported (Farrell et al., 2001). These preliminary results suggest that despite extended storage and growth of spoilage bacteria to high levels (approximately 10^6 CFU/mL) (Fig. 5b), there were no obvious signs of protein degradation.

4. Conclusion

This is the first study in which pyrosequencing was used to analyse the bacterial populations of a carbonated dairy product and of fresh and spoiled sweet whey. Sweet whey was found to be spoiled by organisms that typically spoil milk. Raw Mozzarella whey was spoiled by *Pseudomonas* sp., *Serratia* sp., and other *Enterobacteriaceae* family members, whereas pasteurised Cheddar and Mozzarella whey was spoiled by *Bacillus* sp. (two of three samples studied) or *Pseudomonas* sp. Cheese starter bacteria did not play a role in whey spoilage. CO_2 was effective in delaying spoilage of pasteurised whey stored at 15°C and raw whey stored at 4°C. CO_2 did not select for spoilage organisms different from those in the non-carbonated control. Reduced bacterial growth resulting from CO_2 treatment suggests it is possible to consolidate several batches of carbonated whey before transporting them to off-site facilities for processing. In addition, carbonation can help reduce refrigeration costs as a higher storage temperature can be used. These advantages mean that carbonation has potential to be incorporated in whey processing. Further studies on a larger scale will provide additional support for its feasibility and effectiveness.

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Figure captions

Fig. 1. Rarefaction analysis of pyrosequencing reads obtained from whey samples used in this study. The samples were stored at 15°C unless otherwise specified.

Fig. 2. Analysis of raw whey. (a) Pyrosequencing analysis of bacterial populations of raw Cheddar whey (denoted by C) and raw Mozzarella whey (denoted by M). Genus or species names are shown only for OTUs with an occurrence of 2% or more in at least one sample. The rest of the OTUs are grouped into "others". Bar patterns denote the phyla to which the named taxa belong: solid – Firmicutes; diagonal stripes – Proteobacteria. (b-e) Bacterial growth and pH profiles of raw Mozzarella whey stored at 15 and 4°C.

Fig. 3. Analysis of pasteurised mixed whey stored at 15°C. (a) Pyrosequencing analysis of bacterial populations. Genus or species names are shown only for OTUs with an occurrence of 2% or more in at least one sample. The rest of the OTUs are grouped into "others". Bar patterns denote the phyla to which the named taxa belong: solid – Firmicutes; diagonal stripes – Proteobacteria; grid – Thermi. (b, c) Bacterial growth and pH profiles. The acidified sample was acidified with lactic acid to a pH similar to that of carbonated whey.

Fig. 4. Analysis of pasteurised Cheddar whey stored at 15°C. (a) Pyrosequencing analysis of bacterial populations. Genus or species names are shown only for OTUs with an occurrence of 2% or more in at least one sample. The rest of the OTUs are grouped into "others". Bar patterns denote the phyla to which the named taxa belong: solid – Firmicutes; diagonal stripes – Proteobacteria; vertical stripes – Actinobacteria; horizontal stripes – Bacteroidetes. (b, c) Bacterial growth and pH profiles. Colony counts on M17 are almost identical to those

on NA and so are omitted in (b) for clarity. The acidified sample was acidified with lactic acid to a pH similar to that of carbonated whey.

Fig. 5. Analysis of pasteurised Mozzarella whey stored at 15°C. (a) Pyrosequencing analysis of bacterial populations. Genus or species names are shown only for OTUs with an occurrence of 2% or more in at least one sample. The rest of the OTUs are grouped into "others". Bar patterns denote the phyla to which the named taxa belong: solid – Firmicutes; diagonal stripes – Proteobacteria; vertical stripes – Actinobacteria; horizontal stripes – Bacteroidetes; checks – Planctomycetes. (b, c) Bacterial growth and pH profiles.

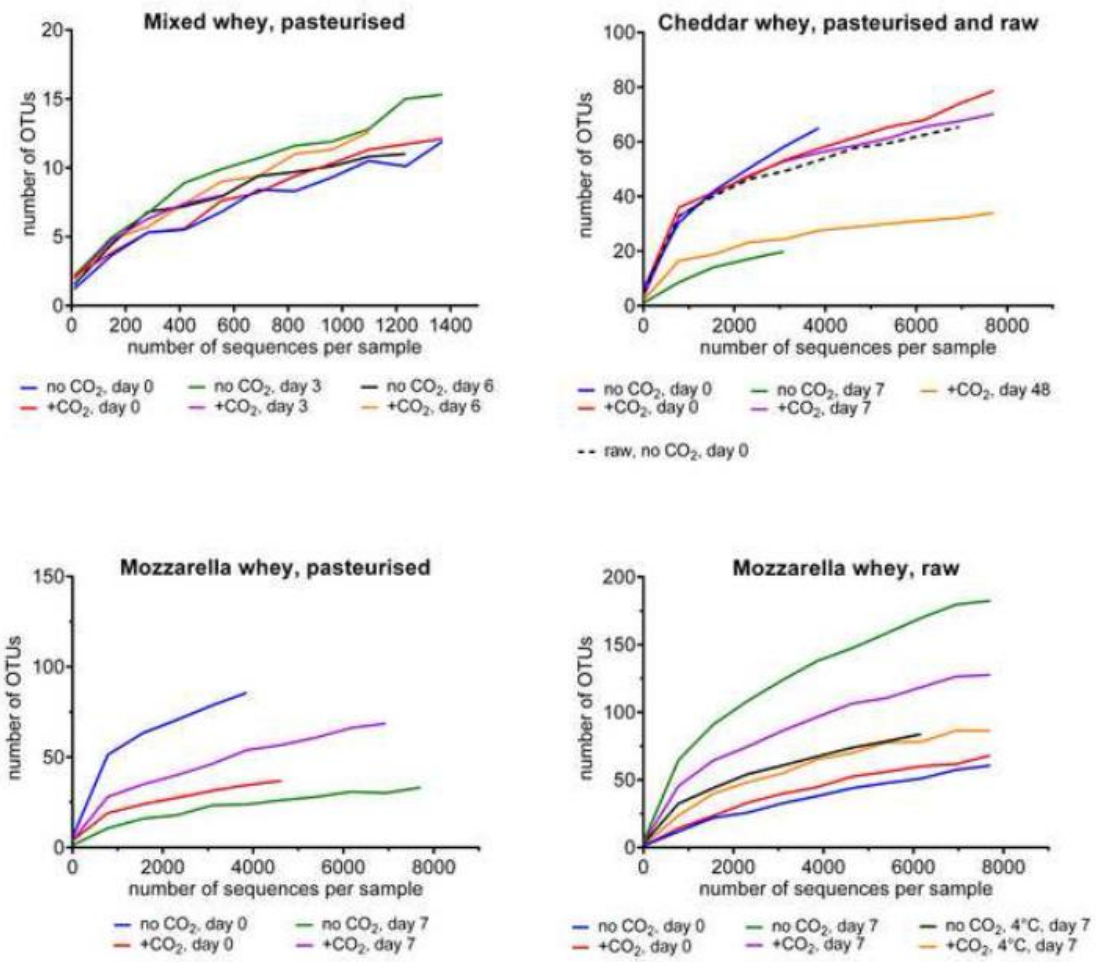

Figure 1

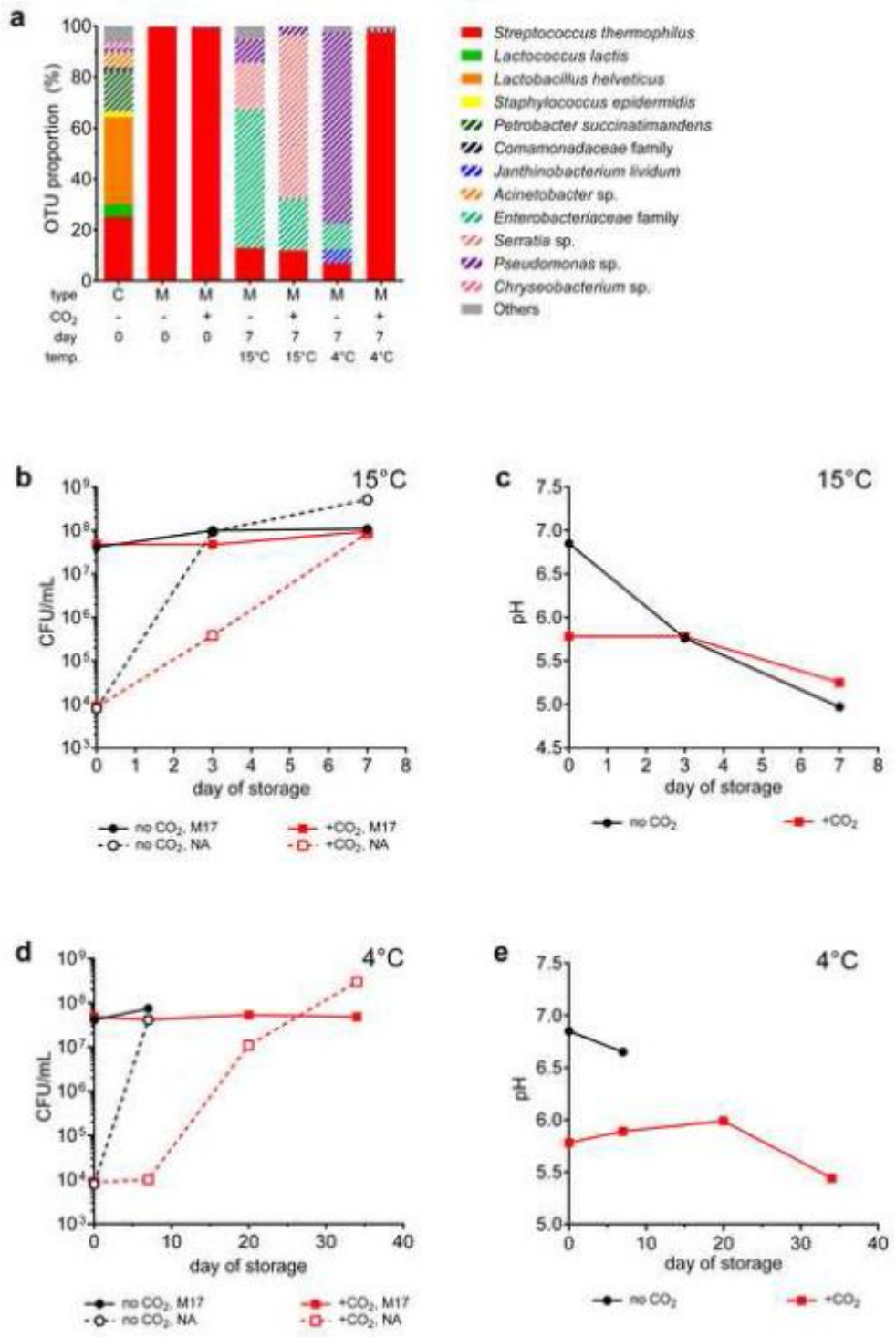

Figure 2

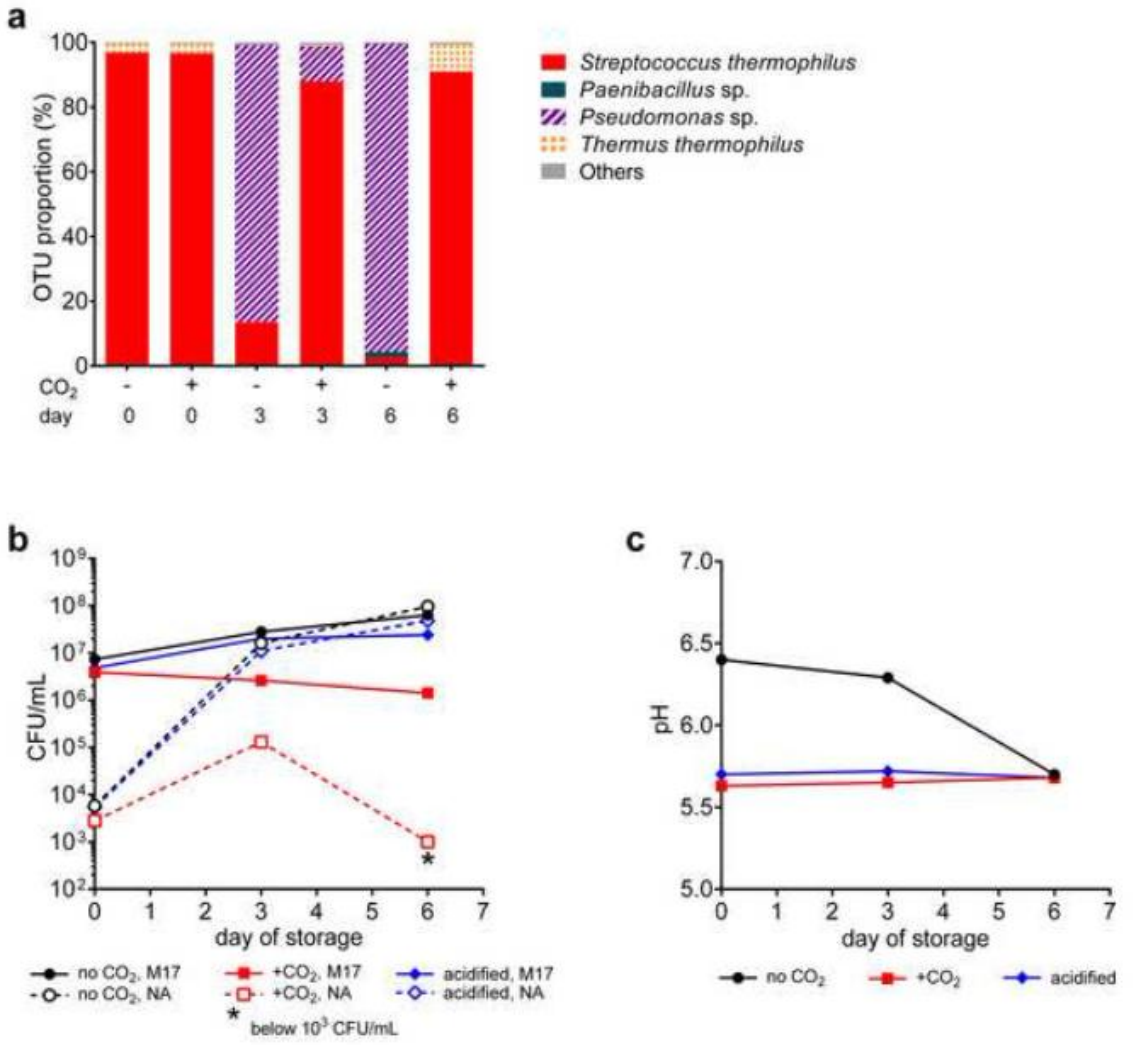

Figure 3

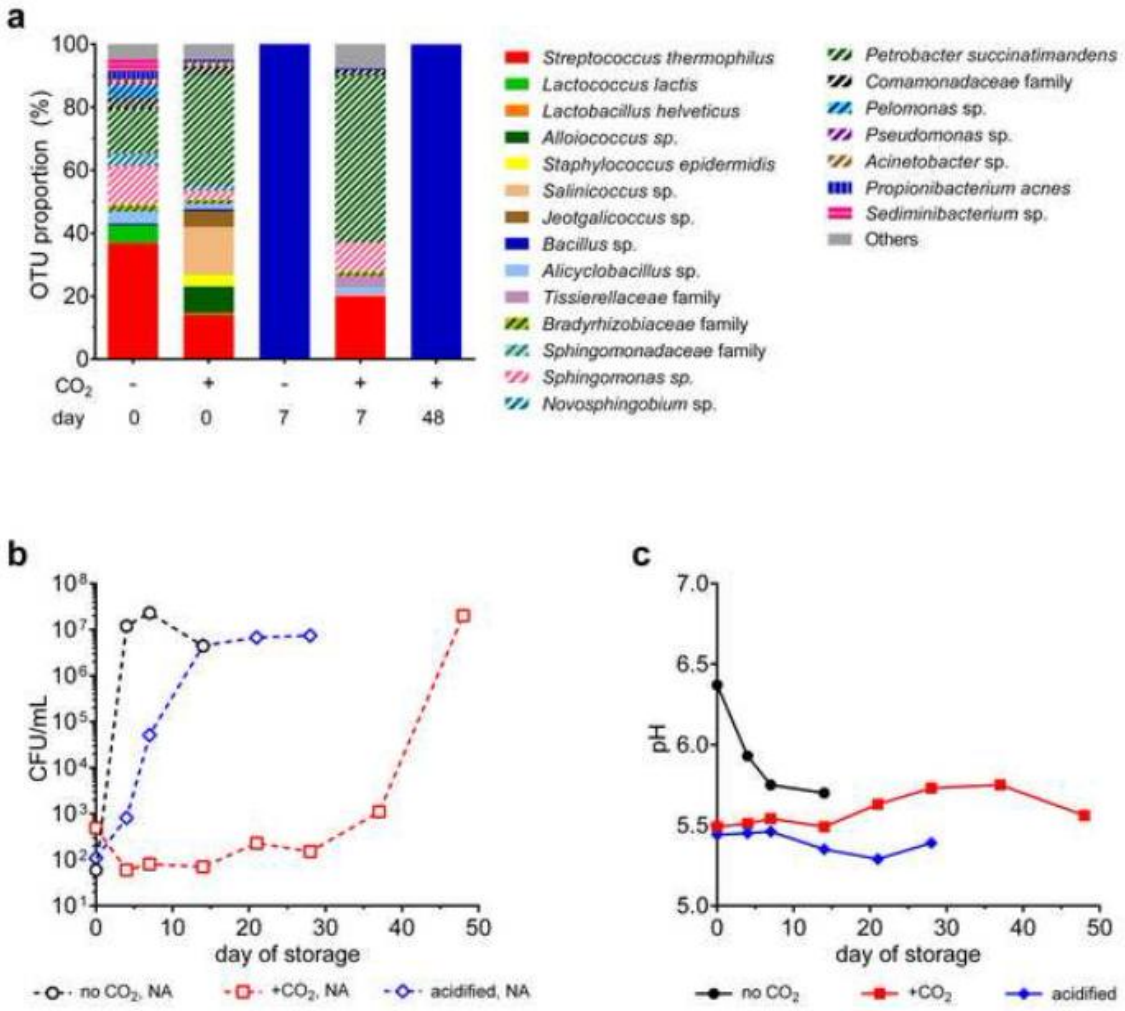

Figure 4

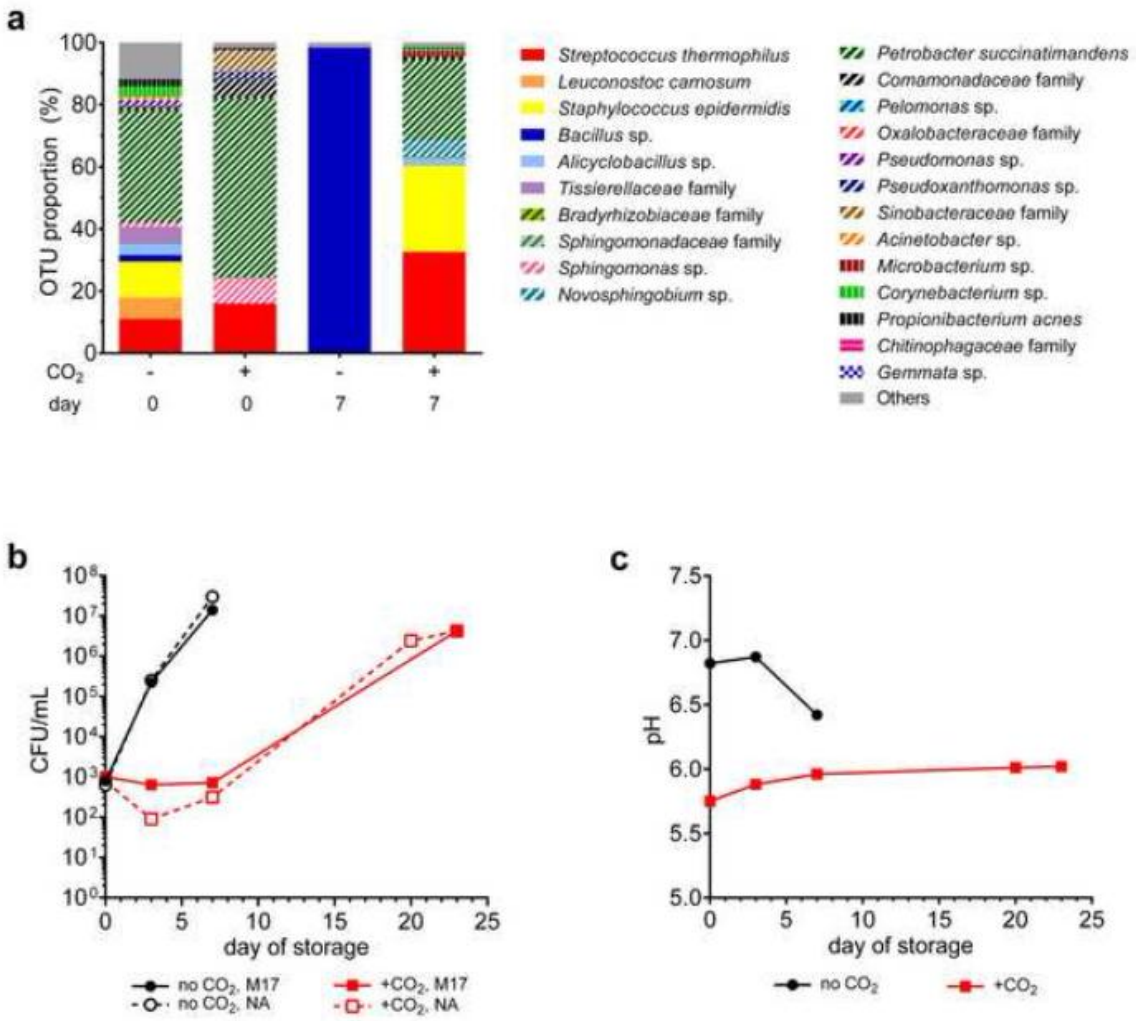

Figure 5

Table 1

Characteristics of pyrosequencing data.

Sample type	Number of samples analysed	Number of reads after quality filtering			
		Total	Minimum	Maximum	Mean
Mixed whey	6	13206	570	5226	2201
Cheddar whey	8	59854	3395	10502	7482
Mozzarella whey	12	109652	3360	18448	9137

Highlights

- Fresh and spoiled whey, with and without CO_2, were analysed by pyrosequencing.
- Pasteurised whey was spoiled by *Bacillus* or *Pseudomonas*.
- Raw whey was spoiled by *Pseudomonas*, *Serratia* or other *Enterobacteriaceae*.
- CO_2 delayed spoilage of pasteurised whey stored at 15°C for up to 33 days.
- CO_2 did not select for different spoilage bacteria.