Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Modulation of the bacterial population in commercial cucumber fermentations by brining salt type

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Keywords
cucumber fermentation, lactic acid bacteria, low salt fermentation, microbial diversity, microbiota.

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2019/0263: received 4 October 2018, revised 6 January 2020 and accepted 21 January 2020

doi:10.1111/jam.14597

Abstract

Aims: Differences in the bacterial population of cucumber fermentations brined with no salt, 100 mmol l^{-1} (1·1%) calcium chloride ($CaCl_2$) or 1·03 mol l^{-1} (6%) sodium chloride (NaCl) were studied.
Methods and Results: Changes in the microbiology and chemistry of commercial and laboratory scale cucumber fermentations occurring as a function of time were monitored using colony counts and metagenetic analysis, and a pH probe and high-performance liquid chromatography analysis respectively. Dissolved oxygen and carbon dioxide content were monitored in commercial fermentations. Fermentations brined with calcium chloride ($CaCl_2$) or no salt sustained faster microbial growth and reduction in pH than those brined with 1·03 mol l^{-1} NaCl. *Leuconostoc*, *Lactococcus* and *Weissella* dominated in fermentations brined with no salt or 100 mmol l^{-1} $CaCl_2$ on day 1 as compared to *Weissella* and enterobacteria in fermentations containing 1·03 mol l^{-1} NaCl. Lactobacilli dominated all fermentations by the third day, regardless of salt type, and was followed, in relative abundance by *Pediococcus*, *Leuconostoc*, *Lactococcus* and *Weissella*. From 84 to 96% of the population was composed of *Lactobacillus* by day 7 of the fermentations, except in the no salt fermentations in which a mixed population of LAB remained. The population of LAB found in commercial cucumber fermentations brined with 100 mmol l^{-1} $CaCl_2$ (n = 18) or 1·03 mol l^{-1} NaCl (n = 9) mimicked that of laboratory fermentations. A declining population of aerobes was detected in commercial fermentations brined with $CaCl_2$ on day 1.
Conclusion: A reduced NaCl content in cucumber fermentation enhances microbial diversity.
Significance and Impact of the Study: This study fills a knowledge gap and aids in the design of improved reduced NaCl cucumber fermentations.

Introduction

Cover brines containing an average of 1·03 mol l^{-1} sodium chloride (NaCl) are typically used in commercial cucumber fermentations as part of a strategy to favour the proliferation of lactic acid bacteria (LAB) and delay the growth of the indigenous microbiota sensitive to NaCl. Growth of LAB contributes to the conversion of the glucose and fructose naturally present in cucumbers to lactic acid, concurrent acidification to pH 3·5–3·0, and the likely formation of secondary metabolites and antimicrobial compounds such as peroxides and bacteriocins (Ivey *et al.* 2013). The resulting decrease in pH as a function of acid production by LAB aids in further reducing the diversity of the indigenous microbiota. A recent study to re-assess the microbiota of modern commercial cucumber fermentations, brined with 1·03 mol l^{-1} NaCl, using culture independent and dependent methods

suggest that LAB such as *Lactobacillus pentosus*, *L. plantarum*, *L. brevis*, *Weissella* sp., *Pediococcus ethanolidurans*, *Leuconostoc* sp. and *Lactococcus* sp. prevail during the natural bioconversion (Pérez-Díaz *et al.* 2016). It was observed that *L. pentosus* consistently dominates in commercial cucumber fermentations followed by *L. plantarum* strains able to produce exopolysaccharides. The study by Pérez-Díaz *et al.* (2016) proposed that the closely phylogenetically related lactobacilli, *L. pentosus* and *L. plantarum*, achieve dominance due to their genetic content, including comparatively larger genomes, higher gene counts, unique distribution of the ribosomal clusters across the genome as opposed to close to the origin of replication, and the presence of more predicted amino acids prototrophies and selected biosynthesis-related genes as compared to their competitors. Non-LAB that are excluded in a cucumber fermentation as the result of salting and the increasingly acidic pH include *Pseudomonas*, *Pantoea*, *Stenotrophomonas*, *Acinetobacter*, *Comamonas*, *Wautersiella*, *Microbacterium*, *Flavobacterium*, *Ochrobactrum* and the Enterobacteriaceae, *Citrobacter*, *Enterobacter* and *Kluyvera* (Pérez-Díaz *et al.* 2019).

Given that the selection for the desired LAB in cucumber fermentations is dependent on the supplementation of cover brines with NaCl, it is logical to consider that the reduction in the salt content results in unacceptable fermentation quality as the result of the activity of the undesired microbiota. NaCl-free cucumber fermentation has been proposed by McFeeters and Pérez-Díaz (2010), and tested at the commercial scale (Pérez-Díaz *et al.* 2015), in an effort to reduce the production of significant volumes of waste waters with high NaCl content (>190 ppm). The reduced NaCl system is characterized by the use of a cover brine containing 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} potassium sorbate, after equilibration with the cucumbers, and a *L. plantarum* autochthonous starter culture. Although, such cover brine formulation is applied to conduct complete cucumber fermentations at the commercial scale with minimal impact in quality and safety, an assessment of its impact in the composition of the natural microbiota is lacking. Understanding how the NaCl and $CaCl_2$ contents affect the microbiota of commercial cucumber fermentations is to fill a knowledge gap and facilitate the optimization of reduced NaCl fermentation technology.

This study examines the bacterial population of cucumber fermentations brined with $CaCl_2$ and compares it to that of fermentations brined with no salt or with the typical brine formulation containing 1·03 mol l^{-1} NaCl. Laboratory scale fermentations were performed in one-gallon jars with cover brines containing either NaCl, $CaCl_2$ or no salt. Commercial scale fermentations in 38 000 L tanks brined with either NaCl (n = 9) or $CaCl_2$ (n = 18) were also evaluated. Analyses of fermentation metabolites, microbial counts, pH, dissolved oxygen and carbon dioxide, and sequencing of the 16S rRNA V3/V4 gene region were performed as a function of time.

Materials and methods

Laboratory scale cucumber fermentations

Two lots of cucumbers harvested at geographically distant farms were used for the evaluation of the influence of NaCl, $CaCl_2$ and autochthonous starter culture types in the fermentations (Table 1). Size 2B pickling cucumbers (32–38 mm in diameter) were obtained from a local processor, rinsed with distilled water upon arrival in the laboratory, and visually inspected to remove defective pieces. Cucumbers were packed in 1-gallon glass jars to 50% volume (1·9 kg) in duplicated jars per lot.

Four cover brines were prepared to investigate the effect of salt type on the bacterial population during the fermentation including a 1·03 mol l^{-1} (6%) NaCl cover brine (Morton Canning and Pickling Salt, Chicago, IL), a 100 mmol l^{-1} (1·1%) anhydrous $CaCl_2$ cover brine (Brenntag, Durham, NC) with or without a *L. pentosus* LA0445 starter culture to 6·0 log CFU per ml, prepared as described below, and water with no salt used as a control for spontaneous fermentation. All cover brine types were supplemented with 6 mmol l^{-1} potassium sorbate (Mitsubishi International Ingredients, Atlanta, GA) to reduce the yeast and moulds activity already known to induce instability in reduced NaCl fermentations (Pérez-Díaz *et al.* 2015).

Cover brines were added to 50% of the volume (~1·9 kg) in duplicate jars for each treatment. Jars were closed with commercial metal lug caps that were heated in boiling water for 10 s to soften the plastisol liner. Each lid was equipped with a rubber septum in its centre to allow for sampling of cover brine using a 10 ml syringe attached to a 18G X 1 1/2" needle (Becton Dickinson and Co., Franklin Lakes, NJ). The jars were incubated at 30°C for 14 days. Cover brine samples were aseptically collected from jars on days 1, 3, 7 and 14 for biochemical and microbiological analyses and DNA extraction. All analyses were performed as described below.

Commercial scale cucumber fermentations

Commercial cucumber fermentations were carried out in 28 400 l (7500 gal) open-top, white fiberglass tanks containing between 50–60% size 2B (27–38 mm diameter) and 3A (39–51 mm diameter) whole cucumbers or precut pieces of the fruits, and 50–40% cover brine solution.

Table 1 Description of the laboratory and commercial scale fermentation trials described in this publication

Cover brine formulation								
Salt content	Starter culture	Acetic acid	Lactic acid	Potassium sorbate	Other ingredients	Air purging routine	Cucumber sizes used for the fermentations	
Laboratory scale fermentations in closed jars								
No salt	None	None	None	6·0 mmol l^{-1}	Distilled water	None		
1·04 mol l^{-1} NaCl	None							
0·1 mol l^{-1} $CaCl_2$	None							
0·1 mol l^{-1} $CaCl_2$	*L. pentosus* LA0445						Lot 1	Lot 2
Industrial scale fermentations in open top 40 000 l fiberglass tanks								
0·1 mol l^{-1} $CaCl_2$/3 tanks (data used in Fig6)	*L. plantarum* 10B (Lyoferm)	None	None	6·0 mmol l^{-1}	Distilled water	15 SCFH for 20 h daily	Two tanks with size 3A fruits and 1 tank with size 2B fruits	
0.1 mol l^{-1} $CaCl_2$/15 tanks (data used in Fig7)					distilled water	15–100 SCFH for 20 h daily	Nubs and Relish or size 3A fruits	
1 mol l^{-1} NaCl/tank (data used in Fig6)	*L. plantarum* 10B (Lyoferm)	<5 mmol l^{-1}	None	None	Distilled water	30 SCFH for 20 h daily	Size 3A fruits	
1 mol l^{-1} NaCl/eight tanks (data used in Fig7)	None	<5 mmol l^{-1}	<20 mmol l^{-1}	None	Recycled fefermentation cover	15–100 SCFH for 20 h daily	Nubs and relish, or size 3A fruits	

Table 1 describes the cover brine components and their equilibrated concentrations, fruit sizes used and number of tanks packed for these experiments. Acetic acid was added as a 20% vinegar solution (Fleischmann Vinegar, Republic, MO) to an equilibrated concentration of <20 mmol l^{-1}. Anhydrous $CaCl_2$ and potassium sorbate were sourced from Brenntag (Durham, NC) and Mitsubishi International Food Ingredients (Atlanta, GA) respectively. Cushion cover brine was added into the tanks prior to the addition of the different sized fruits. In-tank fruits were immediately covered with wooden boards to prevent them from floating after adding the remaining cover brine volume, so that equilibration between the fruits and cover brine solution components would proceed uniformly. The air purging routine used in the tanks for each trial is described in Table 1 and was applied using pre-existing equipment at the processing facility. This equipment was composed of a 3/4" internal diameter feeding tube, an air blower, and a 6″ long, 2″ diameter Kellundite diffuser tube equipped with a 3/4″ PVC nipple (model A-400 FAO-10, vendor no. F23088; Filtros Ltd, East Rochester, NY). Rain water was mixed-in by air purging circulation. Tanks were replenished with cover brine prepared at the equilibrated concentrations as needed, and mostly after cover brine water evaporation. Tanks of cucumbers with $CaCl_2$ cover brine were inoculated to 10^6 CFU per ml using the starter culture strain specified in Table 1. The starter culture was prepared as described by Pérez-Díaz and McFeeters (2011), with a minor modification. Jars were refrigerated for 48 h to achieve pH equilibration, after adding a 1 mol l^{-1} sodium hydroxide solution (Sigma-Aldrich, St Louis, MO) to a pH of 4·8 and prior to inoculation. Tanks packed with the traditional NaCl cover brine formulation were not inoculated.

On each sampling day, approximately 50 ml of cover brine samples were taken from an average of 3·5 ft below the cover brine surface via a perforated pipe placed next to the air purging system in the tank. Cover brine samples were collected after ~100 ml of the cover brines had moved through the sampling tubing. The sampling apparatus consisted of a siphon PVC pump (BSP-1000; CBS Scientific Inc., San Diego, CA) connected to a 1/2" diameter Tygon® SE-200 Inert Tubing (Saint-Gobain Performance Plastics, Akron, OH). The samples were placed in Corning Centristar 50 ml sterile conical tubes (cat. # 430829; Corning Inc., Corning, NY) and immediately transported to our laboratory for same day processing. Cover brine samples were subjected to microbiological and biochemical analyses and DNA extraction as described below. Dissolved oxygen concentration was measured by submerging the corresponding probes in the cover brine on site, as described below. Given the

distance between the participating processing facilities it was not always possible to sample all tanks on the same schedule. Thus, the number of samples per fermentation day fluctuated and is described in each figure.

Microbial analyses of cucumber fermentations

Aseptically collected samples were serially diluted in 0·85% NaCl solution and plated on MRS agar (Difco Laboratories, Detroit, MI) containing 1% of a 0·1% cycloheximide stock solution (Oxoid, Basingstoke, UK) for the enumeration of presumptive LAB, in particular lactobacilli. Yeast and mold agar (YMA) supplemented with 0·01% chloramphenicol (Sigma-Aldrich) and 0·01% chlortetracycline (Sigma-Aldrich) was used for the enumeration of presumptive yeasts. VRBG (Becton Dickinson and Co.) supplemented with 1% glucose was used for the enumeration of presumptive Enterobacteriaceae. Differential reinforced clostridia agar (DRCA) (Becton Dickinson and Co.) supplemented with 1% of a 0·1% cycloheximide stock solution (Oxoid) was used for the enumeration of presumptive *Clostridium* sp. MRS and DRCA plates were incubated at 30°C anaerobically for 48 h in a Coy Anaerobic Chamber (Grass Lakes, MI). YMA plates were incubated aerobically at 30°C for 48 h. Only black colonies were counted from DRCA plates and numbers recorded as presumptive *Clostridium* sp. VRBG plates were incubated at 37°C aerobically for 24 h prior to counting. Purple and pink colonies on VRBG were counted and recorded as presumptive Enterobacteriaceae. Colonies from agar plates were enumerated using a Flash & Go Automated Colony counter (cat. 90006010; IUL Instruments, Barcelona, Spain).

The significant differences among the colony counts for treatments were determined by an ANOVA: two factor with replication and a Tukey HSD test using the Data Analysis Tool Pak in Microsoft Excel. Differences between treatments based on time and treatment were assessed using a P-value $\leq 0{\cdot}05$.

Measurements of dissolved carbon dioxide and oxygen in commercial cucumber fermentations

Dissolved oxygen was measured on-site and in-tank, upon sample collection, at variable depths through the air purging system pipe. A HACH HQd40 dissolved oxygen probe attached to an HQd portable multi-meter (HACH, Düsseldorf, Germany) and equipped with a 3·66 m cable was used to measure dissolved oxygen after adjusting for 0 or 6% salinity, as needed. The probe was calibrated and maintained following the manufacturer instructions.

Dissolved carbon dioxide was measured from cover brine samples (5 ml) collected from the surface of the commercial fermentation tanks using a gastight syringe (Hamilton, 10 ml, cat# LG-07939-54). Cover brine samples were injected and stored in plastic vacutainers with Hemogard closure (Pulmolab, 10 ml, BD #366643). Upon sample processing, a 20% acetic acid solution in the form of vinegar (Fleischmann Vinegar) was used as the carbon dioxide-liberating substance (Fleming *et al.* 1973). Aliquots of 3 ml of 20% vinegar were injected into the vacutainer tubes filled with the cover brine samples followed by the application of even and vigorous agitation for 10 s, to accelerate the release of the targeted gas immediately prior to the injection in a benchtop Map-Pak Combi Gas Analyzer (AGC Instruments Co. Clare, Ireland). The values obtained from the instrument in total percent of CO_2, were converted to the partial pressure of the gas in the headspace using the following equation:

$$P_{CO_2} = (\%\ CO_2/100) \times (P_s - P_{wv} - P_{AAV});$$

where P_{CO2} represents the CO_2 partial pressure in the headspace, P_s represents the standard barometric pressure (1 atm.), P_{wv} is the water vapour pressure at 30°C (0·0418 atm) and P_{AAV} represents the acetic acid partial pressure at 30°C (0·028 atm).

CO_2 content in mg per 100 ml of cover brine was then calculated using the Henry's law where;

$$CO_2\ \text{mg per 100 ml of cover brine} = (P_{CO_2}/K_H)\ (MW_{CO_2}) \times 100)$$

and where K_H represents the Henry's law constant (25·8 atm mol^{-1} at 30°C; Sander 2015; Cohen 1989), and MW corresponds to the molecular weight for CO_2 (44·01 g mol^{-1}).

Analysis of significant differences among the treatments CO_2 and dissolved oxygen measurements were determined by an ANOVA: Two factor with replication and a Tukey HSD test using the Data Analysis Tool Pak in Microsoft Excel. A difference between treatments based on time and treatment was assessed using a P-value $\leq 0{\cdot}05$.

Monitoring of cucumber fermentation metabolites and pH measurement

Cover brine samples were spun for 10 min at 18 512 ***g*** at room temperature (Eppendorf Centrifuge 5810R; Fisher Scientific, CA) to remove cells and particulate, so that clarified supernatants could be used for pH measurement and high-performance liquid chromatography (HPLC) analysis.

pH of samples at different time points were measured with an Accumet® Research 25 pH meter (Ficher Scientific, Pittsburgh, PA) equipped with a Gel-Filled Pencil-Thin pH Combination Electrode (Accumet; Fisher Scientific). The significant differences among the treatments pH measurements were determined by an ANOVA: Two factor with replication and a Tukey HSD test using the Data Analysis Tool Pak in Microsoft Excel. A difference between treatments based on time and treatment was assessed using a *P*-value ≤0·05.

The concentrations of organic acids and sugars were measured by HPLC analysis using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA) for the separation of components and a Shimadzu HPLC system (UFLC; Shimadzu Scientific Instruments, Durham, NC) with accompanying software. Separation was performed at 65°C using 0·01 N H_2SO_4 for the mobile phase with a flow rate of 0·9 ml min^{-1} An ultraviolet light detector (210 nm, RID-10A, Shimadzu) was used for quantification of malic, succinic, lactic, propionic, butyric and benzoic acids. In addition, acetic acid, glucose, fructose, ethanol and glycerol were quantified using a refractive index detector (SPD-20A, Shimadzu) that was connected in series.

Total genomic DNA extraction

Cells were harvested from 10 ml of fermentation cover brine samples by centrifugation at 15 275 ***g*** for 10 min at room temperature (Eppendorf Centrifuge 5810R; Fisher Scientific, CA). Cell pellets were resuspended in 490 µl sterile 0·85% NaCl solution and treated with 10 µl of 2·5 mmol l^{-1} propidium monoazide (PMA) stock solution (1·3 mg ml^{-1} PMA in 20% DMSO; Biotium Inc., Hayward, CA) to eliminate dead bacterial and extracellular DNA as described by Pan and Breidt (2007). PMA-treated samples were stored as cell pellets at −20°C until DNA extraction. Total genomic DNA was extracted from PMA-treated cell pellets using a MasterPure™ DNA Purification Kit (Epicentre, Madison, WI). Bacterial cells were lysed enzymatically using 2 mg ml^{-1} Lyzosyme (Sigma-Aldrich). DNA concentrations were quantified using PicoGreen dsDNA reagent (Invitrogen; Life Technologies, Carlsbad, CA) on a 96-well plate reader and mixed at equimolar concentrations. The 16S rRNA V3 and V4 gene regions were amplified by polymerase chain reaction (PCR) from the total genomic DNA samples using the Bakt_341F/Bakt_805R primers (Sinclair *et al.* 2015). Primers were barcoded as described by the manufacturer for the Illumina MiSeq sequencing technology. Sequencing services were obtained from the Carver Biotech Laboratory at the WM Keck Center for Comparative and Functional Genomics (Chicago, IL).

16S rRNA amplicon sequencing data processing

There were 25 samples collected from laboratory scale cucumber fermentations that included four different types of fermentation cover brines. The 25 cover brine samples analysed represented four time points for two fermentations brined with water, four time points for two fermentations brined with 1·03 mol l^{-1} NaCl, four time points for two fermentations brined with 100 mmol l^{-1} $CaCl_2$ and three time points for two inoculated fermentations brined with 100 mmol l^{-1} $CaCl_2$. Similarly, there were 47 samples collected from commercial cucumber fermentations, which included 15 samples from nine commercial fermentations brined with 1·03 mol l^{-1} NaCl and 32 samples derived from 18 inoculated commercial fermentations brined with 100 mmol l^{-1} $CaCl_2$ (Table 1). Cover brine samples were collected on days 1, 2, 3, 6, 7, 10 and 14 of the fermentations. Not all tanks were sampled on all days.

The sequences were 2 × 250 bp and were quality trimmed with Trimmomatic (ver. 0.36) where any reads with lengths <36 bp were removed (Bolger *et al.* 2014). Any primer sequences remaining in the trimmed PE reads were removed using MacQIIME 1.9.1-20150604 (Caporaso *et al.* 2010a) and the R1/R2 reads were merged using fastq-join with the default parameters (Aronesty 2011). Merged reads that contained bases with Phred quality scores <20 were removed. Any samples that had a low sequencing depth (fewer than 1000 reads) were removed. This included six of the 25 laboratory fermentation samples. The 19 remaining samples were included in the statistical analyses and their sequencing depths can be seen in Table S1. Only two of the 47 samples from the commercial scale fermentations were removed due to low sequencing depth, both with cover brines containing NaCl. Two samples that had cover brines containing $CaCl_2$ were removed due to being outliers. These two samples had relative abundance profiles that were completely different from the other $CaCl_2$ samples and they clustered separately from the rest of the $CaCl_2$ samples in a bootstrapped UPGMA tree. The 43 commercial scale samples that remained were included in the statistical analyses and their sequencing depths can be seen in Tables S2 (NaCl, 13 samples) and S3 ($CaCl_2$, 30 samples).

VSearch was used to generate the operational taxonomic unit (OTU) table (Rognes *et al.* 2016). Initially, the sequences were dereplicated and singletons were removed. The sequences were sorted on abundance and clustered at 97%, where the most abundant read from each cluster was selected as the cluster centroid. Next, chimeras were identified and removed from the list of centroids, resulting in a set of representative sequences.

The reads were mapped to the representative sequences to produce an OTU table. Taxonomic assignments were made using the SILVA (ver. 128) database (Quast *et al.* 2013; Yilmaz *et al.* 2014) and in generating a phylogenetic tree for downstream analyses, PYNAST was used to align the sequences to the SILVA (ver. 128) core alignment sequences (Caporaso *et al.* 2010b). OTUs that represented <0·005% of the total sequences or chloroplasts were removed from the OTU table.

All analyses were conducted using MacQIIME 1.9.1-20150604. The relative abundances for the 19 laboratory fermentation samples and the 43 commercial fermentation samples are shown in Figs 3 and 9, 10 respectively. The phylogenetic tree used in the alpha and beta diversity analyses was generated using FastTree (Price *et al.* 2009). The alpha diversity was calculated using a step size of 5000 with 50 iterations at each step. Based on the alpha diversity metrics Phylogenetic Diversity, Chao1 and Observed OTUs, rarefaction levels of 30 000 and 20 000 sequences per sample were selected for the laboratory and commercial fermentation batches respectively and used for the beta diversity analyses. The alpha diversity values are shown in Table S1 for the laboratory fermentation samples and in Tables S2 and S3 for the commercial fermentation samples. The beta diversity was examined through PCoA plots based on the weighted and unweighted UniFrac distance matrices.

To determine whether there were significant differences between treatments or time points, two-sided two-sample t-tests were used and nonparameteric *P*-values were calculated using 1000 Monte Carlo permutations. Initially, the weighted and unweighted UniFrac distance matrices were calculated at the specified rarefaction levels using jackknifing. This generated 10 weighted and 10 unweighted UniFrac distance matrices. A distance matrix containing the mean values for each set of weighted and unweighted UniFrac distance matrices was then generated and used in the significance calculations.

Results

Characterization of laboratory scale cucumber fermentations brined with $CaCl_2$, NaCl or no salt

Colony counts from culture plates and pH

Supplementation of fermentation cover brines with $CaCl_2$ and no salt resulted in higher plate counts for the population of presumptive lactobacilli, enumerated from MRS, as compared to the treatment supplemented with 1·03 mol l^{-1} NaCl (Fig. 1). The means for colony counts from MRS plates were found to be significantly different among all treatments based on an ANOVA-Tukey HSD test ($P \leq 0{\cdot}05$), except for the comparison between the two treatments containing 100 mmol l^{-1} $CaCl_2$. A quicker

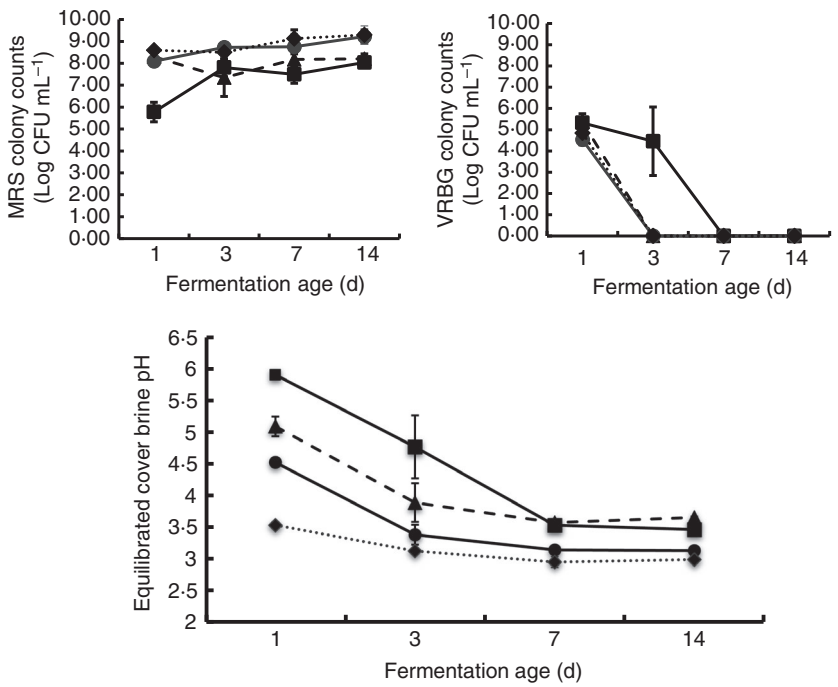

Figure 1 Presumptive lactobacilli colony counts from MRS (left panel) and VRBG (right panel) and cover brine pH measured from laboratory scale cucumber fermentations brined with 1 mol l^{-1} NaCl (■), no salt (▲), 100 mmol l^{-1} $CaCl_2$ (●) or 100 mmol l^{-1} $CaCl_2$ and a starter culture (♦). Data presented corresponds to the average and standard deviations of independent duplicates.

cover brine acidification, with at least −0·5 pH unit difference, was observed in the cover brines of fermentations supplemented with $CaCl_2$ as compared to those brined with no salt or 1·03 mol l^{-1} NaCl (Fig. 1). All pH value means were found significantly different among the four treatments tested ($P \leq 0{\cdot}05$). The combination of cover brine supplementation with $CaCl_2$ and a starter culture resulted in the lowest fermentation cover brine pH, 24 h after tanking, of at least 1 pH unit below the value measured in non-inoculated fermentations brined with $CaCl_2$ (Fig. 1b). Since potassium sorbate, a yeast inhibitor, was added to all the fermentation cover brines to an equilibrated concentration of 6 mmol l^{-1}, the colony counts from YMA plates were below the detection level from all the jars tested. Colony counts from VRBG were reduced from 5·0 ± 0·45 log CFU per ml on day 1 to below the detection limit by day 3 in three out of four treatments, except for that containing 1·03 mol l^{-1} NaCl, a treatment that was significantly different from all others based on the ANOVA-Tukey HSD test ($P \leq 0{\cdot}05$) (Fig. 1). The fermentations brined with NaCl remained at 4·5 ± 1·6 log CFU per ml by the third day, likely due to the longer generation time and lag phase for LAB in such treatment (Fig. 1). Supplementation of fermentation cover brines with $CaCl_2$ resulted in up to 1 log CFU per ml reduction in colony counts from VRBG by day 1 (Fig. 1). No significant differences in the colony counts from VRBG were calculated among the treatments containing no salt or 100 mmol l^{-1} $CaCl_2$ ($P \leq 0{\cdot}05$). Presumptive *Clostridium* sp. were not detected from DRCA plates in any treatment.

Metabolite concentrations

The analysis of fermentation metabolites suggested that substrate conversion as a function of time were substantially different between the lots brined with 1·03 mol l^{-1} NaCl and those brined with 100 mmol l^{-1} $CaCl_2$ after 14 days. For the cover brines with 1·03 mol l^{-1} NaCl, 10·4 ± 9·8 mmol l^{-1} of glucose and 13·9 ± 12·8 mmol l^{-1} of fructose remained. For the brines with 100 mmol l^{-1} $CaCl_2$, 2·8 ± 0·5 mmol l^{-1} of glucose and 4·4 ± 1·3 mmol l^{-1} fructose remained. The lowest residual sugar concentrations were measured from fermentations brined with no salt at <1·5 mmol l^{-1} each. The variability in the speed of the fermentation was also reflected in the rate of lactic acid production. Lactic acid was produced from 91·7 to 105·5 mmol l^{-1}, where the jars brined with 1·03 mol l^{-1} NaCl were at the low end and the no salt system was at the high end of the range. An unknown compound with the same retention time as acetic acid was detected to 16·1 ± 0·9 mmol l^{-1} in one cucumber lot but not the other. However, changes in acetic acid concentrations were not detected as a function of time. No propionic or butyric acids were detected in the fermentation cover brine samples. Residual amounts of malic acid were measured from all treatments in <1·05 ± 0·4 mmol l^{-1}.

Microbiota abundances

The raw read sequences are available from the National Center for Biotechnology Information with SRA accession number SRP157093. The average number of sequences for the 19 samples collected from the laboratory scale cucumber fermentations was 60 426 ± 10 141 (77·1-% ± 2·5 of the starting sequences) with a median length of 427 bp after the quality control and merging steps. The beta diversity of the samples was examined using the unweighted and weighted UniFrac distance matrices (Lozupone and Knight 2005), where unweighted UniFrac reflects the existence of OTUs and weighted UniFrac takes abundance into account. Although the beta-diversity unweighted UniFrac PCoA plot shows no obvious pattern or separation by salt type or content (treatment), the weighted UniFrac plot does show a slight separation along PC2 between the samples brined with 1·03 mol l^{-1} NaCl and the remaining 15 samples (Fig. 2). Pairwise comparisons between treatments based on the weighted and unweighted UniFrac distance matrices show no significant differences. If instead of treatment, one looks at pairwise comparisons between time points, then significant differences for the unweighted UniFrac are found between day 1 *vs* day 3 and day 1 *vs* day 7 with Bonferroni-corrected—*P*-values of 0·010. Similar results are observed in Fig. 3, where *Lactobacillus* makes up only a small percentage of day 1 samples (<1%) compared with a much larger percentage in samples taken later in the fermentation process. A relatively high proportion of Leuconostocaceae is apparent on samples collected on

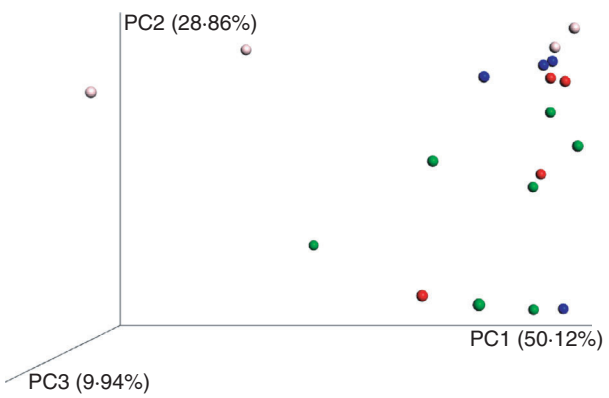

Figure 2 PCoA plot for the weighted UniFrac metric coloured by treatment for the laboratory scale cucumber fermentations. Samples brined with NaCl are orange (n = 4), with $CaCl_2$ are red (n = 4), with $CaCl_2$ and a starter culture (SC) are blue (n = 4), and with no salt are green (n = 7). [Colour figure can be viewed at wileyonlinelibrary.com]

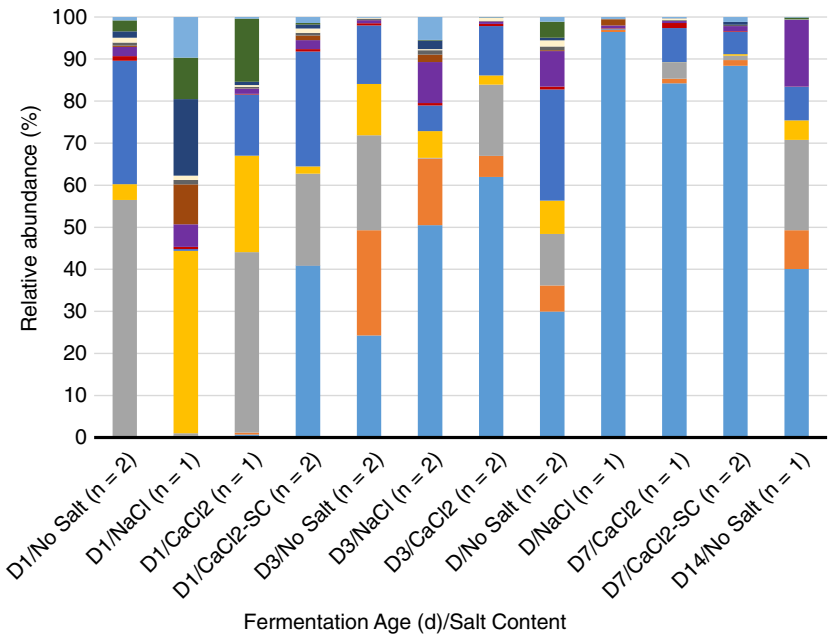

Figure 3 Results from the 16S *rRNA* V3/V4 gene region amplicon sequencing for cover brine samples collected from laboratory scale cucumber fermentations brined with 6 mmol l^{-1} potassium sorbate and no salt, 1·03 mol l^{-1} NaCl or 100 mmol l^{-1} $CaCl_2$. A starter culture (SC) was added to some of the fermentations brined with 100 mmol l^{-1} $CaCl_2$. The relative abundance is shown on the genus level and the bar plots are averaged over replicates for each day/treatment. If the relative abundance of a genus is <0·5% when averaged over the 19 laboratory scale cucumber fermentation samples, then it is not listed and is instead included in the '<0·5%' category (*Lactobacillus*; *Weissella*; *Enterobacter*; *Enterobacteriacea*; *other*; less than 0·5%; *Pediococcus*; *Lactococcus*; *Pantoea*; *Acinetobacter*; *Leuconostoc*; *Citrobacter*; *Raoultella*; *Pseudomonas*). [Colour figure can be viewed at wileyonlinelibrary.com]

day 1 of fermentations, whereas Lactobacillaceae dominates in the remaining days.

Dominance of *Leuconostoc*, *Lactococcus* and *Weissella* was observed in samples fermented with no salt or with 100 mmol l^{-1} $CaCl_2$ (Fig. 3) during the first day of the fermentation. Interestingly, a greater proportion of Gram-negative bacteria was initially observed in samples brined with 1·03 mol l^{-1} NaCl for day 1 with *Enterobacter*, *Pantoea*, *Acinetobacter* and *Pseudomonas* dominating this sector of the population (Fig. 3). By day 3 of the fermentations, the samples contained between 24 and 62% *Lactobacillus*, with the no salt treatment harbouring the lowest percentage. *Pediococcus*, *Leuconostoc*, *Weissella* and *Lactococcus* were the direct competitors of *Lactobacillus* regardless of the cover brine salt content for day 3. The absence of NaCl and $CaCl_2$ supported a more balanced composition of the microbiota with an almost equal distribution for the LAB mentioned above—for day 3. About 84–96% of the population was composed of *Lactobacillus* by day 7 of the fermentation, except in the absence of NaCl and $CaCl_2$, where a mixed population was still dominant and the lactobacilli amounted to 30%. The remaining population was primarily composed of *Lactococcus* and *Leuconostoc* on day 7 of the fermentations across treatments (Fig. 3). Supplementation of cucumber fermentations brined with $CaCl_2$ with a starter culture translated into the dominance of *Lactobacillus* from day 1 (Fig. 3).

Characterization of commercial cucumber fermentations brined with $CaCl_2$ or NaCl

Colony counts from culture plates and pH

Figure 4 shows that the microbial growth was faster in inoculated cucumber fermentations brined with $CaCl_2$ as compared to those brined with NaCl during the first 4 days. Significantly different colony counts from MRS were statistically confirmed using a *T*-test for days 2 and 4, but not for day 5 and beyond. No significant differences in colony counts from MRS plates were found among the two cover brine types using an ANOVA-Tukey HSD test ($P \leq 0{\cdot}05$). pH values shown in Fig. 4 demonstrate an apparent quicker acidification in the absence of NaCl as of day 4 of the fermentation. However, a statistically significant difference was not found among the pH values obtained from samples of the two cover brine types as a function of time. Given that it takes at least 36–48 h for a commercial fermentation tank to fully equilibrate, it is impractical to precisely measure the pH from a cover brine sample prior to day 4. Colony counts

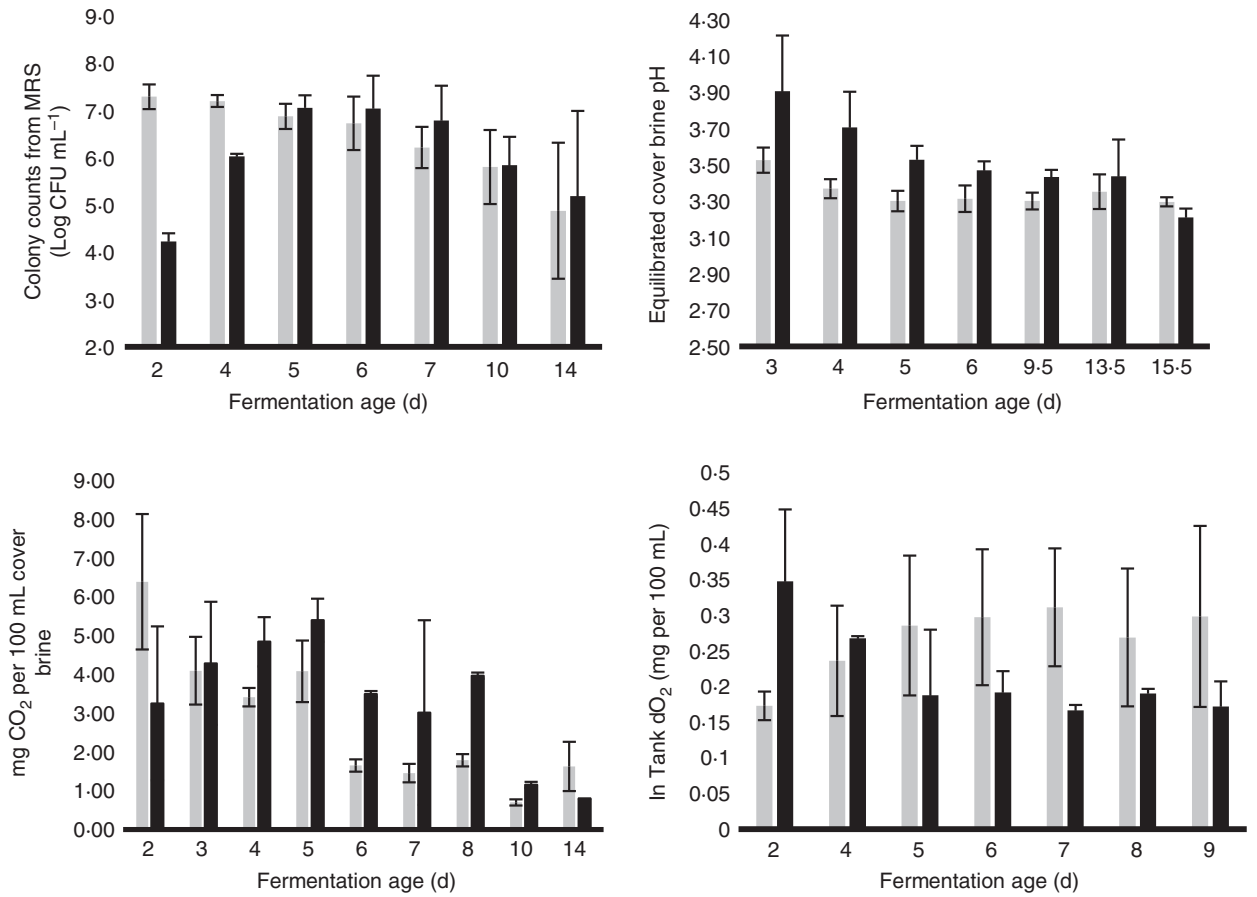

Figure 4 Colony counts from MRS plates, pH, dissolved oxygen and carbon dioxide measured from commercial cucumber fermentations. Colony counts and pH were measured from three commercial fermentations brined with 1·03 mol l^{-1} NaCl (■) and 10 vessels brined with 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} sodium benzoate with a starter culture (■). The dissolved oxygen and carbon dioxide measurements were obtained from 4 and 6 commercial vessels brined with 1·03 mol l^{-1} NaCl (■) or 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} sodium benzoate and a starter culture (□) respectively.

from YMA corresponding to fresh cucumbers were at 4·3 ± 0·9, while the numbers fluctuated from 2·91 ± 0·4 log CFU per ml on day 4 to 4·21 ± 0·6 log CFU per ml on day 6 of the fermentations brined with 1·03 mol l^{-1} NaCl and remained stable through day 14. Colony counts from YMA plates inoculated with brine samples collected from cucumber fermentations brined with $CaCl_2$ were below the detection limit until after day 10, reaching 2·71 ± 0·2 log CFU per ml^{-1} on day 14, likely due to the disappearance or reduction of sorbic acid to below inhibitory levels after day 10 (Fig. 6). The concentration of sorbic acid in samples collected from commercial fermentations brined with $CaCl_2$ declined by 6·6 ± 1·3 mmol l^{-1} on day 14, with 65% of the decline occurring between days 1 and 3. Colony counts from VRBG plates were 3·38 ± 0·53 log CFU per ml for all the industrial scale fermentations on day 1 and too few to counts after 3 days.

Metabolite concentrations

Analysis of the commercial fermentations' metabolites suggest that glucose and most of the fructose, naturally present in cucumber, were utilized (Fig. 5). Although, a complete utilization of glucose was observed in fermentations brined with $CaCl_2$ by day 4, residual fructose was detected on day 14. Faster sugars utilization in commercial fermentations brined with $CaCl_2$ resulted in a quicker production of lactic acid to 83·8 ± 8·8 mmol l^{-1} on day 10. In line with slower microbial growth and reduction in pH is the detection of glucose and fructose in cover brines containing NaCl (Fig. 5). An average of 10·5 ± 2·9 mmol l^{-1} glucose and 18·3 ± 3·1 mmol l^{-1} fructose were the highest concentration points on days 3 and 5 respectively in commercial fermentations brined with 1·03 mol l^{-1} NaCl (Fig. 5). Residual sugars were measured from cover brines containing NaCl on day 14. Up to 78·4 ± 21·5 mmol l^{-1} lactic acid was detected in

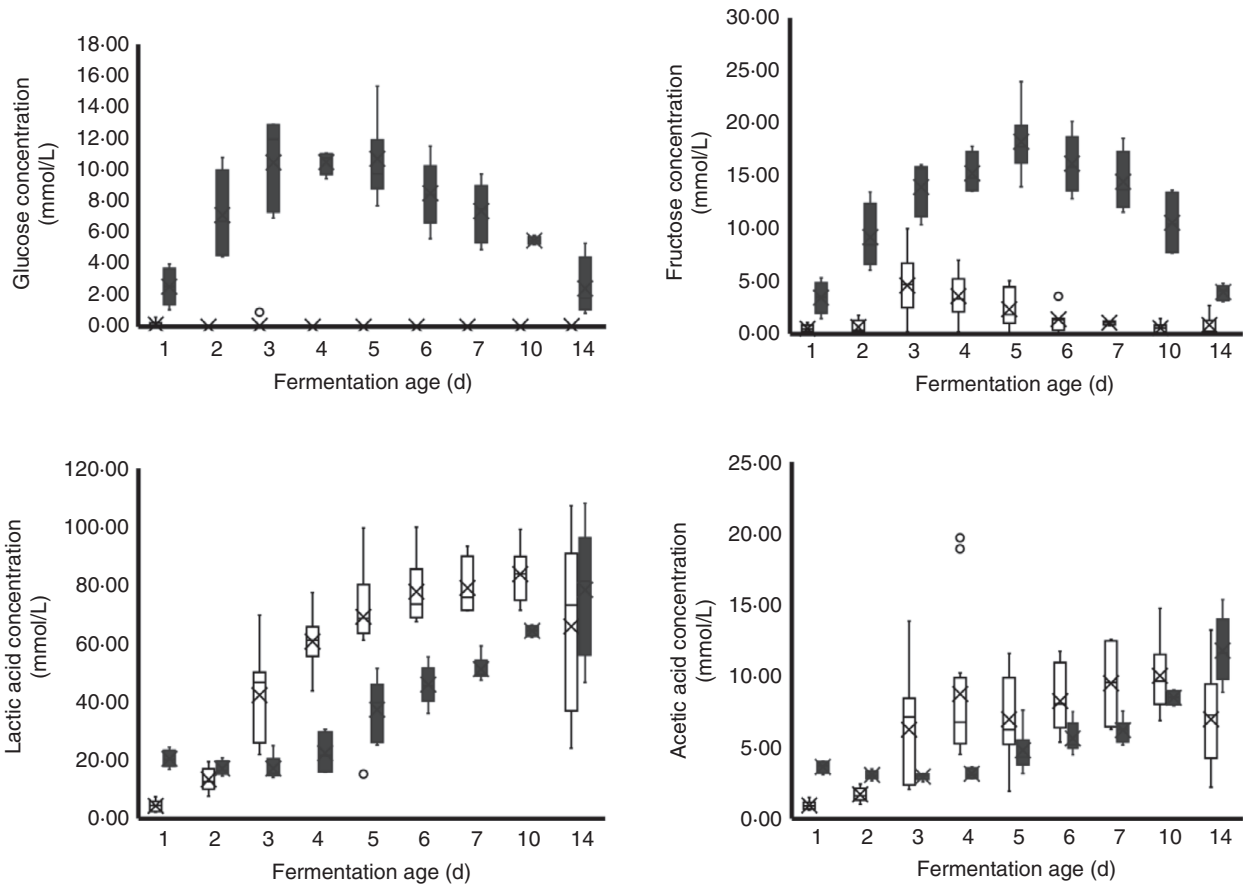

Figure 5 Metabolites of commercial cucumber fermentations brined with either 1·03 mol l^{-1} NaCl or 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} sodium benzoate. Organic acids and sugars concentrations were measured from fermentation cover brines. Samples per time point fluctuated between 6 and 14 and represent duplicates of three tanks containing NaCl cover brine (black) and seven fermentations brined with $CaCl_2$ (white).

commercial fermentations brined with NaCl, which is 5·3 mmol l^{-1} lower that the average production in the $CaCl_2$ counterpart. Acetic acid was produced in all fermentations to an average of 8·7 ± 3·7 mmol l^{-1} (Fig. 5). Residual and undetectable levels of malic acid were detected in the cover brine samples collected from fermentations brined with $CaCl_2$ or NaCl by day 5 (Fig. 5). In average, 1·8 ± 0·8 mmol l^{-1} succinic acid was detected in fermentation cover brine samples, however an increasing trend beyond day 3, representative of production, was lacking (Fig. 5). Figure 5 shows that a substantially higher ethanol concentration, averaging at 13·6 ± 6·1 mmol l^{-1}, was consistently detected in samples collected from commercial fermentations brined with $CaCl_2$ as compared to the NaCl containing samples (6·2 ± 3·8 mmol l^{-1}). A decline in sorbic acid concentration was observed in all fermentations as a function of time (Fig. 6).

Abundances of the bacterial populations

The average number of sequences for the 43 samples collected from the industrial scale fermentations was 45 869 ± 12 578 (78·0% ± 2·7 of the starting sequences) having a median length of 427 bp after the quality control and merging steps.

Lactobacillus dominated fermentations packed with the NaCl cover brine with a relative abundance of at least 95% for day 1, 2 and 3 (Fig. 9). This contrasted with the fermentations brined with $CaCl_2$ for the same time period, in which *Lactobacillus* (46–64%), *Leuconostoc* (8·5–23%), *Lactococcus* (15–21%) and *Weissella* (1–6%) prevailed. The microbiota on the first day of the fermentations brined with $CaCl_2$ were more diverse than on days 2 and 3 (Fig. 9). A reduction in diversity is expected from the production of acid as part of the fermentation, which excludes those microbes that thrive in neutral but not acidic pH and favours the acidophilic LAB.

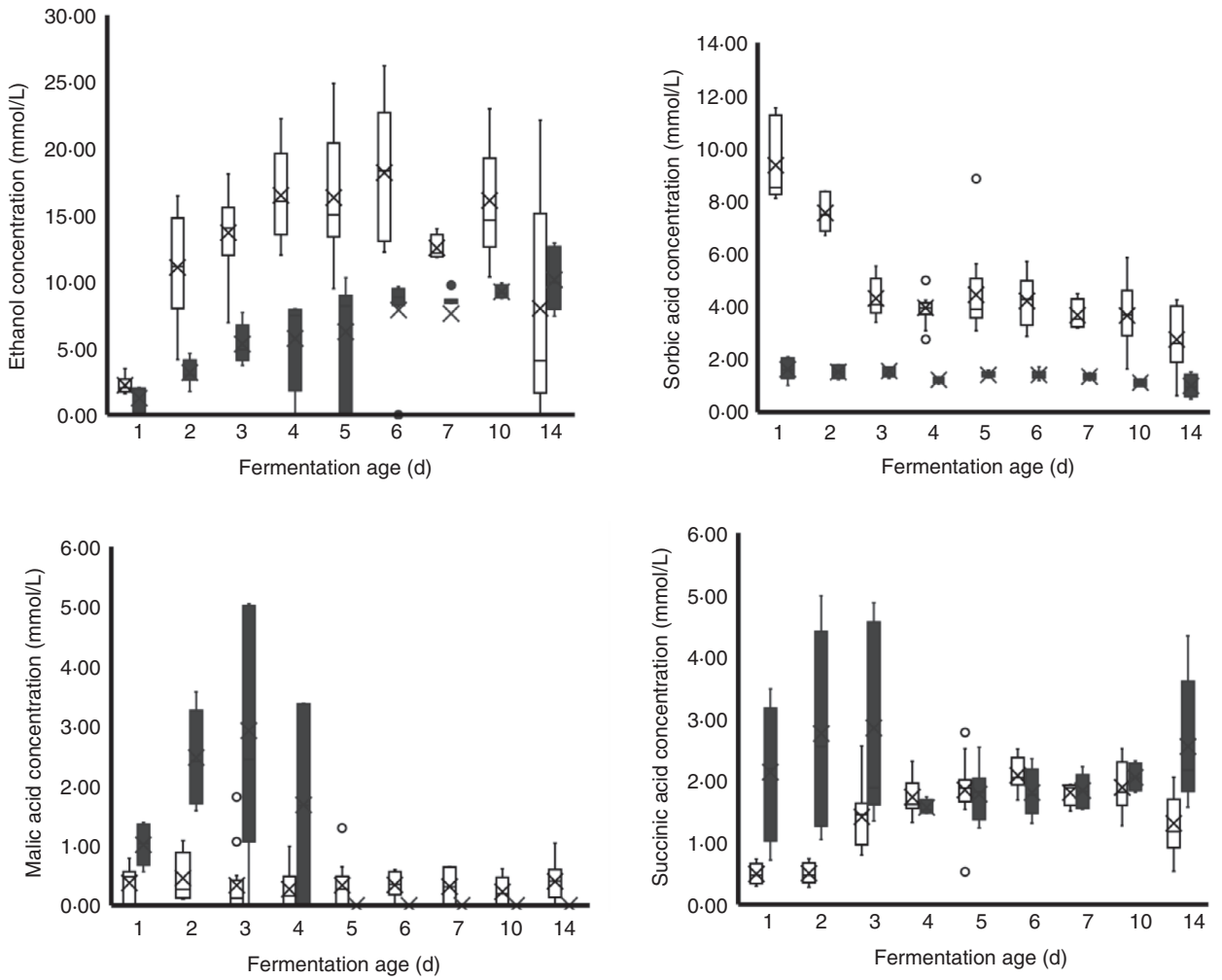

Figure 6 Metabolites of commercial cucumber fermentations brined with either 1·03 mol l^{-1} NaCl or 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} sodium benzoate. Ethanol and sorbic acid concentrations were measured from fermentation cover brines. Samples per time point fluctuated between 6 and 14 and represent duplicates of three tanks containing NaCl cover brine (black) and seven fermentations brined with $CaCl_2$ (white).

The composition of the microbiota found in the first three days of commercial fermentations were also noted in the remaining days studied (Fig. 10). *Lactobacillus* continued to dominate in commercial fermentations brined with NaCl. Figure 10 shows that fermentations brined with $CaCl_2$ and supplemented with a *Lactobacillus plantarum* starter culture were dominated by *Lactobacillus*. While the relative abundance for *Leuconostoc* was <0·25% in commercial fermentations brined with NaCl, its presence in its $CaCl_2$ counterparts declined from 14% on day 6 to 1% on day 14. The relative abundance of *Pediococcus* declined from 5% on day 6 to 0·9% on day 14 in commercial fermentation cover brine samples containing NaCl, while it persisted in the later $CaCl_2$ samples with a relative abundance of 3% on day 14. *Lactococcus*, which had a very small relative abundance (<0·25%) in cover brine samples containing NaCl, remained with an average relative abundance of about 12% in the $CaCl_2$ fermentations for day 6 through day 14. While the *Citrobacter* relative abundance declined from 5% on day 6 to <0·5% by day 14 in cover brine samples containing $CaCl_2$, levels of *Acetobacter* increased to a relative abundance of 10% by day 14. The differences shown in Figs 9 and 10 between the fermentations brined with either 1·03 mol l^{-1} NaCl or 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} potassium sorbate can also be clearly observed on a PCoA plot (Fig. 7). Figure 7 is based on the unweighted UniFrac distance matrix and the separation between the NaCl and $CaCl_2$ samples reflects the different OTUs in the two treatments. When abundance is taken into account in the weighted UniFrac plot (Fig. 8), it can be seen that there is still a divide between the two treatments. However, there is also

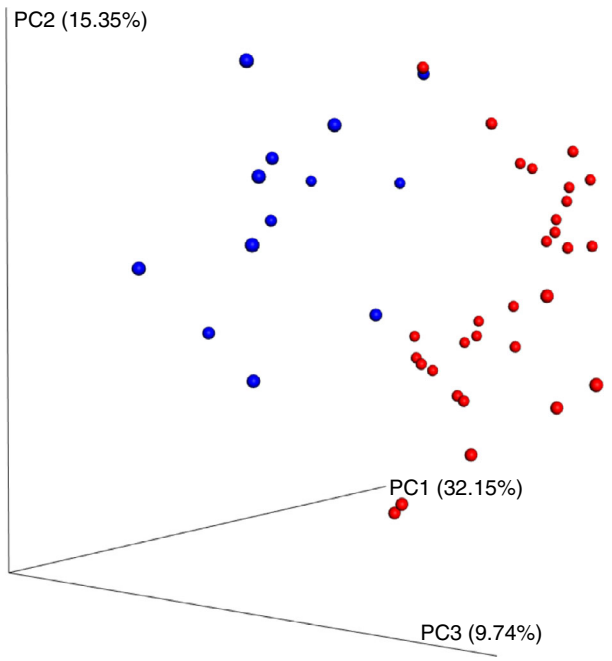

Figure 7 PCoA plot for the unweighted UniFrac metric coloured by treatment for the commercial cucumber fermentations. The NaCl treatment samples are blue (n = 13) and the $CaCl_2$ treatment samples are red (n = 30). [Colour figure can be viewed at wileyonlinelibrary.com]

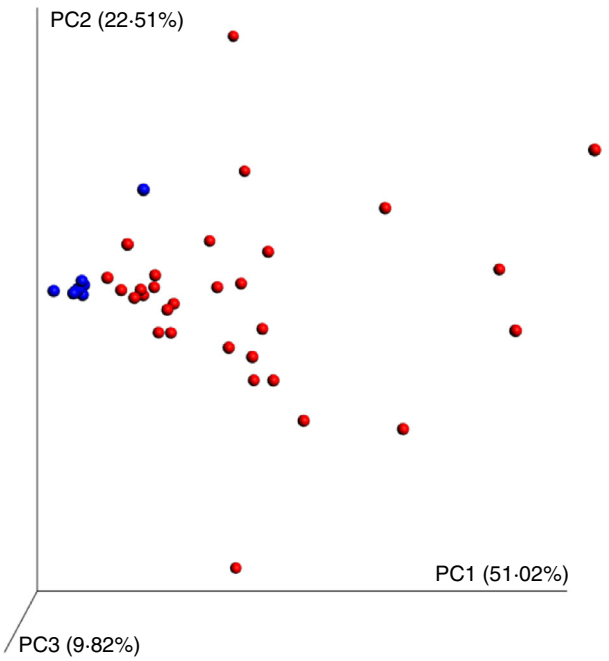

Figure 8 PCoA plot for the weighted UniFrac metric coloured by treatment for the commercial cucumber fermentations. The NaCl treatment samples are blue (n = 13) and the $CaCl_2$ treatment samples are red (n = 30). [Colour figure can be viewed at wileyonlinelibrary.com]

a difference in the type of clustering observed between the two treatment groups. For the fermentations brined with NaCl, the samples cluster tightly together (with the exception of one sample). For the fermentations brined with 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} potassium sorbate, the samples are much more spread out than is observed for the NaCl samples. This likely reflects the similarity in both composition and abundance of the NaCl samples from day 1 through day 14, whereas considerably more variability is observed for the $CaCl_2$ samples in this time period. A pairwise comparison between the NaCl and the $CaCl_2$ treatments based on the mean unweighted UniFrac distance matrix results in a Bonferroni-corrected P-value of 0·010. This suggests that samples within a treatment are significantly more similar to each other than samples between treatments and this ties in with the compositional differences observed between treatments in Figs 9 and 10. A similarly low p-value is not observed for the weighted UniFrac metric.

Dissolved oxygen and carbon dioxide concentrations

Figure 4 shows levels of carbon dioxide measured in fermentation cover brine samples containing $CaCl_2$, which were visibly, substantially higher than those observed for the counterpart brined with NaCl on day 2. Such a pattern flips between days 6 and 10 of the fermentations. No significant difference was calculated among the carbon dioxide values collected from fermentations brined with $CaCl_2$ or NaCl using a ANOVA-Tukey HSD Test ($P \leq 0{\cdot}05$). The trends for dissolved oxygen are the opposite of those observed for dissolved carbon dioxide (Fig. 4). Although, generally the values for dissolved oxygen corresponding to fermentations brined with $CaCl_2$ differ from those measured in the counterparts brined with NaCl, no significant difference was calculated based on a ANOVA-Tukey HSD Test ($P \leq 0{\cdot}05$), presumably due to the large fluctuations in the measurements among replicates.

Cucumber fermentations brined with NaCl and subjected to air purging rates between 15 and 65 SCFH presented bloater defect between 7 and 3%, as determined by the processor from the visual inspection of longitudinally cut fruits (data not shown). However, bloater defect in cucumber fermentations brined with $CaCl_2$ and packed with size 3A cucumbers was measured at 27%.

Discussion

The development of low NaCl fermented products is a required technology if we are to achieve a reduction in the consumption of sodium worldwide. Several studies have evaluated the use of other chloride salts, such as $CaCl_2$ and potassium chloride (KCl), to achieve such a goal. The reduction of sodium in dry fermented sausages

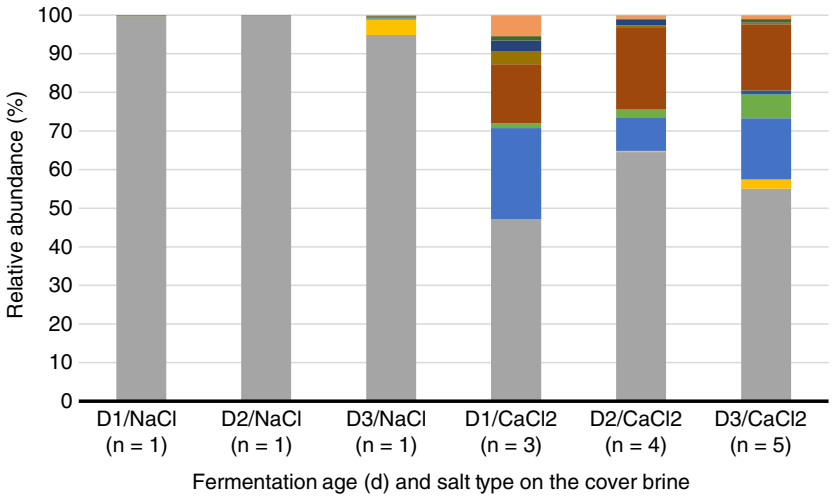

Figure 9 Bacterial ecology of the initial stage of commercial cucumber fermentations (days 1 through 3) brined with either 1·03 mol l^{-1} NaCl or 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} potassium sorbate and a *Lactobacillus plantarum* LA0445 starter culture. The relative abundance is shown on the genus level and the bar plots are averaged over replicates for each day/treatment. If the relative abundance of a genus is <0·5% when averaged over the 43 commercial cucumber fermentation samples, then it is not listed and is instead included in the 'less than 0·5%' category (*Lactobacillus*; *Weissella*; *Acetobacter*; *Acinetobacter*; *Pediococcus*; Leuconostocaceae; Other; *Citrobacter*; less than 0·5%; *Leuconostoc*; *Lactococcus*; *Pseudocitrobacter*). [Colour figure can be viewed at wileyonlinelibrary.com]

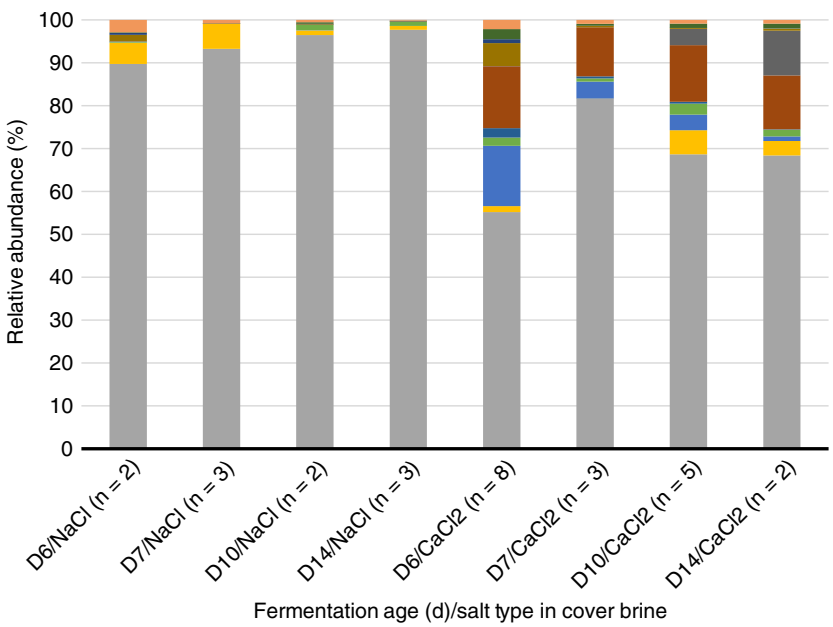

Figure 10 Bacterial ecology of the late stage of commercial cucumber fermentations (days 6 through 14) brined with either 1·03 mol l^{-1} NaCl or 100 mmol l^{-1} $CaCl_2$ and 6 mmol l^{-1} potassium sorbate and a *Lactobacillus plantarum* LA0445 starter culture. The fermentations brined with NaCl were not inoculated and the brine used was recycled. Freshly prepared cover brine was used in the tanks brined with $CaCl_2$. The relative abundance is shown on the genus level and the bar plots are averaged over replicates for each day/treatment. If the relative abundance of a genus is < 0·5% when averaged over the 43 commercial cucumber fermentation samples, then it is not listed and is instead included in the 'less than 0·5%' category (*Lactobacillus*; *Weissella*; *Acetobacter*; *Acinetobacter*; *Pediococcus*; Leuconostocaceae; Other; *Citrobacter*; less than 0·5%; *Leuconostoc*; *Lactococcus*; *Pseudocitrobacter*). [Colour figure can be viewed at wileyonlinelibrary.com]

using a mixture of chloride salts results in enhanced acidification, higher water activity, reduced Micrococcaceae counts, less consumer acceptability due to lack of saltiness, a compromised texture profile and increased lipid oxidation (Gimeno *et al.* 1998, 1999; Zanardi *et al.* 2010; Dos Santos *et al.* 2015).

Incorporation of $CaCl_2$ in vegetable fermentation cover brines is a more viable alternative to partially enable NaCl reduction in processing as compared to sausages. The use of 1·8–11% $CaCl_2$ in combination with reduced NaCl in olive fermentation cover brines does not impact lactic acid production, and accelerates acidification and the die off of Enterobacteriaceae (Bautista-Gallego *et al.* 2010; Panagou *et al.* 2011). However, the addition of $CaCl_2$ in black olive fermentations can result in a reduction in consumer acceptability due to bitterness (Panagou *et al.* 2011). The $CaCl_2$ concentration considered for inclusion in olive fermentations (1·8–11%) are above the levels recommended for inclusion in the typical cucumber fermentation or the NaCl-free cucumber fermentation (Guillou and Floros 1993; Bautista-Gallego *et al.* 2010; McFeeters and Pérez-Díaz 2010; Panagou *et al.* 2011). The optimized salts and preservative concentrations for cucumber fermentation were defined using a response surface model at 3% NaCl, 0·28% $CaCl_2$ and 0·3% potassium sorbate so that a vigorous fermentation free of mould and yeast with tissue firmness retention for 6 months could be achieved (Guillou and Floros 1993). No impact on sugars utilization in cucumber juice fermentations was observed as the result of inorganic cation supplementation, including 10–360 mmol l^{-1} $CaCl_2$ (Lu *et al.* 2002). However, glucose and fructose utilization were lower in the presence of divalent cations as compared to monovalent cations in cucumber juice (Lu *et al.* 2002).

The inception of a NaCl-free cucumber fermentation has been possible in the United States as part of a strategy to reduce chloride and sodium ions in wastewaters derived from processing. While NaCl is eliminated from the new fermentation process, it is still added to finished products to meet consumer's expectations and product identity standards. Inclusion of 100 mmol l^{-1} $CaCl_2$ in the new cover brine formulation to replace NaCl is possible due to the desalting step typically incorporated during processing following fermentation and prior to packing. The decalcification step reduces $CaCl_2$ to levels typically added in finished products, thus preventing the development of bitterness. Although, some compromises on firmness retention have been documented in fermented cucumbers brined with $CaCl_2$ as the only salt (McMurtrie and Johanningsmeier 2018), consumers rejection to the products manufactured with the $CaCl_2$ cover brine have not been documented.

This study demonstrates that supplementation of cucumber fermentations with a cover brine containing $CaCl_2$ as the only salt induces faster microbial growth as compared to cover brines containing no salt or NaCl (1·01 mol l^{-1}) (Figs 1 and 4). Utilization of a starter culture in cucumber fermentations brined with $CaCl_2$ results in faster microbial growth and quicker decline in cover brine pH (Figs 1 and 4), which consequently induces a faster decline of presumptive Enterobacteriaceae, as observed by others in olive and sausage fermentations (Gimeno *et al.* 1998; Bautista-Gallego *et al.* 2010; Panagou *et al.* 2011). The fermentation metabolites profile is also more consistent when $CaCl_2$ cover brine and a starter culture are used (Fig. 5) in cucumber fermentations as compared to the NaCl counterpart.

This study further describes the differences in the fermentation microbiota packed with NaCl-free cover brines and those containing $CaCl_2$. In packing cucumbers in the laboratory with cover brines containing different salt type and content, it became apparent that a main consequence of salting is the modulation of the relative abundance of distinct members of the bacterial populations, in particular on day 1 of the fermentation (Figs 3, 7 and 8). Additionally, in the system brined with NaCl, *Weissella*, a heterofermentative lactic acid bacterium, had to compete with a number of Gram-negative bacteria that are likely to compromise the quality of the finished product (Pérez-Díaz *et al.* 2018). It became apparent that *Leuconostoc* and *Lactococcus* are substantially different to *Weissella* with regards to NaCl sensitivity (Fig. 3). As expected, inoculation of fermentations brined with $CaCl_2$ with a *L. pentosus* starter culture resulted in the displacement of the indigenous bacterial populations (Fig. 3). *Lactobacillus* and *Pediococcus* were noted in most fermentations by day 3, suggesting the need for a longer lag phase as compared to those for *Leuconostoc*, *Lactococcus* and *Weissella* (Fig. 3) detected on day 1. Clearly, addition of NaCl or $CaCl_2$ in cover brines resulted in the dominance of *Lactobacillus* in laboratory scale cucumber fermentations by day 7. Conversely, a lack of NaCl and $CaCl_2$ in the fermentation cover brines yielded comparatively more microbial diversity with less acid production even after 14 days (Fig. 3). No indication of spoilage was observed in the first 14 days post-brining, suggesting the fermentations were effective during the first 2 weeks in closed jars. Commercial scale fermentations brined with $CaCl_2$ and a starter culture or with NaCl resembled the laboratory scale fermentations with regards to pH, colony counts from MRS plates and the fermentation metabolites.

Of interest is the observation that the numbers of *Acetobacter* increased as a function of time in commercial fermentations brined with $CaCl_2$, but not in fermentations brined with NaCl or laboratory scale fermentations (Fig. 10). While Acetobacteriaceae have not been detected in fresh cucumbers or fermentation cover brines samples collected on day 1, they have been isolated from fermentation cover brine samples collected on day 3 to 1·0% relative abundance (Pérez-Díaz *et al.* 2018). *Acetobacter peroxydans*, *Acetobacter aceti* and *Acetobacter pasteurianus*

have been found in commercial cucumber fermentation cover brine samples at pH below 3·4 (Medina-Pradas *et al.* 2016). In this study, the emergence of *Acetobacter* occurs on day 10 of the fermentations brined with $CaCl_2$, after sorbic acid has disappeared and an average of 16·06 mmol l^{-1} ethanol, 9·64 mmol l^{-1} acetic acid and 83·82 mmol l^{-1} lactic acid had been produced (Figs 5, 6 and 10). Moreover the increase in the relative abundance of *Acetobacter* coincides with a slight reduction in lactic and acetic acid concentrations to an average of 73·16 and 6·98 mmol l^{-1} respectively at a pH close to 3·30, in the presence of 0·3 mg per 100 ml of dissolved oxygen and after the levels of CO_2 reached its minimum at 0·70 mg per 100 ml of cover brine (Figs 4 and 10). Together these observations confirm that commercial cucumber fermentations undergo spoilage after the majority of the glucose and fructose is metabolized and a pH of 3·30 is reached. Such spoilage is led by *Acetobacter* sp., the only other genera detected in fermentation cover brine samples after the main sugars are converted to acids and ethanol, as described by Medina-Pradas *et al.* (2016).

It is concluded that the implementation of the environmentally friendly cucumber fermentation brined with $CaCl_2$ at the commercial scale can enable the production of pickles with a more diversified community of potentially probiotic LAB, which is of interest as we move towards personalized nutrition. Cucumber fermentations brined with $CaCl_2$ enable the enhancement of safety, given the early inhibition of potentially pathogenic Enterobacteriaceae as the result of faster acidification, as compared to fermentations brined with 1·03 mol l^{-1} NaCl. However, a more diversified community of LAB in cucumber fermentations brined with $CaCl_2$ could result in a higher incidence of bloater defect from the production of carbon dioxide by heterofermentation.

Acknowledgements

The authors thank Ms Lisa Moeller and Ms. Caitlin Alberts with Mount Olive Pickle Company, Mt Olive, NC, USA for assisting with fermentation cover brine sample collection.

Conflict of Interest

No conflict of interest is declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary sheet for the number of reads and alpha diversity metrics corresponding to the 16S *rRNA* amplicon sequencing data for laboratory scale cucumber fermentation cover brine samples.

Table S2. Summary sheet for the number of reads and alpha diversity metrics corresponding to the 16S *rRNA* amplicon sequencing data for commercial scale cucumber fermentation cover brine samples containing 1·03 mol l^{-1} NaCl.

Table S3. Summary sheet for the number of reads and alpha diversity metrics corresponding to the 16S *rRNA* amplicon sequencing data for commercial scale cucumber fermentation cover brine samples containing 100 mmol l^{-1} $CaCl_2$, 6 mmol l^{-1} potassium sorbate and a starter culture.