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Identification of potential causative agents of the CO_2-mediated bloater defect in low salt cucumber fermentation☆

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ABSTRACT

Development of bloater defect in cucumber fermentations is the result of carbon dioxide (CO_2) production by the indigenous microbiota. The amounts of CO_2 needed to cause bloater defect in cucumber fermentations brined with low salt and potential microbial contributors of the gas were identified. The carbonation of acidified cucumbers showed that 28.68 ± 6.04 mM (12%) or higher dissolved CO_2 induces bloater defect. The microbiome and biochemistry of cucumber fermentations ($n = 9$) brined with 25 mM calcium chloride ($CaCl_2$) and 345 mM sodium chloride (NaCl) or 1.06 M NaCl were monitored on day 0, 2, 3, 5, 8, 15 and 21 using culture dependent and independent microbiological techniques and High-Performance Liquid Chromatography. Changes in pH, CO_2 concentrations and the incidence of bloater defect were also followed. The enumeration of *Enterobacteriaceae* on Violet Red Bile Glucose agar plates detected a cell density of 5.2 ± 0.7 log CFU/g on day 2, which declined to undetectable levels by day 8. A metagenomic analysis identified *Leuconostocaceae* in all fermentations at 10 to 62%. The presence of both bacterial families in fermentations brined with $CaCl_2$ and NaCl coincided with a bloater index of 24.0 ± 10.3 to 58.8 ± 23.9. The prevalence of *Lactobacillaceae* in a cucumber fermentation brined with NaCl with a bloater index of 41.7 on day 5 suggests a contribution to bloater defect. This study identifies the utilization of sugars and malic acid by the cucumber indigenous *Lactobacillaceae, Leuconostocaceae* and *Enterobacteriaceae* as potential contributors to CO_2 production during cucumber fermentation and the consequent bloater defect.

1. Introduction

Bloater defect, commonly occurring in cucumber fermentation, is defined as the formation of hollow cavities inside the fruit with different types and shapes including honeycomb, lens and balloon (Corey et al., 1983a, 1983b; Etchells et al., 1974). Honeycomb bloaters are characterized by small cavities (2 to 5 mm diam.) that formed around the immature vulnerable seeds in the cucumbers (Etchells et al., 1968). The lens lesion in a bloated cucumber is characterized by a lenticular shape and occurs along the longer axis (Etchells et al., 1968). The formation of a balloon lesion in a bloated and fermented cucumber separates the carpel generating a large cavity as a result of gas pressure (Etchells et al., 1968). Bloater damage leads to subtantial substantial losses of yield and quality of fermented cucumbers by compromising the appearance of finished products (Fleming et al., 1973a).

The production of gas, mostly carbon dioxide (CO_2), was identified as the main cause of bloater defect in cucumber fermentation by Veldhuis and Etchells (1939). Measurements of the gaseous components of spoiled commercial cucumber fermentation brines were compared to that found in the hollow cavities of bloated and fermented cucumbers. The gases found in both fractions included CO_2, hydrogen (H_2) and oxygen regardless of the sodium chloride (NaCl) content of the fermentations. It has been observed that bloater defect is initiated when 60 mg of CO_2 per 100 mL of cover brine accumulates in the tissue in the

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presence of 1.2 M (6.7%) NaCl and 48.8 mM (0.4%) sodium acetate at 26.7 °C (Fleming et al., 1973a). Etchells et al. (1973) recommended the maintenance of the CO_2 concentration in controlled cucumber fermentations below 20 mg of CO_2 per 100 mL to prevent bloater damage. A systematic study is lacking to define the minimum CO_2 concentration needed to induce bloater defect under various fermentation conditions, particularly with respect to low salt brining.

Known sources of CO_2 in cucumber fermentations include tissue respiration and microbial activity inclusive of yeasts, molds and bacteria (Etchells and Bell, 1950; Etchells et al., 1968; Fleming et al., 1973a, 1973b; McFeeters et al., 1984). Tissue respiration was shown to be insufficient to cause bloater defect (Fleming et al., 1973b; Zhai and Pérez-Díaz, 2017). Only 30 mg of CO_2 per 100 g of cucumbers were detected in closed jars packed with pasteurized, non-inoculated and unfermented cucumbers in the presence of 1.2 M (6.7%) NaCl and 61.1 mM (0.5%) sodium acetate at room temperature (23 ± 1 °C) (Fleming et al., 1973b). A CO_2 concentration of 114 mg per 100 g of cucumbers was measured in the equivalent pasteurized jars in which a fermentation proceeded (Fleming et al., 1973b). Thus, the microbial production of the gas is considered the main factor causing bloater defect (Etchells and Jones, 1941; Jones et al., 1941).

More bloater defect is observed in fermented cucumbers brined with the environmentally friendly calcium chloride ($CaCl_2$) salt and no NaCl as compared to the counterparts brined with 1.06 M (6%) NaCl (McMurtrie et al., 2019; Pérez-Díaz et al., 2015). A bloater index of 16 to 47 was calculated from controlled cucumber fermentations brined with $CaCl_2$, calcium hydroxide ($Ca(OH)_2$) and 0 to 700 mM (4%) NaCl, inoculated with a starter culture and acidified to pH 4.7 with acetic acid (Zhai et al., 2018). Data derived from a metagenetic analysis of the microbial population in such cucumber fermentations suggest that *Lactobacillaceae* prevail with a minimum relative abundance of 72%. The most abundant microbes in such cucumber fermentations included *Lactobacillus* and *Pediococcus* (Zhai and Pérez-Díaz, 2017). These LAB were followed by *Enterobacteriaceae* on days 3 and 10 and *Leuconostocaceae* after day 7 (Zhai and Pérez-Díaz, 2017). Moreover, cucumber fermentations brined without $Ca(OH)_2$ support the presence of *Enterobacteriaceae* even after day 10 post-packing at 30 °C (McDonald et al., 1991). Although such microbes are theoretically capable of producing CO_2, there is no systematic evaluation implicating them in bloater defect in low salt cucumber fermentations or the typical commercial bioconversion brined with 6% NaCl. An understanding of the causative agent (s) of fermented cucumber bloater defect in cucumber fermentations brined with $CaCl_2$ instead of NaCl or their combination is still lacking. We aimed to determine the amount of CO_2 needed in fermentations brined with $CaCl_2$, as the primary salt, to cause bloater defect and identify the causative agent(s) of the CO_2-mediated defect. The approach taken to determine the amount of the gas needed to cause bloater defect included the acidification of cucumbers with brines containing $CaCl_2$ as the primary salt to which CO_2 was added as sodium bicarbonate ($NaHCO_3$). Cucumber fermentations brined with $CaCl_2$ and NaCl or NaCl alone were studied to elucidate potential causative agents of bloater defect. The microbiome and biochemistry of the fermentations were studied as a function of time using metagenomic analysis and High-Performance Liquid Chromatography (HPLC). Production of CO_2 and bloater defect were also measured as a function of time to establish an association of the emergence of the defect with the changes in the fermentation.

2. Materials and methods

2.1. Carbonation of acidified cucumbers

Size 2B (1.25 to 1.5 in. diameter) cucumbers were obtained from a local processor (Mt. Olive Pickle Company, NC, USA) and brined to achieve preservation by acidification as described by Pérez-Díaz and McFeeters (2008). The acidification cover brine contained 100 mM

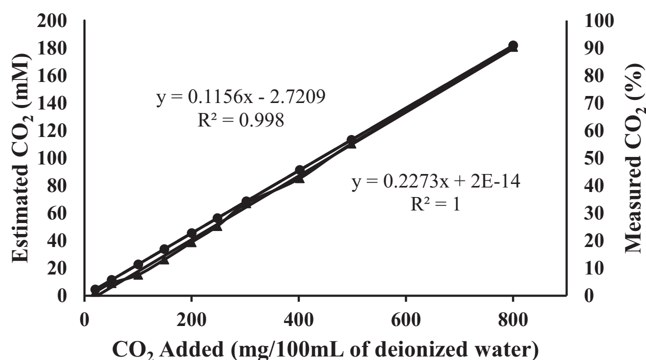

Fig. 1. Unit conversion for carbon dioxide (CO_2) % to mg/100 mL of water or mM. Average of CO_2 concentration measured from triplicate samples of a $NaHCO_3$ solution in deionized water at a pH of 3.3 ± 0.3 (closed triangles). The estimated molarity of CO_2 (mM) was calculated stoichiometrically from the mg of $NaHCO_3$ added per 100 mL of water (closed circles). The equations shown beside the lines represent the linear relationship of CO_2 in mg/100 mL of water with CO_2 in mM (solid line) or % (dashed line).

anhydrous $CaCl_2$ (Brenntag, Durham, NC, USA), 12 mM sodium benzoate (Sigma-Aldrich, St. Louis, MO, USA), and sufficient acetic acid in the form of vinegar (Fleischmann's Vinegar Company Inc., Cerritos, CA, USA) to adjust the pH to 3.5. The amount of acetic acid needed to adjust the pH of brined cucumbers to 3.5, in each treatment containing between 0 and 80% CO_2, were determined by titrating a slurry of the size 2B cucumbers from the experimental lot with each cover brine type individually. Cucumbers were packed in jars in a 50:50 ratio (*w*/w). In the center of the jar, metal lug caps were punched to make a hole and equipped with a 12 mm rubber septa that fitted in the hole. The rubber septa inserted in the metal lug caps functioned as an entry point for sampling using a syringe equipped with a Luer-Lok Tip and BD PrecisionGlide needle (BD, Franklin Lakes, NJ, USA). The modified metal lug caps were boiled for 15 s. immediately prior to closing the jars to activate the plastisol and achieve a vacuum seal. The content of the closed jars was allowed to equilibrate for 4 days prior to adding $NaHCO_3$ to generate CO_2 (Sigma-Aldrich). $NaHCO_3$ and hydrogen are converted to CO_2, sodium and water at a dissociation constant (pKa_1) of 6.3 and theoretically all bicarbonate is converted to CO_2 (gas) at a pH of 3.5 (Harned and Davis, 1943; PubMed CID: 516892). After adding known amounts of powdered $NaHCO_3$ to reach the desired CO_2 concentrations and the needed vinegar to adjust the pH, the cucumber jars were incubated at 30 °C for 7 days. The prolong incubation was included to enable the full equilibration of the liquid phase with the cucumbers, which was expected to enable the penetration of the $NaHCO_3$ into the tissue.

2.2. Measurement of CO_2 from carbonated, acidified cucumbers and cucumber fermentations

The amount of dissolved CO_2 in each jar was determined from a 2 mL cover brine sample collected with a 10 mL gas-tight syringe inserted through the rubber septa on the metal lug caps. The cover brine samples retrieved from the jars were injected into 15 mL plastic vacutainers with Hemogard closure (Pulmolab, 10 mL, BD #366643, Northridge, CA, USA). The CO_2 in the cover brine samples was released into the vacutainer's headspace by adding 3 mL of a vinegar solution containing 20% acetic acid followed by vigorous shaking. The CO_2 concentration in the vacutainer headspace was measured by a nondispersive infrared sensor using a Map-Pak Combi Gas Analyzer (AGC Instruments, Co., Clare, Ireland). The sampling needle on the Map-Pak Combi Gas Analyzer was inserted through the vacutainer Hemogard closure and the readings were recorded in %. Percent CO_2 (%) was converted to mM and/or mg CO_2/100 mL of water using Fig. 1.

Fig. 1 was developed by measuring CO_2 concentrations in %, as

described above, from deionized water solutions containing known amounts of $NaHCO_3$. $NaHCO_3$ was added to 0, 20, 50, 100, 150, 200, 250, 300, 400, 500 and 800 mg CO_2/100 mL of water in individual vacutainers in triplicate. The average of the triplicated measurements in % were plotted in the secondary y-axis on Fig. 1, against the concentration added in mg of CO_2 per 100 mL of water (x-axis). The calculated mM from the mg of CO_2/100 mL solution added were also plotted on the primary y-axis in Fig. 1. The trendlines and R^2 were calculated and are displayed in Fig. 1.

2.3. Assessment of bloater defect from carbonated, acidified cucumbers and fermented cucumbers

Cucumbers were aseptically collected from the jars and cut longitudinally to observe bloater formation and calculate bloater index. Fermented or acidified cucumbers were cut longitudinally using aseptic technique to observe the internal formation of hollow cavities at the end of the experimentation. The bloater index was calculated using the scale developed by Fleming et al. (1977) and modified by Zhai and Pérez-Díaz (2017). Briefly, the calculation of bloater index considers the percent of cucumbers in a jar that were affected by bloater damage and the injury type as well as its size. The numerical value for tissue damage of the varied injury type and size were applied as defined by Fleming et al. (1977) and Zhai and Pérez-Díaz (2017).

2.4. Determination of pH in the carbonated, acidified cucumber jars and fermented cucumbers

The pH of acidified or fermented cucumbers was measured from cover brine samples collected after equilibration. Sample collection proceeded as described above for the measurement of dissolved CO_2, using an Accumet pH meter (cat. 13–636-AR25B, Accumet™ AR25 pH/mV/°C/ISE, probe cat. 13–620-290, Fisher Scientific™, Hampton, NH, USA).

2.5. Cucumber fermentations

Cucumber fermentations were brined as described by Zhai and Pérez-Díaz (2017). The fermentation cover brine contained 50 mM anhydrous $CaCl_2$ (Brenntag) as the primary salt, and 690 mM NaCl (Morton, New Iberia, LA, USA), so that they will be diluted to 25 mM and 345 mM after equilibration with the cucumbers. The buffer capacity of the system was enhanced by adding 40.4 mM $Ca(OH)_2$ (Fisher Scientific, Waltham, MA, USA) and 91.6 mM acetic acid in the form of 20% vinegar (Fleischman's Vinegar Company Inc.). These two molecules would have increased the content of calcium acetate in the system (Etchells et al., 1973), a buffer that was expected to enable the completion of the fermentation itself without hindrance by the acidic pH (Zhai and Pérez-Díaz, 2017). The pack-out ratio for cucumbers and the cover brine per jar was 50:50 (*w*/w), so all ingredients would have equilibrated to half of the amounts added. The initial fermentation pH was 4.7 ± 0.1. A starter culture of *Lactobacillus pentosus* LA 445 was used in one out of two treatments (Treatment 1) to evaluate its influence in CO_2 production. The control fermentation treatment was brined with 1.06 M NaCl instead of $CaCl_2$, as the primary salt, and no $Ca(OH)_2$ or vinegar, so that the experimental treatments could be related to a typical commercial cucumber fermentation. All treatments were supplemented with 6 mM potassium sorbate (Mitsubishi International Food Ingredients, Dublin, OH, USA) to inhibit yeasts growth. Three lots of size 3B (1.75 to 2.0 in. diameter) cucumbers were used for the experiment, so that triplicates of each treatment could be packed with a distinct cucumber lot. Each jar was closed with a metal lug cap containing plastisol, which was activated by boiling for 15 s to achieve a vacuum seal. The center of the jar metal lug caps was punched to make a hole that would fit a rubber septa with a 12 mm diameter. The rubber septa served as an entry point for sampling using a syringe equipped with a Luer-Lok Tip and BD PrecisionGlide needle (BD) without opening the jar. The packed jars were incubated at 30 °C for 21 days. The fermentation jars were sampled on days 2, 3, 4, 5, 8, 15 and 21. Bloater index calculations and microbial and chemical analyses were performed at each sampling point as described.

2.6. Scope of the microbiological analysis

Equal volume of cucumbers and cover brine samples were blended and homogenized for microbiological and chemical analyses. The microbial analyses included plating on selective and differential media, isolation of *Enterobacteriaceae* on day 3 post-packing and metagenomic analysis. Selected colonies growing on HiCrome *Klebsiella* agar (HCK) and *Pseudomonas* Isolation agar (PIA) inoculated with homogenates prepared with samples collected on days 2 and 3 were streaked for purification, isolated and identified using 16S rDNA partial sequencing. A 10 mL aliquot of the fermented cucumbers homogenate collected at each time point was subjected to a propidium monoazide (PMA) treatment and total DNA extraction, as described below, for the metagenomic analysis.

2.7. Enumeration of colonies from cucumber fermentation samples

Cucumber fermentation homogenates were prepared by blending cucumbers and cover brine in a 50:50 ratio (w:v) for 1 min at maximum strength (Waring Co., Torrington, CT, USA). The blended samples were homogenized in plastic sterile bags equipped with a 250 μm porosity filter located perpendicular to the two plastic layers at about 1.5 in. from one of the sides of the bag (Interscience Laboratories Inc., Woburn, MA, USA). The homogenization occurred at medium strength for 30 s using a Stomacher 400 (Tekmar Company, Cincinnati, OH, USA). The homogenates were plated on Violet Red Bile Glucose agar (VRBG), deMan, Rogosa and Sharpe agar (MRS) supplemented with 10 mL cycloheximide/L (0.1% solution, SRO222C, Oxoid Ltd., Basingstoke, Hants, England), PIA and HCK for the enumeration of *Enterobacteriaceae*, homo- and heterofermentative LAB, *Pseudomonas/Citrobacter* and *Klebsiella*, respectively. The homogenates were plated using an Eddy Jet 2 W spiral automated plater (IUL Instruments, Barcelona, Spain). VRBG plates were incubated at 37 °C aerobically for 24 h. The HCK and PIA plates were incubated at 37 °C aerobically for 48 h. MRS plates were incubated at 30 °C aerobically for 48 h. Colonies were enumerated using a Flash & Go Automated Colony counter (Neutec Group, Inc., Barcelona, Spain).

2.8. Metagenomic analysis

Aliquots of 10 mL of the homogenized samples were collected on days 2, 3, 5 and 8 of the fermentations for metagenomic analysis. The homogenates were centrifuged at 10,000 rpm for 10 min at 22 °C (Eppendorf Centrifuge 5810R, Fisher Scientific, Fremont, CA, USA) to form pellets that were subjected to PMA treatments performed as described by Pan and Breidt (2007). To do so, the cell pellets were resuspended in 490 μL of a sterile 0.85% NaCl solution and treated with 10 μL of a 2.5 mM PMA stock solution (1.3 mg/mL PMA dissolved in 20% DMSO; Biotium, Inc., Hayward, CA, USA) to eliminate dead bacterial and extracellular DNA. DNA extractions were conducted on the treated pellets using the InstaGene Matrix DNA extraction kit (Bio-Rad Laboratories, Hercules, CA, USA) as described by the manufacturer. Extracted DNA was shipped to CosmosID Laboratories (Rockville, MD, USA) for sequencing analysis using the 3 M SE100 service. Data processing was conducted using the CosmosID proprietary platform.

2.9. Colony identification by the partial sequencing of the 16S rDNA

Colonies growing on PIA and HCK media with unique morphologies were isolated and streaked on the same enumerating medium for purification prior to identification using 16S rDNA sequencing. Pure colonies

were subjected to genomic DNA extraction using an InstaGene Matrix DNA extraction kit (Bio-Rad Laboratories) that was used as described by the manufacturer. The 16S rDNA conserved region V3-V4 was amplified from the extracted genomic DNA using 2 μM of primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi et al., 1998). Polymerase chain reaction (PCR) amplification was conducted with 1 μL of genomic DNA with concentrations of 30 to 1000 ng/μL and 1 μL of each primer, mixed with 12.5 μL of a master mix (Bio-Rad Laboratories) and water to a total volume of 25 μL. The PCR amplification steps consisted of 1 cycle of 1 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 2 min at 57 °C, and 2 min at 72 °C, with a final extension step of 7 min at 72 °C. Amplicons were stored at 4 °C for 2 days prior to sequencing using Sanger Dideoxy Sequencing technology by Eton Bioscience Inc. (Durham, NC, USA). Sequence data were then analyzed using the BioEdit software (www.mbio.ncsu.edu/bioedit). The DNA sequences obtained were subjected to a quality check, where each bp score had to be equal to or greater than 20. Sequences were subjected to the basic local alignment search tool (BLAST) as described by Pérez-Díaz et al. (2019) to determine the isolates identity. The sequences can be located in the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) with the accession numbers listed in Table 4.

2.10. Analyses of fermentation biochemistry

Homogenate samples collected from the fermentation were stored at −20 °C and exposed to two freeze-thaw cycles prior to analysis by HPLC to release the sugars from the plant matrix. For each freeze-thaw cycle, samples were subjected to a 24 h storage at −20 °C followed by a 4 h incubation at 22 ± 1 °C for thawing. After the last thawing, samples were spun using a Brushless Microcentrifuge (Denville 260D, Denville Scientific, Inc., Holliston, MA, USA) at 12,000 rpm for 10 min at 22 °C. The samples were spun twice to remove particulate prior to analysis by HPLC. Quantification of organic acids and sugars were done using the HPLC method published by McFeeters and Barish (2003) with some modifications using an Aminex HPX-87H resin column of 300 × 7.8 mm (Bio-Rad Laboratories). The operating conditions of the system included a column temperature of 65 °C and a 0.01 N sulfuric acid eluent set to flow at 0.9 mL/min. An SPD- 20A UV–vis detector (Shimadzu Corporation, Canby, OR, USA) was set at 210 nm at a rate of 1 Hz to quantify malic acid. A RID-10A refractive index detector (Shimadzu Corporation) connected in series with the diode array detector was used to measure lactic acid, acetic acid, glucose, fructose and ethanol. The external standardization of the detectors was done using eight gradient concentrations of the standard compounds. The concentration of each compound in each sample was calculated based on the height of the chromatograph peak at specific retention times as compared to each compound standard calibration curve using the LabSolutions workstation (Shimadzu Corporation).

2.11. Statistical analysis

Standard deviations were calculated for every mean value shown in tables and figures. Measurements obtained from acidified or fermented cucumbers were expected to present substantial standard deviations given the natural fluctuation in the concentrations of sugars, malic acid and other chemical constituents in the fresh fruits (Lu et al., 2002). The chemical composition as well as the microbiota indigenously present in cucumbers varies in response to field conditions such as soil composition and extend of irrigation as well as the physiological stage of the fruits. Thus, significant differences among the treatments were determined by LSMeans Tukey HSD using JMP Pro 12 (SAS Institute, Inc., Cary, NC, USA). A difference between treatments based on date was considered in the statistical analysis and the interactions between treatments and sampling times were also included. For all data sets, means denoted by different letters represent a significant difference $P \leq 0.05$ (ANOVA).

Table 1
Bloater index for acidified cucumbers caused by increasing carbon dioxide (CO_2) concentration derived from $NaHCO_3$ at pH 3.5 ± 0.2, about 3 pH units below the pKa_1 (6.3). The data shown represents the average and standard deviation of technical duplicates performed with the same lot of size 2B (1.25 to 1.5 in.) cucumbers. Levels not connected by the same letter within a column are significantly different.

Estimated [CO_2] from the $NaHCO_3$ Added (% / mM)	CO_2 in Cover Brine Samples (%)	Estimated [CO_2] from the % value (mM)	Bloater Index	Acidified Cover Brine pH
0/0	2.75 ± 0.21^{C}	10.75 ± 5.77^{C}	0^{B}	3.60 ± 0.05^{AB}
20/44.67	19.50 ± 2.69^{B}	43.69 ± 10.63^{B}	37.3 ± 10.4^{A}	3.46 ± 0.06^{B}
40/83.99	40.15 ± 3.61^{A}	84.29 ± 12.44^{A}	48.3 ± 5.7^{A}	3.67 ± 0.01^{A}
60/123.31*	11.85 ± 0.35^{B}	28.65 ± 6.04^{B}	32.7 ± 10.8^{AB}	3.56 ± 0.05^{AB}
80/162.63*	14.25 ± 1.06^{B}	33.37 ± 7.43^{B}	39.2 ± 12.0^{A}	3.51 ± 0.05^{AB}

* The corresponding jars experienced gas and liquid loss due to effervescence.

3. Results and discussion

3.1. Amount of CO_2 needed to induce bloater defect in the absence of NaCl and the presence of 100 mM $CaCl_2$

Carbonated, acidified cucumbers were used as a model system to determine the amount of CO_2 needed to cause bloater defect in the absence of NaCl. The acidification of cucumbers was expected to suppress CO_2 production by the indigenous microbiota (Pérez-Díaz and McFeeters, 2008), thus enabling an accurate determination of the amount of the gas needed to cause bloater defect. The stability of the pH value in the jars as the result of acidification indicated lack of a fermentation activity (Table 1). No CO_2 production was detected in control jars of acidified cucumbers as a function of time suggesting the absence of the microbial production of CO_2.

Table 1 and Fig. 2 show that 20% CO_2 in solution is sufficient to induce a bloater index of 37.3 ± 10.4 at a pH of 3.46 ± 0.06 in the absence of NaCl. Such CO_2 percentage equals to 44 mM (180 mg/100 mL) based on the standard curve shown in Fig. 1. Table 1 also shows that the bloater index increased as the CO_2 concentration increased from 0 mM to 20 and 40 mM. The actual CO_2 concentration in the treatments designed to contain 60% and 80% of the gas were lower than expected at 11.85% and 14.25%, respectively, due to effervescence and the likely loss of the gas from the jars before closure (Table 1). However, bloater damage occurred when the CO_2 concentration measured from the cover brine was at 11.85 ± 0.35% (28.65 ± 6.04 mM) indicating concentrations below 20% in cover brines are likely sufficient to induce the defect (Table 1). This observation suggests that the utilization of only 28 mM glucose or fructose or malic acid in a cucumber fermentation would be sufficient to cause bloater defect. Fig. 1 shows that 28.65 ± 6.04 mM CO_2 equals to 126.05 ± 26.60 mg of CO_2 per 100 mL of cover brine, which is substantially higher than the 60 mg CO_2 per 100 mL of cover brine identified by Fleming et al. (1973a) as sufficient to cause bloater defect in fermentations brined with NaCl. The substantial difference in the CO_2 concentration needed to observe bloater defect is likely the result of the dissimilar cover brine formulations used in the studies and possibly the divergent techniques used to measure CO_2. The solubility of CO_2 is reduced in the presence of NaCl making it more readily available in the gas form to cause the defect when the gas is present in lower amounts (Fleming et al., 1973a). A gas analyzer equipped with a nondispersive infrared sensor was used in this study to measure CO_2, while Fleming et al. (1973a) used a volumetric method described in the (Association of Official Agricultural Chemists (AOAC), 1965 sections 11.053 through 11.055 applying the microdiffusion principle of Conway (1957). This experiment generated a new tool to assess bloater defect in

Fig. 2. A representation of bloater defect observed in acidified cucumbers exposed to 20% (left) or 40% (right) CO_2 derived from $NaHCO_3$ at pH 3.5 ± 0.2.

new and/or improve fermentation processes using the commercially available gas analyzers.

3.2. Microbial contributor of bloater defect in cucumber fermentation

Microbes implicated by others in bloater defect in cucumber fermentations brined with 1.06 M or more NaCl include yeasts, molds and heterofermentative lactic acid bacteria (LAB), particularly those able to decarboxylate malic acid, such as *Lactobacillus plantarum* (Etchells and Bell, 1950; Etchells et al., 1968; Fleming et al., 1973a, 1973b; McFeeters et al., 1982, 1984). Although yeasts were originally associated with bloater incidence, given the ability to produce ethanol and CO_2 from sugars (Etchells, 1941; Etchells and Jones, 1941), it has been shown that the incidence of bloater remains unchanged when cucumber fermentations are brined with 0.1% potassium sorbate to suppress yeast growth (Etchells et al., 1968). Gram-negative *Enterobacteriaceae*, such as *Enterobacter aerogenes* and *Escherichia coli*, and halophilic bacteria can produce H_2 and CO_2, and possibly induce bloater defect (Etchells et al., 1968; Veldhuis and Etchells, 1939). Etchells (1941) demonstrated that *Enterobacter*, particularly *E. cloacae*, can produce H_2 and CO_2 in cucumber fermentation brined with 1.78 M (10%) NaCl and cause bloater defect. The contribution of *Enterobacteriaceae* to gas production is limited to the first few days of the fermentation, given their sensitivity to the acidic pH resulting from the lactic acid fermentation (Etchells et al., 1968; McDonald et al., 1991). The growth of *Enterobacter aerogenes* can be suppressed by adding either acetic acid or calcium acetate in fermentation cover brines (McDonald et al., 1991). The supplementation of fermentation cover brines with acetic acid delays but does not inhibit the growth of LAB, particularly the malic acid decarboxylating culture *Lactobacillus plantarum* (McDonald et al., 1991). Although L. *brevis* was isolated from defective commercial batches of fermented cucumbers and found able to produce sufficient CO_2 during fermentation to cause bloater defect (Etchells et al., 1968), *L. brevis* cannot dominate the indigenous microbiota (Pérez-Díaz et al., 2017). Facultative heterofermentors, such as L. *plantarum* and L. *pentosus*, primarily produce lactic acid in cucumber fermentation and are not expected to produce CO_2 from sugar utilization. However, *L. plantarum* and *L. pentosus* can decarboxylate malic acid forming lactic acid and CO_2 (McFeeters et al., 1982). CO_2 production by a malolactic strain of L. *plantarum* amounted to 12.5 mM in a cucumber fermentation brined with 0.88 M (5%) NaCl at 30 °C, which was sufficient to bring cucumbers to the point of bloating (Daeschel et al., 1985; McFeeters et al., 1984). Malic acid is the main organic acid in fresh cucumbers, with its concentration fluctuating between 14.2 and 23.4 mM depending on the cultivar (Daeschel et al., 1985). The concentration of malic acid in cucumber juice containing 1.06 (6%) M NaCl at pH 5.7 and 30 °C are directly proportional to the amount of CO_2 produced when L. *plantarum* WSO is used as a starter culture (McFeeters et al., 1982). Additionally, it is estimated that the malic acid decarboxylating activity of selected facultative heterofermentors, such as *L. plantarum*, can generate 84 mg of CO_2 per 100 g of cucumbers in a fermentation, which is sufficient to cause bloater defect (Daeschel et al., 1985; Fleming et al., 1973b). Thus, the selection and inoculation of LAB that do not decarboxylate malic acid as a starter culture can help reduce the amount of CO_2 production in cucumber fermentations.

In this study the abundance of specific microbes in cucumber fermentations brined with 25 mM $CaCl_2$ and 345 mM NaCl was associated with the incidence of bloater defect, despite the substantial differences that characterize the composition of the indigenous microbiota in the fresh fruits. Bloater defect was observed by day 2 of fermentation, when the numbers of viable LAB were at an average of 8 log CFU/g, a significantly different level relative to fresh cucumbers (time 0) (Table 3). The population of LAB in the fermentations by day 2 was prevailed by *Leuconostocaceae*, *L. plantarum* and/or L. *pentosus* (Fig. 3). The incidence of bloater defect reached an index of 58.8 ± 23.9 by day 3 (Table 2) in the indigenous fermentations brined with $CaCl_2$, affecting 90% of the 45 cucumbers (15 cucumbers per lot) evaluated (Fig. 4). Bloater index remained stable after day 3 in such treatment indicating that most of the tissue damage occurred in the first 3 days of fermentation. Bloater defect was also observed by day 3 of the fermentations representing the two additional treatments (Table 2).

Aside from LAB, viable presumptive *Enterobacteriaceae*, *Klebsiella* and *Pseudomonas* were detected and enumerated from VRBG, HCK and PIA agar plates on days 2 and 3 of the fermentations, except for presumptive *Klebsiella* in fermentations brined with $CaCl_2$ that were inoculated (Table 3). Several *Enterobacteriaceae* have been isolated from commercial cucumber fermentations brined with 1.06 M NaCl including *Enterobacter*, *Pantoea*, *Citrobacter*, *Erwinia*, *Leclercia* and *Kluyvera* (Pérez-Díaz et al., 2019). Colonies isolated from HCK were identified as *Kluyvera cryocrescens*, *Enterobacter* spp., *Kosakonia cowanii* and *Rahnella victoriana*, but not as *Klebsiella* (Table 4). Similarly, the colonies isolated from PIA were not identified as *Pseudomonas* but as *Serratia*, *Kluyvera* and *Enterobacter* spp. (Table 4). The relative abundance of *Enterobacteriaceae*, *Enterobacter cloacae*, *Enterobacter mori*, *Pantoea agglomerans* and *Klebsiella* spp. fluctuated between 1 and 20% in cucumber fermentations (Fig. 3). These observations confirm that *Enterobacteriaceae* are present in the early stage of cucumber fermentations brined with either 1.06 M NaCl or 25 mM $CaCl_2$ and 345 mM NaCl and could contribute to bloater defect.

The data also suggest that the utilization of not only glucose and fructose but also malic acid by *Lactobacillaceae* and *Leuconostocaceae* during the early stage of a cucumber fermentation contributes to bloater defect. The initial concentration of malic acid, glucose and fructose in cucumber slurries was 18.82 ± 7.06, 57.05 ± 16.10 and 68.10 ± 22.19 mM, respectively. Fig. 5 shows that some of the intrinsic malic acid, estimated at 9.4 mM after equilibration of the cucumbers with the cover brine in the jars, was slightly reduced by day 3 in fermentations brined with NaCl and those brined with $CaCl_2$ that were assisted by a starter

Relative Abundance of Species Found (%)	Lot 1 D 2	Lot 1 D 3	Lot 1 D 5	Lot 1 D 8	Lot 2 D 2	Lot 2 D 3	Lot 2 D 5	Lot 2 D 8	Lot 3 D 2	Lot 3 D 3	Lot 3 D 5	Lot 3 D 8
Brined with 1.06 M NaCl												
Lactobacillus pentosus	47	44	48	48	0	0	0	0	0	0	0	0
Lactobacillus plantarum	49	45	49	51	11	36	1	13	0	33	29	27
Leu. mesenteroides	1	0	0	0	35	16	50	16	2	31	17	62
Leu. pseudomesenteroides	0	1	0	0	44	44	42	44	0	13	2	9
Weissella cibaria	0	0	0	0	9	0	0	0	59	12	13	0
Lactococcus lactis	2	2	0	0	0	1	1	12	0	0	0	0
Pantoea agglomerans	0	5	0	0	0	0	0	0	18	1	4	0
Pantoea sp.	0	3	0	0	0	1	0	0	10	0	1	1
Weissella	1	0	0	0	0	0	0	0	0	0	0	0
Less than 4%	1	1	3	1	2	3	6	14	11	8	34	1
Bloater Index	22.0	10.3	41.7	34.7	17.3	25.3	10.3	28.0	11.7	40.7	56.7	53.0
Malic Acid Utilization (%)	100.0	100.0	100.0	100.0	0.0	50.9	35.2	98.8	0.0	78.4	60.2	77.1

Brined with 25 mM CaCl₂ and 345 mM NaCl	Lot 1 D 2	Lot 1 D 3	Lot 1 D 5	Lot 1 D 8	Lot 2 D 2	Lot 2 D 3	Lot 2 D 5	Lot 2 D 8	Lot 3 D 2	Lot 3 D 3	Lot 3 D 5	Lot 3 D 8
Lactobacillus pentosus	47	0	0	42	1	0	48	0	0	48	0	0
Lactobacillus plantarum	49	11	0	43	1	33	49	1	29	51	13	27
Leu. mesenteroides	1	35	2	0	10	31	0	50	17	0	16	62
Leu.pseudomesenteroides	0	44	0	1	3	13	0	42	2	0	44	9
Weissella cibaria	0	9	59	8	48	12	0	0	13	0	0	0
Lactococcus lactis	2	0	0	1	20	0	0	1	0	0	12	0
Pantoea agglomerans	0	0	18	0	0	1	0	0	4	0	0	0
Pantoea sp.	0	0	10	0	0	0	0	0	1	0	0	0
Less than 4%	1	2	11	4	17	8	3	6	34	1	14	2
Bloater Index	26.7	75.0	78.0	74.7	12.7	31.3	22.7	29.0	32.7	70.0	64.3	74.7
Malic Acid Utilization (%)	100	100	100	100	23.5	82.6	78.3	70.8	34.7	47.2	75.6	63.6

Brined with 25 mM CaCl₂ and 345 mM NaCl & a Starter Culture	Lot 1 D 2	Lot 1 D 3	Lot 1 D 5	Lot 1 D 8	Lot 2 D 2	Lot 2 D 3	Lot 2 D 5	Lot 2 D 8	Lot 3 D 2	Lot 3 D 3	Lot 3 D 5	Lot 3 D 8
Lb. pentosus	0	1	0	41	0	0	2	0	0	46	0	0
Lb. plantarum	0	4	0	43	53	13	2	15	1	48	10	13
Leu. mesenteroides	2	36	1	0	11	40	0	57	5	0	16	15
Leu.pseudomesenteroides	0	4	0	0	0	1	0	1	0	0	0	11
W. cibaria	0	4	67	0	0	15	0	1	36	0	1	1
Lactobacillus	89	0	0	0	1	0	0	0	0	0	1	0
Lb.paralimentarius	0	0	0	0	0	0	0	0	0	0	0	27
Pecto.carotovorum	0	0	0	0	0	0	44	0	0	0	0	0
P.pentosaceus	0	45	0	0	26	0	0	15	34	0	61	11
Enterobacter cloacae	0	0	6	0	0	1	1	0	3	0	0	0
Enterobacter mori	2	0	0	0	0	0	6	0	1	0	0	1
Klebsiella sp.	0	0	5	0	0	2	0	0	1	0	0	0
Pantoea agglomerans	0	1	1	2	0	16	20	0	6	0	1	5
Pantoea sp.	0	0	0	0	0	7	9	0	2	0	0	1
Pseudomonas sp.	0	1	0	6	1	0	0	2	2	2	1	5
Less than 4%	6	4	19	7	8	5	14	9	10	5	8	10
Bloater Index	2.7	42.0	63.7	35.3	5.3	9.0	41.0	18.0	16.0	45.3	9.0	23.3
Malic Acid Utilization (%)	86.0	72.3	82.9	87.6	53.0	45.0	43.8	54.8	31.7	24.4	28.3	38.6

Fig. 3. Relative abundance (%) of the bacterial species detected by the metagenomic analysis of total genomic DNA extracted from homogenates prepared from 6 independent cucumber fermentations brined with 25 mM $CaCl_2$, 345 mM NaCl, 20.2 mM Ca $(OH)_2$ and vinegar. Bloater index and malic acid utilization in the fermentations analyzed is also included. Three fermentations were inoculated with L. *pentosus* LA445. The three control fermentations were brined with 1.06 M NaCl instead and without vinegar or a starter culture. The data shown represent one sample for each treatment and lot as a function of time and is inclusive of 36 individual fermentation jars.

culture. Utilization of malic acid, and presumably basal amounts of sugars, resulted in the production of lactic acid (Fig. 6). More than 40 mM lactic acid formed within the first 3 days of the fermentations brined with $CaCl_2$ (Fig. 6). Modest changes in sugar concentrations were detected within individual lots and treatments, however they are masked within the biological variability among cucumber lots in Fig. 5. A minimal increase in acetic acid concentration was also observed within the biological variability in single lots and treatments (Fig. 6). The malic acid disappearance coincided with an increase in colony counts from *Lactobacilli* MRS agar plates (Table 3), the die off of *Enterobacteriaceae* (Table 3), the presence of *Lactobacillaceae* and *Leuconostocaceae* (Fig. 3) and the formation of lactic acid (Fig. 6). Malic acid continued to disappear in the fermentations brined with 1.06 M NaCl until day 8 (Fig. 5). It is concluded that the utilization of not only sugars, but also malic acid by the LAB contributes to the production of CO_2 and bloater defect in cucumber fermentations, regardless of cover brine composition.

The differences in bloater index in treatments 1 and 3 relative to treatment 2 suggest that the starter culture and 1.06 M (6%) NaCl in the cover brine differentially suppressed the indigenous microbiota (Tables 2 and 3). Colony counts for presumptive *Enterobacteriaceae* were at 2.4 ± 3.5 log CFU/g on day 4 of the fermentations brined with NaCl (Treatment 3) while these microbes were at 1.8 ± 1.8 log CFU/g on day 2 of the fermentations brined with $CaCl_2$ that were inoculated with a

Table 2
Bloater index for fermented cucumbers brined with 6 mM potassium sorbate and either 25 mM $CaCl_2$ and 345 mM NaCl or 1.06 M NaCl. The experimental treatments included cover brines containing $CaCl_2$, NaCl, $Ca(OH)_2$ and vinegar (20%) to adjust the initial pH to 4.7 ± 0.1 (Treatments 1 and 2) or a cover brine containing 1.06 M NaCl and no vinegar for pH adjustment (Treatment 3). Only Treatment 1 was inoculated with L. *pentosus* LA445. The data shown are the averages and standard deviations of triplicates with 3 independent lots. Levels not connected by the same letter within time points (columns) are significantly different.

Treatment	Fermentation time (d)						
	2	3	4	5	8	15	21
1	8.0 ± 7.1^{B}	32.1 ± 20.1^{B}	23.8 ± 11.9 ^{B}	37.9 ± 27.5 ^{B}	25.6 ± 8.9^{B}	29.1 ± 18.5 ^{B}	27.7 ± 2.7 ^{B}
2	24.0 ± 10.3^{A}	58.8 ± 23.9 ^{A}	41.8 ± 24.5 ^{A}	55.0 ± 28.8 ^{A}	59.4 ± 26.3 ^{A}	53.7 ± 31.6 ^{A}	57.8 ± 16.7 ^{A}
3	17.0 ± 5.2^{AB}	25.4 ± 15.2 ^{AB}	57.8 ± 14.3 ^{AB}	36.2 ± 23.6 ^{AB}	38.6 ± 13.0 ^{AB}	49.7 ± 34.4 ^{AB}	48.8 ± 32.5 ^{AB}

starter culture (Table 3). As expected, the absence of NaCl in the cover brine resulted in an accelerated die off of *Enterobacteriaceae* in natural fermentations (Table 3, Treatment 2) (Bautista-Gallego et al., 2010; Gimeno et al., 1999; Panagou et al., 2011; Pérez-Díaz et al., 2020).

Fig. 3 summarizes the data obtained from the metagenomic analysis and shows lot to lot variability. It identifies the presence of L. *plantarum* and L. *pentosus* in all fermentations, but at varied time points. The lactobacilli only prevailed across time points in one fermentation brined with 1.06 M NaCl. In contrast the *Leuconostocaceae* prevailed in two fermentations brined with 1.06 M NaCl and one brined with 25 mM $CaCl_2$ and 345 mM NaCl (Fig. 3). The heterofermentative LAB *Leuconostoc mesenteroides* and *Leuconostoc paramesenteroides* were detected in all fermentations regardless of brined type and inoculation with a higher relative abundance on day 3 of the bioconversions (Fig. 3). *Weissella cibaria*, also a heterofermentative LAB, was more frequently detected to higher relative abundance in fermentations brined with $CaCl_2$ between days 2 and 5 (Fig. 3). These observations contrast with those documented by Pérez-Díaz et al. (2017) for commercial cucumber fermentations brined with 1.06 M NaCl in which L. *pentosus* had a dominating role. In this study, *L. pentosus* did not prevail in eight out of the nine cucumber fermentations studied, even if inoculated with a starter culture of the same species. Together these observations suggest that the lactobacilli or the *Leuconostocaceae* can dominate the first stage of a cucumber fermentation, regardless of the composition of the cover brine. It also evidences that the combined activity of several microbes contributes to the sugar conversion to lactic acid in cucumber fermentations and that the process can be led or initiated by varied LAB. Such scenario implicates that the prevention of bloater defect demands a multifactorial strategy and explains the difficulties in identifying the specific microbial contributors to CO_2 production over the last few decades.

It is noteworthy that the lot-to-lot variability within a treatment could be linked with differences in the bloater defect observed. Bloater index was 10.3 in lot 1 and 25.3 in lot 2 of the fermentations brined with 1.06 M (6%) NaCl on day 3, which related to the presence of *Pantoea* spp. (8%), *L. plantarum* (44%) and *L. pentosus* (45%) in lot 1 and 60% *Leuconostoc* spp. in lot 2 (Fig. 3). The bloater index from lot 1 of the fermentations brined with $CaCl_2$ that were not inoculated was 75.0 on day 3 with an incidence of *Leuconostoccacea* of 88%, while in lot 2 it was at 31.3 and the bacterial family was detected to 56% (Fig. 3). More CO_2

Fig. 4. A representation of bloater defect observed in 3 independent cucumber fermentations brined with 25 mM $CaCl_2$, 345 mM NaCl, 20.2 mM Ca $(OH)_2$ and vinegar without a starter culture (Treatment 2) after 3 days of incubation. The average and standard deviation of the bloater index from the three independent lots was 58.8 ± 23.9.

and higher bloater indexes were detected in the absence of the starter culture in fermentations brined with $CaCl_2$ (Figs. 4 and 7). The use of a starter culture in fermentations brined with $CaCl_2$ promoted a more prominent role for the homofermentor, *Pediococcus pentosaceous*.

Besides substrate utilization, results of the HPLC analysis show the addition of 45.86 ± 5.93 mM acetic acid to adjust the initial fermentation pH to 4.7 in the fermentations brined with $CaCl_2$ and the production of 11 to 24.74 mM acetic acid by the end of the fermentation (Fig. 6). The cucumber fermentations brined with 1.06 M NaCl produced about half of the lactic acid (71.77 ± 15.33 mM) produced in the experimental fermentations brined with $CaCl_2$ (146.05 ± 33.81 mM) and 5.73 ± 2.25 mM acetic acid by day 21, indicating a slower homofermentation and a nominal heterofermentation (Fig. 6). In contrast, 168.61 ± 12.27 mM lactic acid was produced in the cucumber fermentations brined with the calcium salt that were inoculated with a starter culture, evidencing a

Table 3
Colony counts (log CFU/g) from homogenates of fermenting cucumbers and cover brine for presumptive *Enterobacteriaceae*, *Leuconostaceae*, *Lactobacillaceae*, *Pseudomonas* and *Klebsiella* from VRBG, MRS, PIA and HCK agar, respectively. The experimental treatments included cover brines containing $CaCl_2$, NaCl, Ca $(OH)_2$ and vinegar (20%) to adjust the initial pH to 4.7 ± 0.1 (Treatments 1 and 2) or a cover brine containing 1.06 M NaCl and no vinegar for pH adjustment (Treatment 3). Only Treatment 1 was inoculated with *Lactobacillus pentosus* LA445. The data shown are the averages and standard deviations of triplicate homogenate samples prepared with 3 independent lots of cucumbers. Measured values not connected by the same letter within treatment (row) and across treatments (columns) are significantly different.

Treatment	Fermentation time (d)						
	0	2	3	4	8	15	21
	Lactobacilli deMan, Rogosa and Sharpe Agar (MRS)						
1	7.4 ± 0.0^{ABCDE}	8.5 ± 0.1^{A}	8.4 ± 0.1^{A}	8.2 ± 0.1^{AB}	7.8 ± 0.3^{ABCD}	6.5 ± 0.6^{EFG}	4.8 ± 0.7^{H}
2	5.3 ± 0.1^{GH}	8.1 ± 0.2^{ABC}	8.3 ± 0.1^{A}	8.4 ± 0.4^{A}	7.3 ± 0.4^{ABCDE}	6.8 ± 0.6^{DEF}	5.9 ± 0.7^{FGH}
3	5.3 ± 0.1^{GH}	7.0 ± 0.6^{CDEF}	7.5 ± 0.2^{ABCDE}	7.6 ± 0.4^{ABCDE}	7.0 ± 0.3^{BCDEF}	6.4 ± 0.7^{EFG}	5.9 ± 0.6^{FGH}
	Violet, Red Bile & Glucose Agar (VRBG)						
1	5.2 ± 0.7^{A}	1.8 ± 1.8^{ABC}	1.7 ± 2.9^{ABC}	0.9 ± 1.5^{ABC}	BDL	BDL	BDL
2	5.2 ± 0.7^{A}	4.0 ± 0.5^{ABC}	3.1 ± 2.7^{ABC}	0.9 ± 1.5^{ABC}	BDL	BDL	BDL
3	5.2 ± 0.7^{A}	4.6 ± 0.6^{AB}	4.6 ± 1.1^{ABC}	2.4 ± 3.5^{ABC}	0.5 ± 0.9^{BC}	BDL	BDL
	HiCrome *Klebsiella* Agar (HCK)						
1	3.2 ± 1.3^{AB}	BDL	BDL	BDL	BDL	BDL	BDL
2	3.2 ± 1.3^{AB}	3.9 ± 0.5^{AB}	1.9 ± 2.2^{AB}	1.6 ± 2.8^{AB}	BDL	BDL	BDL
3	3.2 ± 1.3^{AB}	4.8 ± 0.3^{A}	4.9 ± 0.8^{A}	2.0 ± 1.9^{AB}	0.8 ± 1.3^{AB}	BDL	BDL
	Pseudomonas Isolation Agar (PIA)						
1	4.8 ± 0.6^{A}	BDL	BDL	BDL	BDL	BDL	BDL
2	4.8 ± 0.6^{A}	BDL	BDL	BDL	BDL	BDL	BDL
3	4.8 ± 0.6^{A}	4.3 ± 1.1^{A}	BDL	BDL	BDL	BDL	BDL

Table 4
Identification of colonies isolated from HCK and PIA media inoculated with homogenates prepared with cover brines and fermenting cucumbers from three independent lots. Only homogenates prepared on days 2 and 3 of the fermentations produced colonies on HCK and PIA. The data shown for each sample was selected from four amplicon replicates based on the DNA sequence chromatograph. The DNA sequences were banked in the National Center for Biotechnology Information database under the accession numbers provided.

Fermentation days	Cucumber Lot	Growth Media / Colony Code	No. of Bases Used	Description of the Identification	% Identity	Accession Number
Treatment 2: Brined with 25 mM $CaCl_2$, 345 mM NaCl, 20.2 mM Ca $(OH)_2$ and vinegar for pH adjustment without inocula						
2	1	HCK / 2	47–501	*Enterobacter cloacae* ATCC 23373	100	MN266314
2	3	HCK / 0	27–695	*Kosakonia cowanii* strain 888–76	99	MN266317
2	2	HCK / 2	27–597	*Kosakonia cowanii* strain 888–76	99	MN266318
Treatment 3: Brined with 1.06 M NaCl and no vinegar for pH adjustment						
2	1	HCK / 2	29–641	*Kluyvera cryocrescens* strain 12,993 (flagella)	100	MN266312
2	1	HCK / 2	25–638	*Enterobacter tabaci* strain YIM Hb-3	99	MN266313
2	3	HCK / 2	24–640	*Kosakonia cowanii* strain 888–76	99	MN266315
2	3	HCK / B	31–643	*Rahnella victoriana* strain FRB 225	99	MN266316
3	1	PIA / 0	29–597	*Serratia odorifera* strain NBRC 102598	99	MN266319
3	1	PIA / 0	30–649	*Serratia odorifera* strain NBRC 102598	99	MN266320
3	2	PIA / 2	27–595	*Kluyvera georgiana* strain ATCC 51603	99	MN266321
3	2	PIA / B	29–597	*Kluyvera georgiana* strain ATCC 51603	99	MN266322
3	3	PIA / 0A1	24–615	*Enterobacter bugandensis* strain 247BMC	99	MN266323
3	3	PIA / 0A2	15–703	*Serratia odorifera* strain NBRC 102598	99	MN266324

faster and more complete homofermentation as a function of the inoculum, which presence in the bioconversion is evidenced by the higher counts from MRS agar plates on day 0 and 2 (Fig. 6 and Table 3). Uninoculated fermentations brined with $CaCl_2$ produced 123.48 ± 34.36 mM of lactic acid (Fig. 6). Although, sugars are mainly converted to lactic acid by the indigenous LAB (8 log of CFU/g) in the fermentation (Table 3 and Fig. 5), a nominal heterofermentation by *Leuconostocaceae* is apparent from the production of 11 to 24.74 mM acetic acid by day 8 (Figs. 3 and 6). Ethanol was produced as early as day 3 and remained at 24.12 ± 2.67 mM throughout the fermentation brined with $CaCl_2$ and no inocula, while below 5 mM ethanol was produced in the other two treatments (data not shown). The differences in cover brine formulations induced differences in the rate of fermentation, where the use of $CaCl_2$ without an inoculum allowed greater microbial diversity by day 2 (Fig. 3), and the use of 1.06 M NaCl in the cover brine delays the progress of the fermentation. However, the sugars and pH were reduced by the end of the bioconversions to near the limit of detection and 3.6 ± 0.1, respectively (Figs. 5 and 7).

In agreement with previously described observations, production of CO_2 was reduced in the presence of the starter culture, and the production of the gas was delayed in the presence of 1.06 M NaCl as compared to fermentations brined with $CaCl_2$. More CO_2 production was observed in the fermentations brined with $CaCl_2$ that were not inoculated reaching 17.7% (estimated at 40 mM) on day 21 (Fig. 7). The highest production of CO_2 in the wild fermentations brined with $CaCl_2$ resulted in the highest bloater index at 24.0 ± 10.3 by day 2 (Table 2 and Fig. 7). Supplementation of such fermentation with a starter culture resulted in a significantly lower CO_2 production and bloater index by day 2 (Table 2). These observations suggest that the abundant proliferation of *Leuconostocaceae* during days 2 and 3 of the bioconversion in the absence of a starter culture and 1.06 M NaCl results in a pronounced bloater defect and that a *L. pentosus* starter culture can only partially

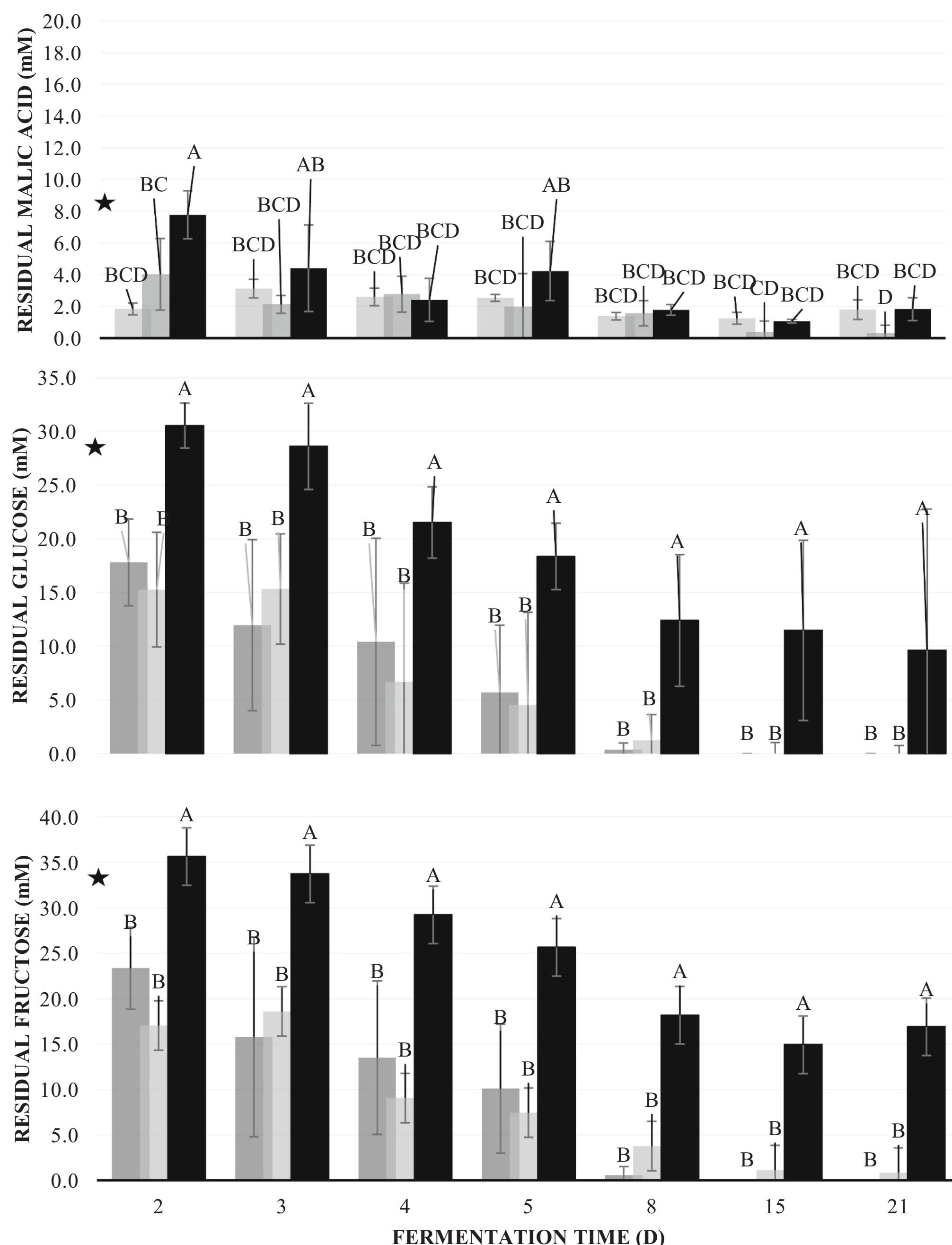

Fig. 5. Utilization of glucose, fructose and malic acid in cucumber fermentations brined with 25 mM $CaCl_2$ and 345 mM NaCl or 1.06 M NaCl. The three treatments tested included cucumber fermentations brined with 25 mM $CaCl_2$, 345 mM NaCl, 20.2 mM Ca $(OH)_2$ and vinegar (■, ■) or a cover brine containing 1.06 M NaCl and no vinegar for pH adjustment (■). Only one treatment was inoculated with *L. pentosus* LA445 (■). The estimated concentration of malic acid, glucose and fructose in the jars initially is identified with a star in each panel. The data shown are the average and standard deviations of triplicate homogenate samples prepared with three independent cucumber fermentations. Levels not connected by the same letter were significantly different.

suppress the formation of hollow cavities in the cucumber tissue. The *Leuconostocaceae* could contribute to bloater defect by utilizing sugars and decarboxylating malic acid.

In summary, the indigenous cucumber microbiota caused bloater defect in all the cucumber fermentations tested after 2 days. Production of 12% CO_2 was sufficient to cause bloater defect in cucumber fermentations brined with low salt. Bloater defects worsen in the fermentations by day 21. Bloater defect not only occurs before day 5 of a cucumber fermentation when *Lactobacillaceae* and *Leuconostocaceae* prevail, but also can occur before day 2 when the indigenous *Enterobacteriaceae* are present. The microbial activity of these bacterial families is likely to contribute to CO_2 production and the consequent bloater defect. It seems that the *Leuconostocaceae* contribute more than the *Enterobacteriaceae* and the *Lactobacillaceae* to CO_2 production and that they do so by utilizing sugars and malic acid. Inoculation of a starter culture decreased bloater defect in fermentations brined with 25 mM $CaCl_2$ and 345 mM NaCl but did not prevent it. Further studies are to determine if a reduction in bloater defect and economical losses for pickle processors can be achieved by the improved control of the contributing microbes.

CRediT authorship contribution statement

Ms. Yawen Zhai contributed to the experimental design, conducted experimentation, collected data, interpreted the results and drafted the manuscript. Dr. Ilenys Pérez-Díaz defined the scientific approach, contributed to the experimental design, assisted with the execution of the experiment and data collection and edited the manuscript.

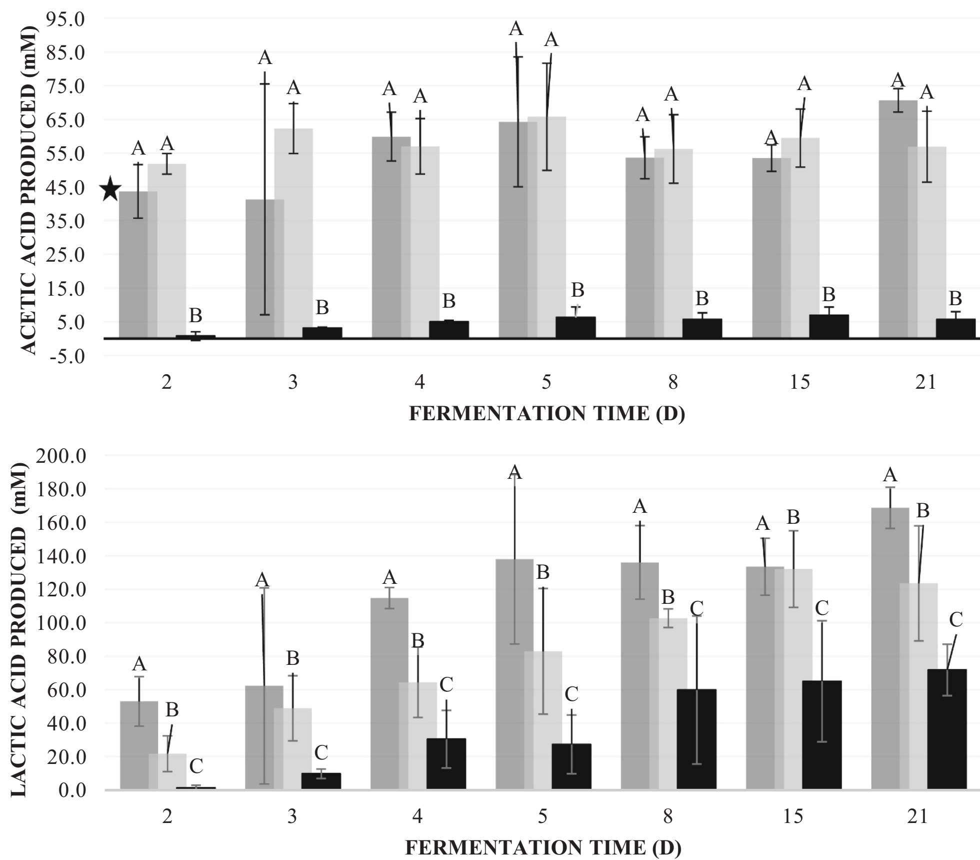

Fig. 6. Production of lactic and acetic acids in cucumber fermentations brined with 25 mM $CaCl_2$ and 345 mM NaCl or 1.06 M NaCl and 6 mM potassium sorbate and an initial pH of 4.7 ± 0.1adjusted with 20% vinegar (45.86 mM acetic acid equilibrated; identified by a star). In addition to potassium sorbate, the three treatments for testing included: cucumber fermentations brined with 25 mM $CaCl_2$, 345 mM NaCl, 20.2 mM $Ca(OH)_2$ and vinegar (■, ■) or a cover brine containing 1.06 M NaCl and no vinegar for pH adjustment (■). Only one treatment was inoculated with L. *pentosus* LA445 (■). The data shown are the average and standard deviations of triplicates with 3 independent lots. Levels not connected by the same letter are significantly different.

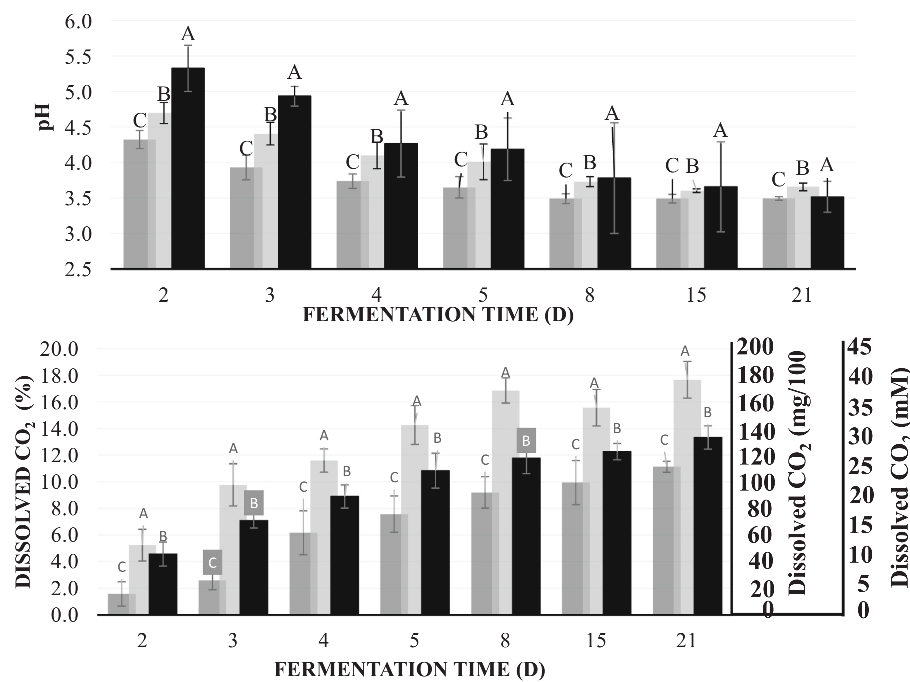

Fig. 7. pH and CO_2 content (in %, mg/100 mL and mM) in cucumber fermentations brined with 25 mM $CaCl_2$ and 345 mM NaCl and 1.06 M NaCl and 6 mM potassium sorbate with an initial pH adjustment to 4.7 ± 0.1 using 20% vinegar (45.86 mM acetic acid equilibrated). In addition to potassium sorbate, the three treatments for testing included: cucumber fermentations brined with 25 mM $CaCl_2$, 345 mM NaCl, 20.2 mM Ca $(OH)_2$ and vinegar (■, ■) or a cover brine containing 1.06 M NaCl and no vinegar for pH adjustment (■). Only one treatment was inoculated with *L. pentosus* LA445 (■). The data shown are the average and standard deviations of triplicate homogenate samples prepared with 3 independent fermentations. Levels not connected by the same letter are significantly different.

Declaration of competing interest

No conflict of interest is declared.

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