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Contribution of *Leuconostocaceae* to CO_2-mediated bloater defect in cucumber fermentation☆

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ABSTRACT

Fermented cucumber bloater defect, caused by the accumulation of microbiologically produced carbon dioxide (CO_2), creates significant economic losses for the pickling industry. The ability of *Leuconostocaceae*, indigenous to cucumber, to grow and produce CO_2 during a fermentation and cause bloater defect was evaluated. *Leuconostocaceae* grew and produced over 40% CO_2 in cucumber juice medium, used as a model for cucumber fermentation. The inoculation of *Leuconostocaceae* to 5 Log CFU/g in cucumber fermentations brined with 25 mM calcium chloride and 6 mM potassium sorbate resulted in no significant differences in bloater defect, colony counts from MRS and VRBG agar plates or the fermentation biochemistry; suggesting an inability of the inoculated bacterial species to prevail in the bioconversion. Acidified cucumbers were subjected to a fermentation inoculated with a *Leuconostoc lactis* starter culture after raising the pH to 5.9 ± 0.4. CO_2 was produced in the acidified cucumber fermentations to 13.6 ± 3.5% yielding a bloater index of 21.3 ± 6.4; while 8.6 ± 0.8% CO_2 and a bloater index of 5.2 ± 5.9 were observed in the non-inoculated control jars. Together the data collected demonstrate that *Leuconostocaceae* can produce enough CO_2 to contribute to bloater defect, if not outcompeted by the leading lactic acid bacteria in a cucumber fermentation.

1. Introduction

Commercial cucumber fermentations are typically carried out in 28,000 to 40,000 L (7500 gal) open-top, white fiberglass tanks containing between 50 and 60% of the fresh fruits and 50 to 40% cover brine solution. Acetic acid (about 14 mM) is added in the typical cucumber fermentation in the form of vinegar along with 1.04 M (6%) sodium chloride and up to 6 mM (0.01%) potassium sorbate. The in-tank fruits are promptly covered with wooden boards to prevent them from floating after adding the cover brine, so that equilibration between the fruits and cover brine solution components proceeds uniformly. Air purging is routinely applied to the bottom of fermentation tanks, so that air bubbles comingle with the carbon dioxide that forms during the fermentation and travel to the top of the cover brine solution preventing bloater defect. Bloater defect is defined as the formation of gas pockets inside the fruit with lesions resembling honeycomb, lens or balloon shapes (Etchells et al., 1974). The accumulation of the gases formed during the fermentation, mostly carbon dioxide (CO_2) but also hydrogen in some cases, in the fermentation vessel increases the internal pressure of the cucumbers, displacing the endo- or mesocarp and forming hollow cavities (Etchells et al., 1968; Fleming et al., 1973b; Fleming and Pharr, 1980). Production of CO_2 by the indigenous microbiota in cucumber fermentation is the culprit in the generation of bloater defect (Etchells and Jones, 1941; Jones et al., 1941). Such defect causes serious economic losses for the pickling industry (Corey et al., 1983; Fleming et al., 1973a).

The main sources for the cucumbers microbial diversity are the irrigation water, the soil, pre-processing washing water and processing equipment upon harvest (Etchells and Goresline, 1940; Pérez-Díaz et al., 2017). The current understanding is that besides *Enterobacteriaceae* such as *Enterobacter* spp. and *Pantoea* spp. among others, lactic acid bacteria (LAB) are naturally present in cucumber fermentations, including *Weissella cibaria*, *Weissella hellenica*, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Enterococcus casseliflavus*,

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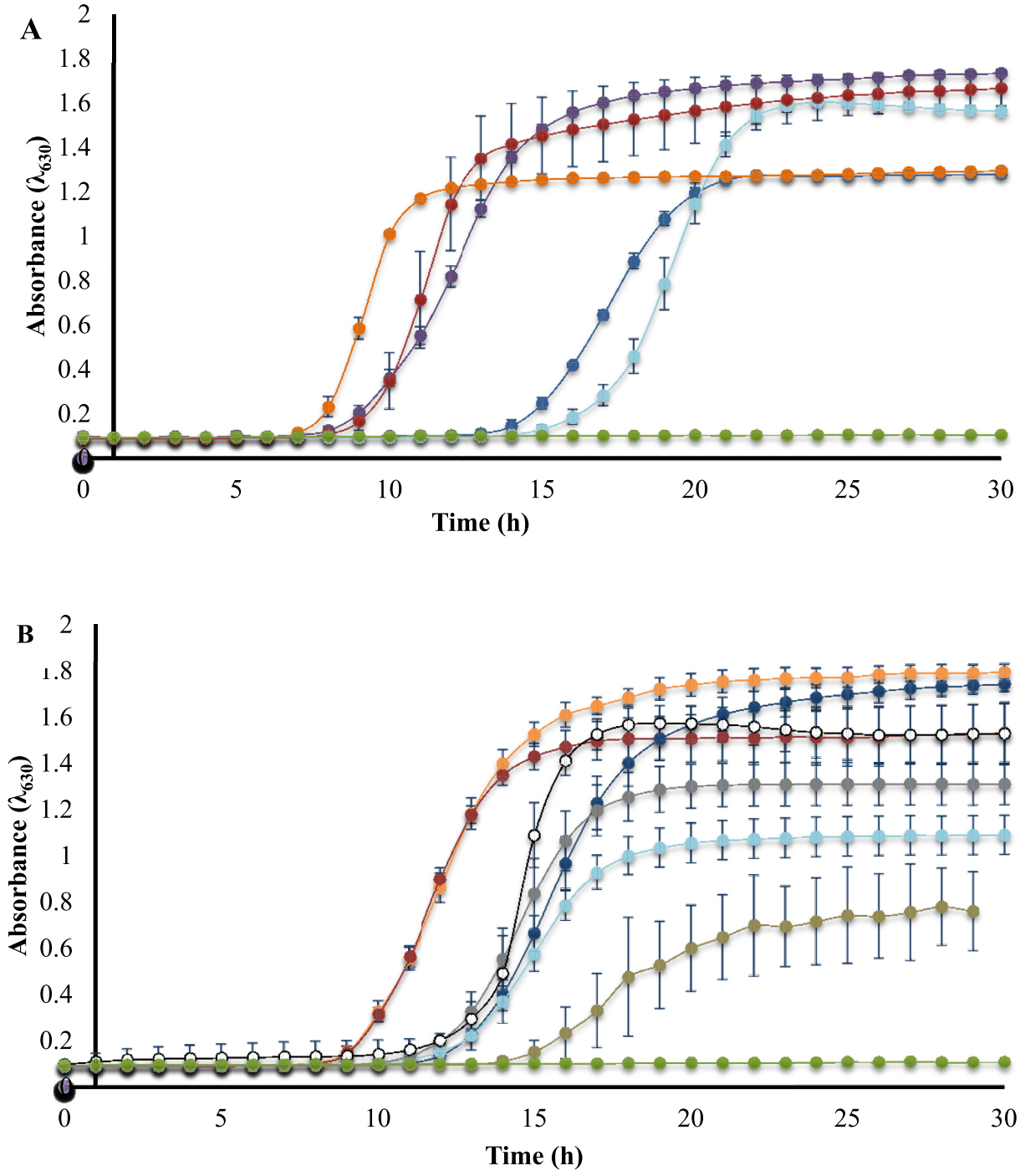

Fig. 1. Growth of *Leuconostocaceae* in CJM prepared from size 3B (1.75–2.0″ diameter) cucumbers and incubated at 30 °C using a 96-well plate. The data shown are the averages and standard deviations of technical duplicates within the same lot of size 3B (1.75–2.0″ diameter) cucumber juice. Panel A shows the *Weissella* spp. tested including *W. hellenica* 1.2.50 (●, purple), *W. paramesenteroides* 3.2.24 (●, blue), *W. paramesenteroides* 7.2.23 (●, aqua blue), *W. cibaria* 3.8.44 (●, orange) and *W. cibaria* 7.8.4 (●, red). Panel B shows the *Leuconostoc* spp. tested including *Lc. lactis* 1.2.28 (●, orange), *Lc. lactis* 1.8.39 (●, red), *Lc. mesenteroides* 1.2.6 (●, blue), *Lc. mesenteroides* 1.2.47 (●, aqua blue), *Lc. fallax* 1.2.22 (●, tan), *Lc. citreum* 1.2.37 (●, gray) and *Lc. holzapfelli* 3.8.12 (○, white). The non-inoculated controls are shown in light gray on both panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Lactobacillus brevis, *Pediococcus pentosaceous*, *Lactobacillus plantarum* and *Lactobacillus pentosus* (Chen et al., 2012; Pérez-Díaz et al., 2017). Microbes specifically implicated in bloater defect include *Enterobacter* spp. in combination with yeasts and the facultative heterofermentors *Lb. plantarum* and *Lb. pentosus* able to decarboxylate malic acid (Etchells and Bell, 1950; Etchells et al., 1968; Fleming et al., 1973a, 1973b; McFeeters et al., 1982; McFeeters et al., 1984; McDonald et al., 1991). *Leuconostoc* species are present in cucumber fermentations brined with 6% sodium chloride (NaCl) on days 1 and 3, including *Lc. mesenteroides*, *Lc. fallax*, *Lc. citreum* and *Lc. holzapfelii* (Pérez-Díaz et al., 2017). *W. mesenteroides* and *W. cibaria* overlap with *Leuconostoc* spp. until *Lactobacillaceae* prevailed on day 7 (Pérez-Díaz et al., 2017). Recently, the *Leuconostocaceae* indigenous to cucumber fermentations were identified as a possible source of CO_2 and cause of bloater defect along with *Enterobacteriaceae* and *Lactobacillaceae* (Zhai et al., *Submitted*). The study demonstrated that bloater defect occurs in cucumber fermentations as

early as day 2 in the presence of *Enterobacteriaceae* and *Leuconostocaceae*. The dominance of *Lactobacillaceae* later in the fermentation further increases CO_2 production and increases bloater defect (Zhai et al., *Submitted*).

The *Leuconostocaceae* are a family of Gram-positive bacteria, including *Fructobacillus*, *Leuconostoc*, *Oenococcus* and *Weissella*, that usually grow in nutrient-rich environments, including vegetable products (Björkroth and Holzapfel, 2006). *Leuconostocaceae* cells are mostly ellipsoidal to spherical, often elongated. The exception to this description is the genus *Weissella* known to form short rods with rounded tapered ends or ovoid rods that occur in pairs or in short chains. *Leuconostocaceae* grow in glucose medium and appear morphologically closer to lactobacilli than to streptococci. Some strains may produce additional acetate instead of ethanol in the presence of oxygen (Holzapfel et al., 2009). *Leuconostoc* spp. are known to be non-acidophilic and prefer to grow at pH 6.5, even though their growth may proceed at pH 4.5. These species are also sensitive to NaCl concentrations at/or slightly below 6.5% (DeBruyne et al., 2007; deVos et al., 2009).

This study aims at understanding the role of *Leuconostocaceae* in bloater defect. The ability of *Leuconostocaceae* to grow and produce CO_2 in cucumber juice medium (CJM), a model system for the fermentation of the fruit, was tested. Selected *Leuconostocaceae* were used as starter cultures for cucumber fermentation to understand their contribution to CO_2 production and bloater defect. Acidified fermentations with an adjusted pH of 5.9 ± 0.4 were also inoculated with *Leuconostocaceae* to determine their role in bloater defect in the absence of microbial competition.

2. Materials and methods

2.1. CJM preparation

CJM was prepared by blending size 3B (1.75–2.0" diameter) fresh pickling cucumbers for 60 s at maximum speed using a commercial blender assembly (Waring Co., Torrington, CT). The cucumber slurries were sieved using cheesecloth to remove the particulate and filter-sterilized using a 0.2 μm filtration unit (Nalgene®-Rapid Flow™, Thermo Scientific, Santa Clara, CA). One lot of fresh cucumbers was used for this experiment. The sterilized CJM was stored at 4 °C until used. The physical and chemical characteristics of cucumber juice were evaluated by Lu and others in 2002. Cucumber juice contains about equal concentrations of glucose and fructose at 30 ± 8 mM and 10 ± 3 mM malic acid with the fluctuations correlating to fruit size. Cucumber juice may have a pH of 6.2 to 5.4 (Lu et al., 2002). The chemical composition of the cucumber juice used in this experiment were in line with those documented by Lu et al. (2002).

2.2. Bacterial cultures

The *Leuconostocaceae* cultures used in this study are described in the legend of Fig. 1. These cultures were isolated from MRS agar plates inoculated with commercial fermentation cover brine samples. Such fermentations were brined with 6% NaCl. The cultures were isolated and identified as described by Pérez-Díaz et al. (2017). The cultures were transferred from frozen stocks to MRS agar (catalog no. 288130; Difco™, Becton Dickinson and Co., Franklin Lakes, NJ) prior to the inoculation of the experimental CJM or cucumber fermentations. MRS agar plates were incubated for 48 h at 30 °C under static conditions. Pure colonies were transferred from MRS agar to MRS broth and incubated at 30 °C for 24 h. The cultures were spun at 10,000 rpm for 10 min at room temperature (Eppendorf Centrifuge 5810R, Fisher Scientific, Fremont, CA) and suspended in the same volume of sterile CJM prior to the inoculation of the experimental media.

2.3. Method for accurate determination of viable counts

Fresh, fermenting or fermented cucumbers were aseptically blended using a commercial blender assembly for 60 s at maximum speed (Waring Co.) and subsequently homogenized at medium speed for 30 s using a Stomacher 400 (Tekmar Company, Cincinnati, OH) and homogenization bags equipped with side filters of 250 μm porosity (Interscience Laboratories Inc, Woburn, MA). Samples were serially diluted in 0.85% NaCl solution using aseptic techniques. The colony counts for presumptive LAB were determined by plating on Lactobacilli deMan, Rogosa and Sharpe (MRS) agar supplemented with 10 mL/L of a 0.1% solution of cycloheximide (SRO222C, Oxoid Ltd, Basingstoke, Hants, England) to exclude the aerobic growth of yeasts and molds. Yeasts and molds were enumerated in Yeast and Mold agar (YMA) supplemented with 0.04% chloramphenicol and 0.04% chlortetracycline to inhibit bacteria. The MRS and YMA plates were incubated at 30 °C for 48 h prior to enumeration. *Enterobacteriaceae* and total viable and culturable microbes were cultivated in Violet Red Bile agar supplemented with 1% glucose (VRBG) and Brain Heart Infusion agar (BHI), respectively. The VRBG and BHI plates were incubated at 37 °C for 24 h and 30 °C for 24 h, respectively, prior to the enumeration of colonies. Spiral plating was done using an Eddy Jet 2W spiral plater (IUL Instruments, Barcelona, Spain) for all media types. Colonies were enumerated using a Flash & Go Automated Colony counter (Neutec Group, Inc., Barcelona, Spain).

2.4. To conduct growth studies of Leuconostocaceae in CJM

A 96-well plate format was used to conduct this experiment. Each well was inoculated to 2 Log CFU/mL in a total culture volume of 200 μL inclusive of 180 μL of CJM and 20 μL of the inoculum. The plate was incubated at 30 °C for 96 h (4 days). The cultures absorbance (λ_{630}) was monitored hourly during incubation using an ELx808 Absorbance Microplate Reader (BioTek, Winooski, VT) to obtain bacterial growth curves and determine the log and stationary phases of growth (Fig. 1). One lot of fresh cucumbers was used to prepare CJM and each isolate was inoculated in triplicate with a single inoculum. The data presented corresponds to technical triplicates.

2.5. Production of CO_2 by Leuconostocaceae in CJM

The ability of five *Leuconostocaceae* to produce CO_2 was observed in CJM contained in vacutainers (Becton Dickinson and Co.) including 4 isolates that grew to an Abs_{630} of 1.6 ± 0.2 in CJM, such as *W. cibaria* 3.8.44, *Lc. lactis* 1.2.28, *Lc. holzapfelii* 3.8.12 and *Lc. mesenteroides* 1.2.47, and *Lc. fallax* 1.2.22 which only reached an Abs_{630} of 0.8 ± 0.2 in the same medium. Two lots of fresh cucumbers were used to prepare CJM. Each batch of CJM was inoculated with an independent inoculum prepared as described in section 2.2 to 2 Log CFU/mL using aseptic techniques, which generated independent duplicates. The *Leuconostocaceae* were inoculated in 24 mL of sterile CJM. Aliquots of 4 mL of the inoculated CJM were aseptically transferred to 6 vacutainers using sterile syringes equipped with Luer-Lok Tip and BD Precision Glide needles (Becton Dickinson and Co.). The sampling times of 9, 12, 14, 18, 24 and 36 h were selected based on the growth curves shown in Fig. 1. Six vacutainers were inoculated per isolate and replicate so that a single culture could be sacrificed per sampling point for a total of 60 experimental tubes and 12 non-inoculated control tubes. The cultures contained in vacutainers were incubated at 30 °C. One mL liquid samples were taken through the Hemogard closure of the vacutainer (Pulmolab, 10 mL, BD #366643, Northridge, CA) using sterile syringes for pH and high-performance liquid chromatography (HPLC) measurements at each sampling time (Tables 1 and 2). The pH for each sample was measured from culture supernatants obtained by spinning the culture aliquots at 12,000 rpm for 10 min at 22 °C (Brushless Microcentrifuge, Denville 260D, Denville Scientific, Inc., Holliston, MA).

Table 1
Changes in pH and CO_2 concentration in cultures of selected *Leuconostocaceae* inoculated in CJM aliquoted in vacutainers and incubated at 30 °C for 36 h. The data shown represent the average and standard deviation of technical duplicates with cucumber juice expressed from one lot of size 3B (1.75–2.0" diameter) cucumbers. Levels not connected by the same letter within a column are significantly different.

Inoculated Species and Genus Bacterial culture ID	Final CJM pH	Estimated mM of Dissolved CO_2
None (Positive Control)	6.0 ± 0.4	6.33 ± 0.14
Weissella cibaria 3.8.44	4.3 ± 0.1^{A}	56.66 ± 14.88^{B}
Leuconostoc lactis 1.2.28	3.9 ± 0.2^{AB}	103.16 ± 1.11^{A}
Leuconostoc holzapfelii 3.8.12	3.8 ± 0.0^{B}	92.05 ± 10.70^{A}
Leuconostoc fallax 1.2.22	3.8 ± 0.1^{B}	56.66 ± 7.37^{B}
Leuconostoc mesenteroides 1.2.47	3.9 ± 0.1^{AB}	78.49 ± 0.97^{AB}

The pH values were measured with an Accumet pH meter (cat. 13-636-AR25B, Accumet™ AR25 pH/mV/°C/ISE, Fisher Scientific) equipped with a thin gel filled probe (cat. 13-620-290, Fisher Scientific). Supernatants were stored at −20 °C after the pH were measured to conduct HPLC analysis at a later time as described in section 2.7. An additional 1 mL sample was collected from each vacutainer at each sampling time for determining colony counts on MRS plates (Fig. 2). The amount of CO_2 formed in each vacutainer was measured from the remaining 2 mL of culture in each vacutainer as described by Zhai and Pérez-Díaz (2017). CO_2 concentrations were recorded in percent (%), derived from the partial pressure of the gas in the headspace, and were converted to mM using the unit conversion curve published by Zhai et al. (Submitted; Table 1).

2.6. CO_2 production and bloater defect in cucumber fermentation by *Leuconostocaceae*

Three independent lots of fresh cucumbers were fermented in closed and sterilized 3.8 L-glass jars with a cover brine containing 25 mM calcium chloride ($CaCl_2$), 20.2 mM $Ca(OH)_2$ and 6 mM potassium sorbate, at equilibrium, with an initial adjusted pH of 5.9 ± 0.4. The jars were filled using a 50:50 pack-out ratio of cucumbers and cover brine by weight. The pH was adjusted with a 33.3 M (20%) acetic acid solution as vinegar. The amount of acetic acid needed for pH adjustment were determined by titrating 2–100 g cucumber slurry samples per lot prepared with 50 g of blended cucumbers of a single experimental lot and 50 g of the experimental cover brine. Potassium sorbate was included in the cover brine formulation to prevent the growth of yeasts indigenous to cucumber fermentations (Etchells et al., 1968) and eliminate the production of CO_2 by the eukaryotes. *Leuconostocaceae* were inoculated to 5 Log CFU/g, mimicking the intrinsic levels in a cucumber fermentation (Pérez-Díaz et al., 2017), into six experimental jars. The six control jars remained not inoculated. There were two jars per lot of fresh cucumbers in the experimental treatment and control treatment. The fermentation jars were incubated at 30 °C and samples were collected after 18 and 36 h of incubation. One jar per treatment and lot was sacrificed at each sampling time (18 and 36 h). CO_2 was measured from the headspace at each sampling time by inserting the needle of the Map-Pak Combi Gas Analyzer (AGC Instruments, Co., Clare, Ireland) through the rubber septa installed on the jar lid and initiating the detection on the instrument (Table 4 and Fig. 3). Cucumbers were aseptically cut longitudinally to assess bloater defect at each sampling time as described by Zhai and Pérez-Díaz (2017) (Table 3). The extent of the defect was quantified by calculating bloater index, which takes into account the number of injuries on the internal cucumber tissue and the acuteness of each injury as described by Wehner and Fleming (1984). Fermented cucumber slurries were prepared (without the fermentation cover brine) for microbiological and chemical analyses conducted as described in sections 2.3 and 2.7, respectively. The pH was measured from homogenized samples obtained as described in section 2.5. Slurry samples (2 mL) were stored at −20 °C, completely thawed at 22 °C for at least 4 h and freeze and thaw a second time to enable the equilibration of water soluble metabolites between the plant material and the juice prior to HPLC analysis. The supernatants of thawed samples were obtained by spinning at 12,000 rpm for 10 min (Brushless Microcentrifuge, Denville 260D, Denville Scientific) at 22 °C. Quantification of organic acid and sugars was done using the HPLC method described in section 2.7.

2.7. Quantification of organic acids and sugars by HPLC analysis

The HPLC method described by McFeeters and Barish (2003) was used using an Aminex 300 × 7.8 mm HPX-87H resin column (Bio-Rad Laboratories, Hercules, CA). The operating conditions of the system included a column temperature of 65 °C and a 0.01 N H_2SO_4 eluent set to flow at 0.9 mL/min. A SPD- 20A UV–vis detector (Shimadzu Corporation, Canby, OR) was set at 210 nm at a rate of 1 Hz to quantify malic acid and succinic 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 (data not shown). The compound concentration for the samples was calculated based on the height peak of each compound in the chromatograph as compared to the corresponding compounds on the standard curves at specific retention times using the LabSolutions Workstation (Shimadzu Corporation).

2.8. Bloater defect and CO_2 production by Lc. lactis 1.2.28 in acidified cucumber fermentations

Size 2B (1.25–1.5" diameter) cucumbers of three lots were used for these fermentations. A sample of 200 g of each fresh cucumber lot was prepared as described in section 2.3 upon arrival to the lab to obtain

Table 2
Metabolites of fermentation by *Leuconostocaceae* in CJM incubated at 30 °C for 36 h. The concentration of malic acid, glucose and fructose in CJM were 14.90 mM, 42.54 mM and 53.43 mM, respectively. The data presented represent the average and standard deviation of technical duplicates with cucumber juice expressed from one lot of size 3B (1.75–2.0" diameter) cucumbers. Levels not connected by the same letter within a column are significantly different. BDL abbreviates below detection limit.

Inocula	Residual Malic Acid (mM)	Substrates Utilized (mM)		Metabolic Products (mM)			
		Glucose	Fructose	Succinic Acid	Acetic Acid	Lactic Acid	Ethanol
None (Positive Control)	14.9 ± 4.0	42.5 ± 0.4	53.4 ± 5.5	2.4 ± 0.3	BDL	1.3 ± 1.8	BDL
Weissella cibaria 3.8.44	BDL^{E}	29.6 ± 0.0^{BC}	9.0 ± 0.8^{A}	13.0 ± 0.8^{B}	8.0 ± 0.6^{C}	25.3 ± 3.5^{B}	19.4 ± 3.3^{A}
Leuconostoc lactis 1.2.28	BDL^{E}	40.7 ± 0.2^{C}	30.6 ± 7.1^{B}	1.1 ± 0.5^{B}	10.0 ± 2.3^{C}	44.1 ± 4.4^{A}	25.8 ± 2.8^{A}
Leuconostoc holzapfelii 3.8.12	BDL^{E}	38.4 ± 0.0^{BC}	50.4 ± 0.2^{C}	8.6 ± 1.10^{B}	29.1 ± 0.8^{A}	43.7 ± 1.6^{A}	BDL^{B}
Leuconostoc fallax 1.2.22	15.6 ± 2.8^{ABC}	29.2 ± 0.1^{B}	48.5 ± 0.3^{C}	0.8 ± 0.1^{A}	20.4 ± 1.3^{B}	28.6 ± 6.8^{AB}	BDL^{B}
Leuconostoc mesenteroides 1.2.47	BDL^{E}	37.7 ± 0.1^{C}	50.7 ± 0.1^{C}	7.4 ± 1.1^{B}	27.2 ± 1.4^{A}	38.7 ± 3.2^{AB}	BDL^{B}

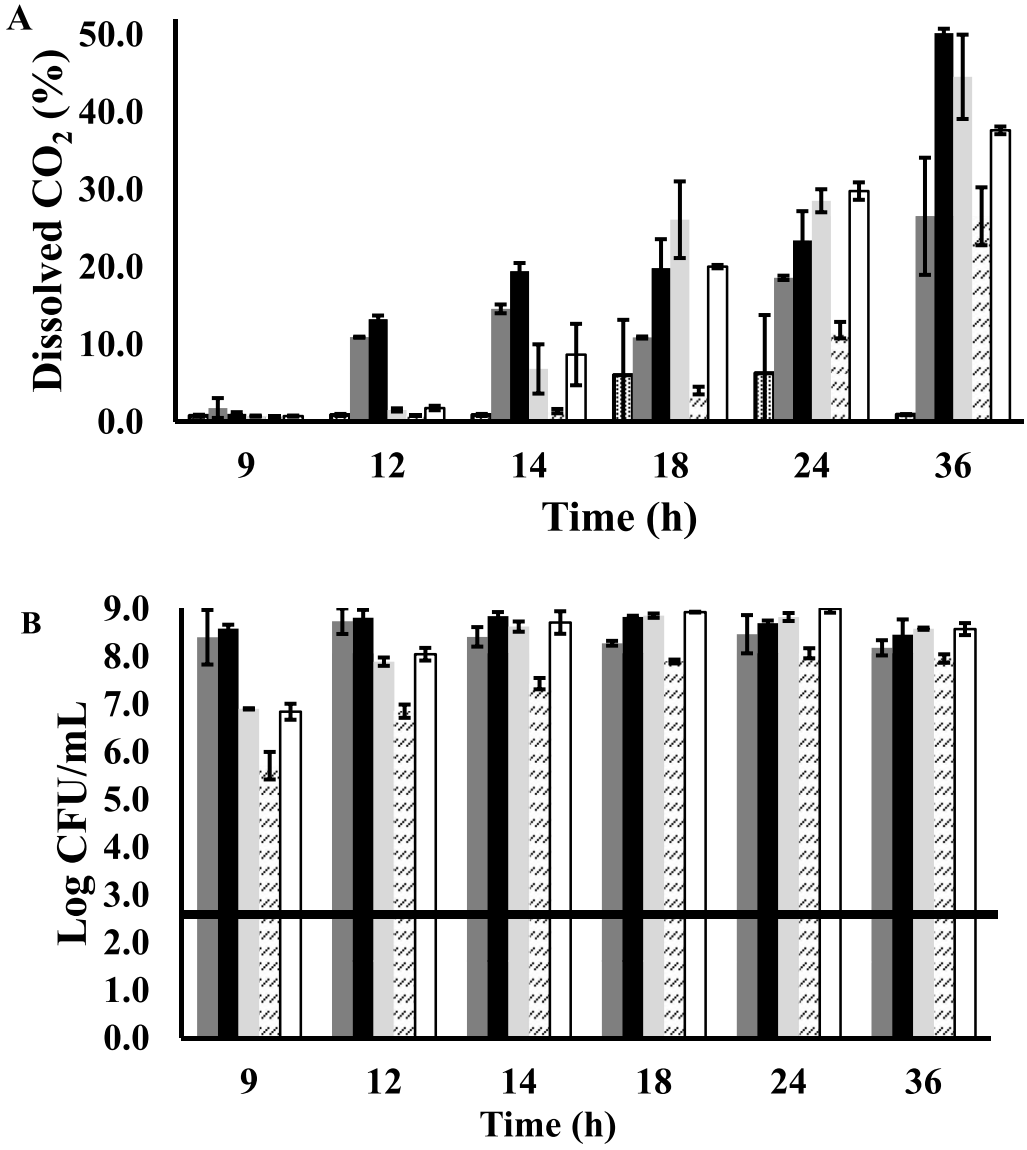

Fig. 2. CO_2 production (panel A) and colony counts (panel B) from cultures of *Leuconostocaceae* in CJM inoculated to 2 Log CFU/mL (horizontal black line). ▒ Control; ■ *W. cibaria*; ■ *Lc. lactis*; ■ *Lc. holzapfelii*; ≈ *Lc. fallax*; □ *Lc. mesenteroides*. The data shown are the average and standard deviation of technical duplicates collected from vacutainers filled with cucmber juice expressed from one lot of size 3B (1.75–2.0″ diameter) cucumbers.

Table 3
Bloater index and colony counts for presumptive *Enterobacteriaceae*, yeasts and molds and lactic acid bacteria from Violet Red Bile-Glucose agar (VRBG) and Brain Heart Infusion agar (BHI), Yeast and Mold agar (YMA) and Lactobacilli deMan, Rogosa and Sharpe agar (MRS), respectively. The culture media were inoculated with a 36 h old cucumber fermentation homogenate. The cucumber fermentations were inoculated with *Leuconostocaceae*. Fresh cucumbers were brined with 25 mM $CaCl_2$, 6 mM potassium sorbate and 20.2 mM $Ca(OH)_2$. The initial fermentation pH was adjusted to 6.0 with 20% vinegar (acetic acid). The data shown represent the average and standard deviation of triplicate samples collected from cucmber fermentaion jars packed with three independent lots of size 2B (1.25–1.5" diameter) cucumbers. Levels not connected by the same letter within a column are significantly different.

Inocula	Inoculation Level (Log CFU/mL)	Colony Counts (Log CFU/mL)				Bloater Index	
		VRBG	MRS	YMA	BHI	18h	36 h
None (Positive Control)	None	5.5 ± 0.7^{A}	7.6 ± 0.3^{A}	1.7 ± 0.4^{B}	7.7 ± 0.3^{A}	0^{B}	1.8 ± 0.8^{A}
Leuconostoc lactis 1.2.28	5	5.3 ± 0.4^{A}	7.6 ± 0.1^{A}	1.8 ± 0.6^{AB}	7.6 ± 0.2^{A}	0^{B}	1.8 ± 0.8^{A}
Leuconostoc fallax 1.2.22	5	5.5 ± 0.7^{A}	7.7 ± 0.2^{A}	1.8 ± 0.2^{AB}	7.7 ± 0.2^{A}	0^{B}	1.3 ± 1^{AB}

initial colony counts. Cucumbers were packed in 3.8 L-glass jars with a cover brine formulated to contain 25 mM $CaCl_2$ and 6 mM potassium sorbate at equilibrium. The cover brine also contained enough acetic acid, added as 20% vinegar, to adjust the initial pH to 3.3 ± 0.1. The amount of acetic acid needed was determined by scaling up proportionally the amount of acetic acid needed to titrate 100 g of cucumber slurries prepared with cucumbers from a single experimental lot and the experimental cover brine mixed in a 50:50 ratio. The initial

Table 4
Metabolites detected from fermented cucumbers collected from jars inoculated with selected *Leuconostocaceae* to 5 Log CFU/mL. Two species *Lc. lactis* 1.2.28 and *Lc. fallax* 1.2.22 were inoculated in cucumber fermentations. Cucumber samples were collected from 18 h to 36 h old cucumber fermentations. Cucumbers were brined with 25 mM $CaCl_2$, 6 mM potassium sorbate and 20.2 mM $Ca(OH)_2$. The initial fermentation pH was adjusted to 6.0 using 20% vinegar (acetic acid). The concentration of malic acid, glucose and fructose in the fresh cucumbers were 14.94 mM, 59.80 mM and 64.90 mM, respectively. The data presented represent the average and standard deviation of triplicate samples collected from jars packed with 3 independent lots of size 2B (1.25–1.5") cucumbers. Levels not connected by same letter within a column are significantly different.

Inocula	Substrates Utilized (mM)			Products Formed (mM)			Estimated CO_2 in the headspace (mM)
	Malic Acid	Glucose	Fructose	Lactic Acid	Acetic Acid	Ethanol	
Initial concentration from fresh cucumber slurries	14.9 ± 0.6	59.8 ± 1.8	64.9 ± 1.6	2.2 ± 0.6	16.6 ± 4.2 (added for acidification)	Below Detection Limit	Not Applicable
18 h old cucumber fermentation							
None	3.3 ± 1.2^{A}	14.8 ± 6.4^{A}	12.2 ± 6.4^{A}	3.6 ± 1.4^{B}	13.7 ± 6.0^{AB}	19.7 ± 2.5^{A}	59.0 ± 1.9^{C}
Leuconostoc lactis 1.2.28	1.9 ± 1.7^{A}	11.3 ± 2.2^{A}	7.6 ± 2.2^{A}	3.3 ± 0.0^{B}	12.1 ± 2.7^{B}	21.4 ± 0.7^{A}	56.7 ± 3.5^{C}
Leuconostoc fallax 1.2.22	4.1 ± 1.2^{AB}	14.1 ± 4.5^{A}	11.9 ± 4.3^{A}	3.3 ± 0.7^{B}	12.2 ± 3.7^{B}	19.3 ± 1.4^{A}	54.8 ± 8.6^{C}
36 h old cucumber fermentation							
None	7.5 ± 0.6^{C}	32.1 ± 3.7^{B}	29.8 ± 4.2^{B}	17.6 ± 2.1^{A}	27.5 ± 6.5^{A}	19.1 ± 2.3^{A}	118.7 ± 6.1^{B}
Leuconostoc lactis 1.2.28	7.6 ± 0.4^{C}	29.2 ± 4.2^{B}	27.4 ± 5.0^{B}	16.0 ± 2.3^{A}	25.7 ± 5.3^{AB}	19.3 ± 2.0^{A}	134.5 ± 5.8^{AB}
Leuconostoc fallax 1.2.22	6.8 ± 1.0^{BC}	29.5 ± 5.7^{B}	27.9 ± 5.8^{B}	15.4 ± 2.0^{A}	26.4 ± 6.4^{AB}	21.5 ± 2.2^{A}	139.1 ± 8.1^{A}

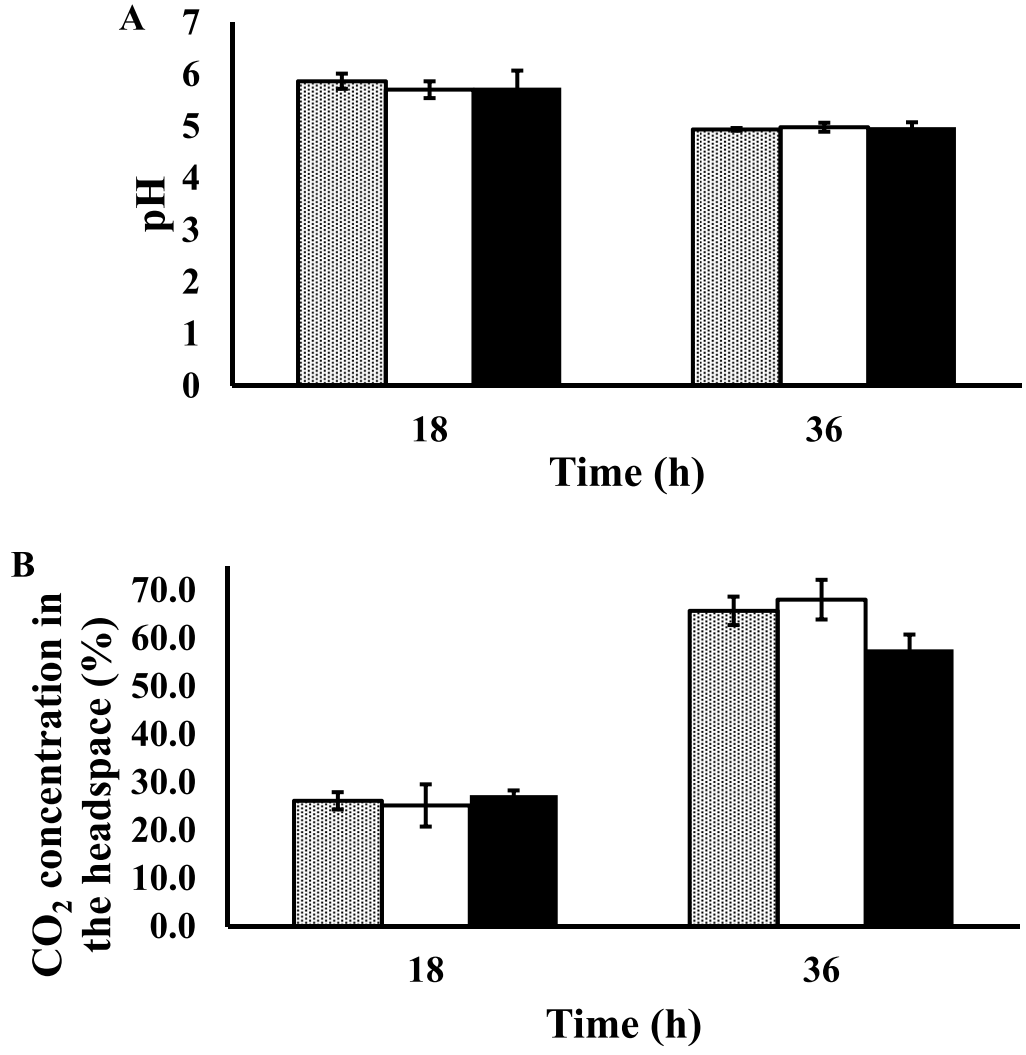

Fig. 3. pH (panel A) and CO_2 production (panel B) on cucumber fermentations inoculated with *Leuconostocaceae* to 5 Log CFU/g. Cucumbers were brined with 25 mM $CaCl_2$, 6 mM potassium sorbate and 20.2 mM $Ca(OH)_2$. The initial fermentation pH was adjusted to 6.0 ± 0.1 using 20% vinegar (acetic acid). (▨) *Lc. lactis*; (□) *Lc. fallax*; (■) Notinoculated control. The data presented represent the average and standard deviation of triplicate samples of fermented CJM prepared with juice expressed from 3 independent lots of size 2B (1.25–1.5″ diameter) cucumbers.

pH was adjusted to 3.3 ± 0.1 to inhibit and eliminate the indigenous acid-sensitive microbiota in the fresh cucumbers (Pérez-Díaz and McFeeters, 2008). The final pH in a cucumber fermentation is usually at 3.3 to 3.5 (Pérez-Díaz et al., 2015), which promotes the die-off of most microbes including the acid-tolerant *Lb. plantarum*. An equal weight of cucumbers and cover brine were added into each jar aiming to include 16 cucumbers per jar. The jars were vacuum sealed with metal lug lids that were boiled for 15 s to prevent leakage of the gas to be produced. The center of the jar lids were punched and equipped with a 12 mm rubber septa that fitted in the punched hole, for sampling using a

syringe equipped with a Luer-Lok Tip and BD Precision Glide needle (Becton Dickinson and Co.). Jars were incubated at 30 °C for 3 days to enable the equilibration of the cucumber and cover brine components to pH 3.3 ± 0.1. During the equilibration period, additional cucumbers were blended and mixed with the fermentation cover brine to conduct titrations with $Ca(OH)_2$ and determine the amount needed to raise the pH back to 5.9 ± 0.4, so that *Leuconostocaceae* could proliferate upon inoculation. *Lc. mesenteroides* is more acid sensitive than *Lb. plantarum* and stops growing at an intracellular pH of 5.4–5.7 (McDonald et al., 1990). Therefore, a fermentation pH slightly above 5.4 was expected to permit the growth of *Leuconostoc* species. One cucumber and the equivalent weight of cover brine were retrieved from each jar using aseptic techniques to determine microbial counts after equilibration on day 4 post-packing to confirm the inhibition or elimination of the indigenous microbiota. The brined cucumbers and cover brine were blended and homogenized prior to plating on VRBG, MRS, YMA and BHI media as described in section 2.3. $Ca(OH)_2$ was added to each jar to the pre-determined concentrations and the jars were incubated for three additional days at 30 °C to enable equilibration before inoculation. Three independent *Lc. lactis* 1.2.28 cultures were diluted as needed with CJM and inoculated to 5 Log CFU/g in the three treatment jars, each containing an independent lot of cucumbers generating independent triplicates. Control cucumber fermentation jars remained not inoculated. Aliquots of 50 mL to 110 mL of 3.33 M acetic acid were added to the fermentation jars to re-adjust the pH to 5.9 ± 0.4. All jars were re-capped with lids equipped with a 12 mm rubber septa to enable sampling with a syringe and needle assembly as described in section 2.6. Daily CO_2 measurements were obtained from the headspace of the jars using the Map-Pak Combi Gas Analyzer as described in section 2.6. The cucumber fermentations were stopped on day 7 after inoculation. Cover brine samples were collected through the rubber septa on the lids with a syringe and needle assembly (Becton Dickinson and Co.) and transferred to vacutainers for CO_2 measurements as described by Zhai and Pérez-Díaz (2017). Fermentation cover brine supernatants were prepared for pH measurements as described in section 2.6. Fifteen fruits per jar were aseptically cut longitudinally for bloater defect assessment. Samples of the cut cucumbers and cover brine (in a 1:1 ratio w/w) were collected from each treatment for microbiological analysis conducted as described in section 2.3. Each slurry sample was stored at −20 °C to conduct HPLC analysis at a later time as described in section 2.7. The final acetic acid production presented in Table 5 was calculated by deducting the total amount of the acetic acid added for pH adjustment.

2.9. Statistical analysis

The significant differences among the treatments were determined by LSMeans Tukey HSD using JMP Pro 12 (SAS Institute, Inc., Cary, NC). A difference between treatments based on date was considered and the interactions between treatments and sampling times were assessed. For all data sets, means denoted by different letters are statistically significantly different $P \leq 0.05$ (ANOVA).

3. Results

3.1. Growth of Leuconostocaceae in CJM and CO_2 production

Most *Leuconostocaceae* reached maximum cell densities in CJM by 12 to 20 h, with maximum optical densities at λ_{630} from 0.8 to 1.8 (Fig. 1). Five representative cultures with different growth patterns were selected for assessment of CO_2 production in CJM contained in vacutainers. *Lc lactis* 1.2.28 and *Lc. holzapfelii* 3.8.12 produced over 40% (equivalent to 90–100 mM) CO_2 in CJM (Fig. 2 and Table 1). The remaining three *Leuconostocaceae*, *W. cibaria* 3.8.44, *Lc. fallax* 1.2.22 and *Lc. mesenteroides* 1.2.47 produced an average of 30% (estimated at 61 mM) CO_2 (Table 1 and Fig. 2). No significant differences among cultures were observed on pH and colony counts from MRS plates (Tables 1 and 2, Fig. 2) by the end of the fermentation in CJM. Table 1 shows that most *Leuconostoc* species ceased growing at an average pH of 3.9 ± 0.1, while *W. cibaria* stopped at a pH of 4.3 ± 0.1.

Table 5
Fermentation biochemistry and microbial colony counts from acidified cucumber fermentation homogenates. The samples were collected on day 4 after packing the jars and on day 7 after the cucumbers in jars were inoculated with *Lc. lactis* 1.2.28. The colony counts from VRBG and YMA were below detection limit (BDL). The concentration of malic acid, glucose and fructose in the fresh cucumbers were 12.56 mM, 50.95 mM and 55.42 mM, respectively. The data presented represent the average and standard deviation of triplicate homogenate samples collected from jars packed with three independent lots of size 2B (1.25–1.5" diameter) cucumbers. Levels not connected by the same letter within a row are significantly different.

Measurement	Not Inoculated Control	Inoculated with *Leuconostoc lactis* 1.2.28
Day 4 after packing the jars (pH equilibrated to 3.3 ± 0.1)		
Residual Malic Acid (mM)	7.75 ± 1.68^{A}	10.25 ± 3.58^{A}
Glucose Utilized (mM)	24.83 ± 5.52^{A}	27.81 ± 2.84^{A}
Fructose Utilized (mM)	27.59 ± 5.80^{A}	30.39 ± 2.41^{A}
Estimated Acetic Acid Formed (mM)	BDL	BDL
Ethanol Formed (mM)	19.51 ± 2.46^{B}	18.90 ± 1.05^{B}
Lactic Acid Formed (mM)	0^{C}	0^{C}
Day 7 after culture inoculation (14 days post-packing)		
Final Fermentation pH	5.95 ± 0.55^{A}	5.88 ± 0.15^{A}
Colony counts (Log CFU/g) from BHI	6.9 ± 0.4^{B}	9.1 ± 0.3^{A}
Colony counts (Log CFU/g) from MRS	8.0 ± 0.3^{B}	9.2 ± 0.2^{A}
Dissolved CO_2 (%)	8.57 ± 0.8^{A}	13.60 ± 3.48^{A}
Estimated CO_2 in mM converted from %	22.19 ± 1.58^{A}	32.09 ± 6.85^{A}
Bloater Index	5.2 ± 5.9^{B}	21.3 ± 6.4^{A}
Residual Malic Acid (mM)	12.70 ± 2.15^{A}	12.73 ± 2.76^{A}
Glucose Utilized (mM)	45.72 ± 2.80^{B}	50.61 ± 0.59^{B}
Fructose Utilized (mM)	48.63 ± 3.14^{B}	49.42 ± 1.27^{B}
Estimated Acetic Acid Formed (mM)	47.33 ± 17.01^{A}	54.67 ± 5.51^{A}
Ethanol Formed (mM)	26.65 ± 1.54^{A}	28.35 ± 2.47^{A}
Lactic Acid Formed (mM)	60.22 ± 14.76^{B}	88.30 ± 6.88^{A}

Table 2 shows the biochemistry of the fermentation by *Leuconoctocaceae* in CJM. *Lc. lactis* 1.2.28, *Lc. holzapfelii* 3.8.12 and *Lc. mesenteroides* 1.2.47 exhausted the glucose; *Lc. lactis* 1.2.28 had about 22 mM of fructose left while *Lc. fallax* 1.2.22 used more fructose than glucose (Table 2). Most of the cultures utilized malic acid, except *Lc. fallax* 1.2.22. *Lc. lactis* 1.2.28 and *Lc. holzapfelii* 3.8.12 produced the highest amount of lactic acid at 43.9 ± 2.7 mM. An average of 28.1 ± 1.5 mM acetic acid was produced by *Lc. holzapfelii* 3.8.12 and *Lc. mesenteroides* 1.2.47 in CJM (Table 2). While *W. cibaria* 3.8.44 produced 20 mM ethanol, it did not complete the fermentation in CJM in 36 h. *Lc. lactis* 1.2.28, was the only other ethanol producer and did not produce acetic acid above 10 mM. *W. cibaria* 3.8.44, *Lc. holzapfelii* 3.8.12 and *Lc. mesenteroides* 1.2.47 produced from 7 to 13 mM succinic acid (Table 2).

3.2. CO_2 production and bloater defect in cucumber fermentation by Leuconostocaceae

Two *Leuconostocaceae* cultures, *Lc. lactis* 1.2.28 and *Lc. fallax* 1.2.22, were selected for this experiment based on the fermentation biochemistry in CJM (Table 2). Contrary to *Lc. fallax* 1.2.22, *Lc. lactis* 1.2.28 utilized malic acid and produced ethanol (Table 2). The inoculation of *Leuconostocaceae* in cucumber fermentations to 5 Log CFU/mL resulted in no significant differences in bloater defect, colony counts or fermentation biochemistry (Tables 3 and 4). About 56% (± 7%) of CO_2 was measured in the fermentation jar headspace (Fig. 3). The formation of lactic acid, acetic acid and ethanol to 16.3 ± 2.3 mM,

26.4 ± 6.2 mM and 20.5 ± 2.1 mM, respectively, suggested that heterofermentation by the indigenous and inoculated *Leuconostocaceae* took place. A bloater index of 1.6 ± 0.9 was observed in all treatments after 36 h of incubation.

3.3. Bloater defect and CO_2 production by Lc. lactis 1.2.28 in acidified cucumber fermentations

The effectiveness of the initial acidification in reducing the indigenous microbiota is demonstrated by colony counts on BHI and MRS plates from the homogenized samples of cucumbers and cover brines at 3 Log CFU/g and 2 Log CFU/g, respectively, on day 4 after packing the jars (data not shown). These colony counts represented 1000X reduction in the microbial counts from the fresh cucumbers at 6 Log CFU/g on BHI plates and 5 Log CFU/g on MRS and VRBG plates. The colony counts did not increase 4 days after adding calcium hydroxide ($Ca(OH)_2$) for pH adjustment (data not shown). Table 5 shows that the total colony counts from BHI were 6.9 ± 0.4 Log CFU/mL in the not inoculated control as compared to 9.1 ± 0.3 in the jars inoculated with *Lc. lactis* 1.2.28 7 days after inoculation. Colony counts for presumptive *Enterobacteriaceae* and yeasts and molds from VRBG and YMA plates were too few to count by the end of the incubation (data not shown). The colony counts from MRS were significantly higher (by almost 1 Log CFU/g) in the jars inoculated with the heterofermentor than the not inoculated jars (Table 5). The inoculation of the acidified cucumber fermentations with *Lc. lactis* 1.2.28 resulted in a CO_2 production of 13.60 ± 3.48% (estimated at 32.09 ± 6.85 mM) with a bloater index of 21.3 ± 6.4 (Table 5), which contrasted with the formation of 8.57 ± 0.8% CO_2 (estimated at 22.19 ± 1.58) and a bloater index of 5.2 ± 5.9 in the not inoculated jars (Table 5). No CO_2 production was detected in the fermentation jars headspace 7 days post-inoculation (Table 5).

4. Discussion

The amount of CO_2 produced by the *Leuconostocaceae* in CJM are two to three times that needed to cause bloater defect. Twelve percent (estimated at 28 mM) CO_2 is needed in fermentation jars to induce injuries in the tissue (Zhai et al., submitted). The ability of *Leuconostocaceae* to produce CO_2 and proliferate in CJM or a cucumber fermentation seems to be limited by pH below 3.9 (Table 3). A similar pH limitation was observed for *Lc. holzapfelii* (De Bruyne et al., 2007). The *Leuconostoc* spp. and *Weissella* spp. isolated from industrial cucumber fermentations utilized both glucose and fructose and most used malic acid (Table 4). Malic acid degradation protects LAB, particularly *Lb. plantarum*, from low pH stress (Garcia et al., 1992). *Leuconostocaceae* show no preference for glucose or fructose utilization from the CJM to produce lactic acid, acetic acid and some succinic acid (Table 2). The fact that *Leuconostocaceae* can produce no more than 28 mM acetic acid and 25 mM ethanol in CJM suggests that these microbes are likely minimally contributing these compounds to cucumber fermentations. Succinate is produced via the reductive tricarboxylic acid cycle which is affected by pH, temperature, the concentration of H_2, CO_2, carbon, nitrogen sources and the metal ions available in the growth medium (Agarwal et al., 2007; Andriani et al., 2019). Although the production of succinic acid is nominal, it seems likely that *Leuconostoc* species can ferment the glucose and fructose naturally present in cucumbers to a mixture of formate, acetate, lactate and succinate, anaerobically, by converting the central intermediate phosphoenolpyruvate into oxaloacetate. Given that acidic pH reduces the CO_2 solubility and succinic acid production is a CO_2 fixing pathway, the production of such acid would favorably reduce the CO_2 concentrations contributed by the same bacterial family, the *Leuconostocaceae* (Samuelov et al., 1991; Zeikus et al., 1999).

The supplementation of cucumber fermentations with a *Leuconostoc* starter culture did not significantly impact the fermentation biochemistry, colony counts from MRS or bloater index. These observations suggest a possible inability of the inoculated species to dominate in a cucumber fermentation or an inability to cause bloater defect (Tables 3 and 4). An acidified cucumber fermentation was considered as an alternate tool to observe the possible influence of *Leuconostocaceae* in bloater defect in the absence of the competing microbiota. This experiment was designed to determine if the inability of a *Leuconostocaceae* starter culture to prevail in a cucumber fermentation prevented a contribution to bloater defect in the previous cucumber fermentation experiment. The acidification of the cucumbers to be fermented to pH 3.3 ± 0.1 was applied to reduce the indigenous microbiota, which was indeed reduced by 3 Log CFU/g. The acidification effectively controlled for acid sensitive gram-negative bacteria and some LAB intolerant to pH 3.3 ± 0.1. *Leuconostoc mesenteroides* is known to survive in acidic conditions as long as its internal pH is above 5.4 to 5.7, while *Lb. plantarum* can tolerate an internal pH of 4.6 to 4.8 (McDonald et al., 1990). Thus, it is likely that the indigenous lactobacilli retained viability in the acidified fermentations after the pH was reduced to 3.3 ± 0.1 and grew after the pH was raised to 5.9 ± 0.4. This is supported by the colony counts from MRS plates and the formation of lactic acid in the non-inoculated jars (Table 5). The possibility that the native *Leuconostocaceae* proliferated in the control jars cannot be excluded given that ethanol and acetic acid were also formed (Table 5).

The inoculation of *Lc. lactis* 1.2.28 in acidified fermentations after the pH was adjusted to 5.9 ± 0.4 brought the CO_2 level to 13.6 ± 3.5%, which is just above the 12% needed to cause bloater defect (Zhai et al., submitted) (Table 5). The bloater index from the jars inoculated with *Lc. lactis* 1.2.28 was at 21.3 ± 6.4, which was significantly higher than that observed in the non-inoculated control jars at 5.2 ± 5.9 (Table 5). Thus, it is concluded that although *Leuconostocaceae* cannot dominate in cucumber fermentations they have the ability to produce enough CO_2 in cucumber fermentations to cause bloater defect. It seems likely that the *Leuconosctocaceae* are not the only or main contributor of CO_2 in cucumber fermentations as it can be outcompeted by other indigenous lactic acid bacteria.

5. Conclusion

It is apparent that *Leuconostocaceae* can produce CO_2 from sugar catabolism in cucumber fermentations brined with 25 mM $CaCl_2$ and 6 mM potassium sorbate and contribute to bloater defect. *Leuconostocaceae* have the ability to produce over 40% CO_2 in CJM. Although, the inoculation of *Leuconostocaceae* in cucumber fermentations to 5 Log CFU/g did not exacerbate bloater defect after 36 h of incubation at pH 5.0, they can contribute to CO_2 production by mixed-acid fermentation and induce the formation of hollow cavities in acidified cucumber fermentations, depicted of the acid resistant indigenous microbiota. Further studies are needed to develop strategies for the control of *Leuconostocaceae* in cucumber fermentations and prevent bloater defect.

Author contributions

Ms. Yawen Zhai designed and conducted the experiments, collected the 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 interpretation and edited the manuscript.

Declaration of competing interest

There is no conflict of interest.

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