Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Evaluation of the use of malic acid decarboxylase-deficient starter culture in NaCl-free cucumber fermentations to reduce bloater incidence

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Keywords
fermented foods, lactic acid bacteria, *Lactobacillus*, microbial physiology, nonthermal processes.

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2017/1544: received 18 August 2017, revised 11 October 2017 and accepted 30 October 2017

doi:10.1111/jam.13625

Abstract

Aims: Accumulation of carbon dioxide (CO_2) in cucumber fermentations is known to cause hollow cavities inside whole fruits or bloaters, conducive to economic losses for the pickling industry. This study focused on evaluating the use of a malic acid decarboxylase (MDC)-deficient starter culture to minimize CO_2 production and the resulting bloater index in sodium chloride-free cucumber fermentations brined with $CaCl_2$.

Methods and Results: Attempts to isolate autochthonous MDC-deficient starter cultures from commercial fermentations, using the MD medium for screening, were unsuccessful. The utilization of allochthonous MDC-deficient starter cultures resulted in incomplete utilization of sugars and delayed fermentations. Acidified fermentations were considered, to suppress the indigenous microbiota and favour proliferation of the allochthonous MDC-deficient *Lactobacillus plantarum* starter cultures. Inoculation of acidified fermentations with *L. plantarum* alone or in combination with *Lactobacillus brevis* minimally improved the conversion of sugars. However, inoculation of the pure allochthonous MDC-deficient starter culture to 10^7 CFU per ml in acidified fermentations resulted in a reduced bloater index as compared to wild fermentations and those inoculated with the mixed starter culture.

Conclusions: Although use of an allochthonous MDC-deficient starter culture reduces bloater index in acidified cucumber fermentations brined with $CaCl_2$, an incomplete conversion of sugars is observed.

Significance and Impact of the Study: Economical losses due to the incidence of bloaters in commercial cucumber fermentations brined with $CaCl_2$ may be reduced utilizing a starter culture to high cell density.

Introduction

Bloater defect in fermented cucumbers leads to significant yield and quality losses for the pickling industry in the United States (Fleming *et al.* 1973a). The mechanism for the development of bloated fermented cucumbers is known to result from the production of carbon dioxide (CO_2) during fermentation, which is trapped in the cucumber tissue, increasing the internal pressure and forming hollow cavities or gas pockets within the vegetables (Etchells *et al.* 1968; Fleming *et al.* 1973a). It is estimated that about 20 mg of CO_2 per 100 ml of cover brine is required for the initial formation of hollow cavities in fermenting cucumbers at ambient temperature (25°C), in cover brines with 1·03 mol l^{-1} sodium chloride (NaCl) (Fleming *et al.* 1973a; Fleming 1984). Bloater defect varies in degree of acuteness and tissue disruption can occur in honeycomb, lens or balloon shape (Wehner and Fleming 1984). The incidence of bloating is determined by calculating the bloating index, which considers

the degree of acuteness (slight, medium and severe) and type of tissue disruption (Fleming *et al.* 1977) (Table 1).

The natural cucumber microbiota, specifically yeasts, was initially considered the primary source of CO_2 production in cucumber fermentations (Jones *et al.* 1941). However, the incidence of cucumber bloating in commercial fermentations in which yeast growth was suppressed was unaltered (Etchells *et al.* 1968). It is now understood that a variety of bacteria have the potential to contribute to the accumulation of CO_2 throughout the fermentation process. In the early stage of cucumber fermentation, the Gram-negative Enterobacteriaceae can produce H_2 and CO_2, which can result in spoilage conducive to bloating (Veldhuis and Etchells 1939). Other contributing microbes include the facultative heterofermenting lactic acid bacteria (LAB) *Lactobacillus plantarum*, which has been found to produce sufficient CO_2 from malic acid decarboxylation (MDC) to induce bloating (McFeeters *et al.* 1982), and the heterofermentor *Lactobacillus brevis*.

It is also known that the combination of cucumber tissue respiration and the microbial metabolic activity may trigger cucumber bloating. About 30 mg of CO_2 per 100 g of tissue forms in unfermented pasteurized and brined cucumbers (Fleming *et al.* 1973a). As the rate of tissue respiration is affected by conditions such as temperature, duration of storage and the physiological state of the vegetable, the amount of CO_2 produced during fermentation can vary (Eaks and Morris 1956).

The use of *L. plantarum* starter cultures deficient in MDC has been proposed as a strategy to reduce bloater incidence in cucumber fermentations brined with 1·03 mol l^{-1} NaCl. Although a reduction in malic acid utilization, CO_2 production and incidence of bloaters is observed with the utilization of an allochthonous starter culture naturally deficient in MDC, *L. plantarum* FS965, in cucumber fermentations brined with 1·03 mol l^{-1} NaCl, the resulting conversion of sugars is incomplete (McFeeters *et al.*1984). The use of an autochthonous MDC-deficient *L. plantarum* starter culture (MU045), however, results in a reduction in bloater incidence and a complete conversion of sugars in cucumber fermentations brined with 1·03 mol l^{-1} NaCl. It is still unknown whether the difference in sugars utilization in cucumber fermentations observed between the allochthonous and autochthonous MDC-deficient starter cultures is due to genetic variation related to isolation niches or strain level differentiation unrelated to horizontal gene transfer. It is relevant to note that *L. plantarum* MU045 is the result of the undirected chemical mutagenesis of the autochthonous culture, *L. plantarum* LA0445 (Daeschel *et al.* 1984; Daeschel *et al.*, 1987), a method that could have introduced multiple mutations and functional impairments in the deriving strain. Nevertheless, fermentations inoculated with *L. plantarum* MU045 are characterized by a prolonged bacterial lag phase and generation time as compared to those inoculated with the parental culture (McDonald *et al.* 1993). It has been proposed to use the autochthonous MDC-deficient culture in an aseptic system, deprived of competition from the wild microbiota to enable the dominance of the starter culture.

Even though a combination of the use of a *L. plantarum* MDC-deficient starter culture, cover brine acidification and air purging of fermentation tanks is an effective strategy to reduce bloater defect in cucumber fermentations brined with at least 1·03 mol l^{-1} NaCl, it is unknown whether such factors are functional in the newly developed low-salt cucumber fermentations brined with 100 mmol l^{-1} calcium chloride ($CaCl_2$) and 6 mmol l^{-1} potassium sorbate without NaCl (McFeeters and Pérez-Díaz 2010; Pérez-Díaz *et al.* 2015). Although commercial cucumber fermentations brined with $CaCl_2$ represent a system with a reduced environmental impact as compared to the traditional system brined with NaCl, a higher incidence of bloaters has been associated with it, in particular, when size 3A and 3B cucumbers are brined (McMurtrie 2016; personal communication with processors). Although, some correlation has been established between a higher incidence of bloaters in fermented cucumbers brined without NaCl and the minimized air purging routine applied in the initial trials to establish

Table 1 Definition of bloater damage type and weight damage value (WDV) used in this study: description of slight, medium and severe bloater damage and WDV corresponding to each bloater type, as used for the calculation of bloater index in this study

	Slight			Medium			Severe		
Bloater category									
WDV	Balloon	Lens	Honeycomb	Balloon	Lens	Honeycomb	Balloon	Lens	Honeycomb
	20	10	5	50	40	35	90	80	75

the low-salt technology (McMurtrie 2016), effective strategies to reduce the incidence of bloater in the novel system remain to be identified.

This study intended to isolate autochthonous MDC-deficient *L. plantarum* strains from commercial cucumber fermentations and evaluate and optimize conditions for growth in the newly developed low-salt preservation system. Autochthonous starter cultures are known to lead a faster reduction in pH in fermented foods, present shorter lag phases, establish more efficiently in their habitat and positively influence quality and nutritional attributes of the finished products as compared to allochthonous cultures (Di Cagno *et al.* 2008, 2009). An autochthonous *L. plantarum* MDC-deficient starter culture was expected to perform optimally in the low-salt system as compared to the weak competitor and chemical mutant *L. plantarum* MU045, derived from an MDC+ culture also isolated from pickles at a pH of 3·0 (McDonald *et al.* 1991, 1993), given that it would have been genetically adapted to such habitat.

Attempts to isolate an autochthonous MDC-deficient *L. plantarum* strains from commercial cucumber fermentations were unsuccessful in this study, so the performance of alternate MDC-deficient autochthonous starter cultures was evaluated in low-salt cucumber fermentations and their influence in bloater incidence determined. The utilization of a mixed culture consisting of *L. plantarum* and *L. brevis* was also evaluated in an attempt to obtain a complete fermentation. Cover brine acidification was also applied to reduce the competitiveness of the indigenous microbiota and enhance the prevalence of the MDC-deficient *L. plantarum* starter cultures.

Materials and methods

Determining cucumber bloater index

Cucumbers were cut longitudinally to observe the extent and type of hollow cavity formation. Acuteness of bloating was classified as slight (5–20 weight damage values (WDV)), medium (35–50 WDV) or severe (75–90 WDV) according to the type of tissue disruption, as described in Table 1. All types of bloater damage (balloon, lens and honeycomb) were considered. Bloater index was calculated as described by Fleming *et al.* (1977) using the WDV shown in Table 1.

Screening for malic acid decarboxylase-deficient *L. plantarum*/*pentosus* cultures in commercial cucumber fermentations and pure cultures identification

Cover brine samples were obtained from 478 commercial cucumber fermentation tanks subjected to purging immediately prior to sample collection. Two tank yards were included in this screening: one located in Mount Olive, NC, which contributed 95% of the cover brine samples, and the second located in Chaska, MN, which contributed 5% of the samples. Commercial fermentations were performed as described by Pérez-Díaz *et al.* (2015) using recycled and fresh NaCl cover brines for those vessels in North Carolina and Minnesota, respectively, with variable size cucumbers. Samples were collected in 50-ml conical centrifuge tubes (cat. # 430829; Corning Incorporated, Corning, NY) using Falcon sterile serological pipettes (Corning Incorporated, Durham, NC) and aseptic techniques, from the surface of cover brines of actively fermenting tanks (days 3–14 of the fermentations). Cover brine samples were delivered to our laboratory in 24 h or less by ground or air transportation at room temperature and processed immediately upon arrival. Cover brine samples were serially diluted with saline solution prior to plating on Lactobacilli MRS agar plates (cat. # 288130; Difco™, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0·001 cycloheximide (v/v) (0·1% stock solution; Oxoid, Basingstoke, England), to prevent yeasts growth. Colonies were transferred from Lactobacilli MRS agar plates to the differentiating MD medium to screen for MDC deficiency, based on a colour change in the agar (Daeschel *et al.* 1985), using replica plating with a sterilized velvet. The MD medium was incubated at 30°C for 3–4 days under anaerobic conditions to detect yellow-green colonies characteristic of the desired MDC-deficient cultures. Pure cultures of potential MDC-deficient lactobacilli were obtained by streaking in Lactobacilli MRS medium. Isolates were transferred to MD broth to confirm the MDC-deficient phenotype and the derived cultures identified by 16S *rRNA* gene sequencing. DNA extraction from each pure culture in MRS broth was conducted using an InstaGene Matrix DNA extraction kit (cat # 732-6030; Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. Extracted DNA was used for the partial amplification of the 16S *rRNA* gene sequence for identification. The PCR mixture contained 2X master mix (Bio-Rad), 10 μl of the resulting total genomic DNA extracted from each isolate, and 0·6 μmol l^{-1} of primers 8f (5′-AGA GTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCT TGTTACGACTT-3′) (Wilson *et al.* 1990). The PCR steps consisted of 4 min at 94°C followed by 30 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 until mailed for sequencing by Eton Bioscience Inc. (Durham, NC) using the Sanger DNA sequencing technology. Sequence data were formatted and analysed using BIOEDIT software (www.mbio.ncsu.edu/bioedit). Only bases that had quality scores ≥20 were used for the alignment. The sequences obtained were subjected to the basic local alignment search tool (Altschul *et al.* 1990; Benson *et al.* 1997)

Table 2 Description of malic acid decarboxylase-deficient (MDC−) *Lactobacillus plantarum* strains identified as candidates for starter cultures for cucumber fermentations brined with $CaCl_2$ instead of NaCl

Cultures ID	Identified	Source and MDC phenotype	References
LA 0445	*L. plantarum*	Cucumber fermentation pH 3·3/MDC$^+$	McDonald *et al.* (1993)
MU 045	*L. plantarum*	Chemically mutated LA0445/MDC$^-$	Daeschel *et al.* (1987)
FS965 & FS963	*L. plantarum*	Cheddar cheese/natural MDC$^-$	National Institute for Research in Dairying (Reading, England); McFeeters *et al.* 1984; Daeschel *et al.* 1984
LA1204 & LA990	*L. plantarum*	Sauerkraut fermentation/natural MDC$^-$	Lu *et al.* (2003)

Table 3 Microbiological and biochemical analysis of cucumber fermentations brined with $CaCl_2$ inoculated with malic acid decarboxylase-deficient *Lactobacillus plantarum* strains. Values shown represent the means of duplicates and standard errors. Levels not connected by same letter are significantly different

Cultures tested	Cover brine pH	Colony counts from MRS plates on day 7 (log of CFU per ml)	Metabolites concentration (mmol l^{-1}) on day 14				
			Glucose	Fructose	Lactic acid	Acetic acid	Malic acid
No inoculum	4·61 ± 0·03^{A}	BDL^{J}	26·81 ± 0·08^{A}	34·50 ± 0·09^{A}	6·78 ± 0·08^{I}	5·79 ± 0·01^{CD}	12·75 ± 0·03^{A}
LA 0445	3·02 ± 0·0^{EF}	7·85 ± 0·08^{BC}	5·15 ± 0·19^{E}	6·66 ± 0·03^{F}	81·31 ± 2·13^{BC}	4·26 ± 0·08DE	1·91 ± 0·02^{F}
FS 965	3·23 ± 0·01^{B}	7·11 ± 0·33DE	12·80 ± 0·10^{B}	23·66 ± 0·09^{B}	48·91 ± 0·26^{H}	7·15 ± 0·03^{C}	12·12 ± 0·04^{BC}
FS 963	3·03 ± 0·02DE	8·09 ± 0·04^{AB}	5·56 ± 0·43^{D}	6·40 ± 0·40^{E}	76·78 ± 0·62^{E}	4·08 ± 0·01^{E}	1·09 ± 0·03^{F}
LA 990	3·08 ± 0·01^{CD}	8·58 ± 0·05^{A}	0 ± 0·0^{FG}	13·81 ± 0·35^{C}	79·11 ± 0·38DE	4·19 ± 0·01^{E}	1·52 ± 0·02^{E}
LA 1204	3·11 ± 0·03DE	7·48 ± 0·09^{CD}	0 ± 0·0^{FG}	13·02 ± 0·25^{D}	72·41 ± 0·61^{F}	4·08 ± 0·0^{E}	1·39 ± 0·02^{EF}
MU 045	3·06 ± 0·01DE	7·48 ± 0·30^{CDE}	14·23 ± 0·0^{BC}	14·54 ± 0·0^{D}	53·97 ± 0·0^{G}	23·20 ± 0·0^{B}	10·51 ± 0·0^{D}

using the 16S ribosomal RNA sequence database to determine the identity of the isolates.

Evaluation of MDC-deficient cultures as candidates for starter cultures and cover brine acidification in cucumber fermentations brined with $CaCl_2$ instead of NaCl

Table 2 describes the MDC-deficient isolates selected as candidates for starter cultures for low-salt cucumber fermentations evaluated in this experiment. Cultures were transferred from frozen stocks maintained in the U.S. Department of Agriculture-Agricultural Research Service, Food Science Research Unit culture collection (Raleigh, NC) into Lactobacilli MRS broth and incubated at 30°C for 24 h prior to inoculation of cucumber fermentation jars. Cells were harvested from MRS broth by spinning the cultures at 4629 ***g*** for 5 min (Centrifuge 5810; Eppendorf, Hamburg, Germany). The pellets were washed twice with 150 mmol l^{-1} NaCl solution prior to resuspension in the same solution. All cultures were diluted to 10^6 CFU per ml to achieve an inoculation level of 10^4 CFU per ml in a cucumber fermentation model system (CJM) consisting of 50% cover brine and 50% cucumber juice (w/w). The cover brine contained 100 mmol l^{-1} anhydrous $CaCl_2$ (Brenntag, Durham, NC) and 6 mmol l^{-1} potassium sorbate (Mitsubishi International Food Ingredients, Atlanta, GA). The cucumber juice was prepared by blending size 2A cucumbers (3·1–3·8 cm. diam.) acquired from a local company (Mt. Olive Pickle Co., Mount Olive, NC). The blended cucumber juice was centrifuged for 30 min at 15 557 ***g*** (Centrifuge 5810; Eppendorf) to remove particulate prior to mixing with the cover brine and then filter sterilized using a 0·2 μ PES filter membrane (Nalgene; Nalge Nunc International, Rochester, NY). The initial pH of the CJM was adjusted with 5 mmol l^{-1} malic acid to 4·6 to aid with the selection of the MDC-deficient cultures. Consequently, the total malic acid content in the CJM was 12 ± 1 mmol l^{-1} as measured by high-performance liquid chromatography (HPLC) analysis conducted as described below. Malic acid also served as a buffer, given that a cytoplasmic proton is consumed per decarboxylated molecule (Radler 1966; Caspritz and Radler 1983; Passos et al. 2003). The cucumber juice was aliquoted in 8 oz. jars that were sealed with metal lug caps equipped with rubber septa, so that samples could be collected using a gas-tight syringe and needle assembly. Jars were

filled with 100 ml of CJM and inoculated with six cultures individually (Table 3). Uninoculated jars were used as control. Duplicate jars per treatment were incubated at 30°C for fermentation. Fermentations were biochemically and microbiologically monitored as described below on days 7 and 14.

For the evaluation of the effect of cover brine acidification on the performance of selected MDC-deficient cultures, cucumber fermentations were conducted in jars packed as described above. Cover brines were acidified with a 3·33 mol l^{-1} acetic acid solution added as vinegar, a 9·44 mol l^{-1} lactic acid solution or a 3 mol l^{-1} phosphoric acid solution to pH 4·6. A negative control treatment was also set with no acids added. Jars were inoculated with the MDC-deficient cultures, *L. plantarum* FS965 or MU045 and the malic acid decarboxylating *L. plantarum* LA0445 as control (Table 4). Same size jars were used, and each treatment was performed in duplicate. The jars were incubated at 30°C to ferment. Counts of lactobacilli and the fermentation biochemistry were analysed as described below from samples collected every 3–4 days.

Evaluation of cucumber fermentations inoculated with mixed starter cultures to obtain the complete conversion of sugars to lactic acid while reducing bloater incidence

46-oz size jars were packed with size 2A fresh cucumbers (3·1–3·8 cm. diam.) to 50% of the total jar volume by weight. The remaining 50% of the volume in each jar was filled with cover brine containing 200 mmol l^{-1} anhydrous $CaCl_2$ (Brenntag) and 12 mmol l^{-1} potassium sorbate (Mitsubishi International Food Ingredients) by volume, so that upon equilibration there would be 100 mmol l^{-1} of the salt and 6 mmol l^{-1} of the preservative in solution. The initial pH of fermentation was set at 4·6 after acidifying with phosphoric acid (diluted to 3 mol l^{-1} from CAS. 7664-38-2, 85520; Sigma-Aldrich Co., St. Louis, MO). The volume of the acid to be added to the cover brine was determined by titrating three samples of 100 ml of a 50 : 50 cucumber slurry suspension, made by blending the same size 2A fresh cucumbers used for experimentation, and cover brine. The cucumber slurry and cover brine were mixed by volume.

For the evaluation of cucumber fermentations inoculated with mixed starter cultures of MDC-deficient cultures and *L. brevis* ATCC 14869, jars were inoculated to 5 or 7 log of CFU per ml of *L. plantarum* FS965 alone or in combination with 2 log of CFU per ml of *L. brevis,* as indicated in Table 5. The inocula were prepared as described above. The MDC-deficient strain FS965 was inoculated to at least 2 log CFU per ml higher than the colony counts for the lactobacilli naturally present in the fresh cucumbers as determined by plating on MRS, as recommended by Breidt and Fleming (1992). Each treatment was tested with two lots of cucumbers. Uninoculated jars were used as control. Jars were incubated at 30°C for the duration of the experiment. Counts of lactobacilli on MRS plates and the fermentation biochemistry were monitored on days 0, 3, 7, 14 and 21, as described below. The bloater index of fermented cucumbers was determined at the ending point (day 21).

Evaluation of CJM and cucumber fermentation biochemistry (pH and HPLC)

Cover brine samples (1 ml) were collected with gas-tight needle and syringe assembly (Hamilton Co., Reno, NV, USA) through the rubber septa placed on the metal lug caps at the time points indicated in the text above. Samples were spun at 18 514 ***g*** for 10 min (Brushless Microcentrifuge, Denville 260D; Denville Scientific, Inc., Holliston, MA) at room temperature twice to remove particulates prior to pH measurement and HPLC analysis. The supernatants pH was measured with an Accumet pH meter (cat. 13-636-AR25B; Accumet™ AR25 pH/mV/°C/ISE, probe cat. 13-620-290; Fisher Scientific™, Hampton, NH), prior to storage at −20°C until HPLC analysis was conducted to measure organic acids and sugars. Cover brine supernatants were transferred to HPLC vials for analysis. Quantification of organic acid and sugar

Table 4 Changes in metabolites in acidified cucumber fermentations brined with $CaCl_2$ after 14 days of incubation at 30°C. Cucumber fermentations were acidified with lactic, acetic or phosphoric acid. The MDC-deficient and MDC+ starter cultures were used for this experiment. Values shown represent the mean of duplicate for each treatment with the three acids and standard deviations. Levels not connected by the same letter are significantly different

Starter cultures used	Substrate utilization (mmol l^{-1})			Lactic acid produced (mmol l^{-1})
	Malic acid	Glucose	Fructose	
No inoculum	–	–	–	$4{\cdot}44 \pm 1{\cdot}4^{E}$
Lactobacillus plantarum FS965	–	$15{\cdot}1 \pm 2{\cdot}4^{C}$	$15{\cdot}7 \pm 1{\cdot}8^{B}$	$63{\cdot}9 \pm 6{\cdot}4^{D}$
L. plantarum MU045	$6{\cdot}7 \pm 0{\cdot}3^{C}$	$20{\cdot}5 \pm 2{\cdot}7^{B}$	$21{\cdot}2 \pm 1{\cdot}6^{C}$	$85{\cdot}1 \pm 6{\cdot}7^{C}$
L. plantarum LA0445	$10{\cdot}3 \pm 0{\cdot}4^{D}$	$24{\cdot}6 \pm 1{\cdot}9^{D}$	$25{\cdot}6 \pm 3{\cdot}2^{E}$	$104{\cdot}3 \pm 6{\cdot}3^{A}$

Table 5 Biochemistry and bloater index associated with cucumber fermentations brined with $CaCl_2$ and inoculated with mixed starter cultures. Fermentations were acidified with phosphoric acid to pH 4·6. The mixed starter cultures contained *Lactobacillus plantarum* and *Lactobacillus brevis*. Analyses were performed with samples collected after 14 days of incubation. Values shown represent the means of three runs, including two independent replicates with different lots of cucumbers and two technical replicates of one lot, and standard errors. Levels not connected by the same letter are significantly different

Starter culture used	Inoculation level (log CFU per ml)	Lactic acid produced (mmol l^{-1})	Substrates residual (mmol l^{-1})		Bloater index
			Glucose	Fructose	
No inoculum	–	$55{\cdot}34 \pm 10{\cdot}28^{ABC}$	$6{\cdot}22 \pm 0{\cdot}91^{A}$	$2{\cdot}06 \pm 0{\cdot}70^{ABC}$	$6{\cdot}1^{A}$
L. plantarum FS965	7	$58{\cdot}09 \pm 12{\cdot}48^{AB}$	$5{\cdot}41 \pm 1{\cdot}04^{A}$	$2{\cdot}39 \pm 0{\cdot}56^{ABC}$	$2{\cdot}5^{D}$
L. plantarum FS965	5	$44{\cdot}82 \pm 3{\cdot}30^{ABC}$	$4{\cdot}66 \pm 0{\cdot}07^{A}$	$0{\cdot}93 \pm 0{\cdot}47^{BC}$	$5{\cdot}2^{B}$
L. plantarum FS965	5	$43{\cdot}80 \pm 3{\cdot}32^{ABC}$	$4{\cdot}57 \pm 0{\cdot}02^{A}$	$1{\cdot}43 \pm 0{\cdot}26^{BCD}$	$3{\cdot}3^{C}$
L. brevis ATCC 14869	2				

concentrations were performed using the HPLC method published by McFeeters and Barish (2003) using an Aminex 300 × 7·8 mm HPX-87H resin column (Bio-Rad Laboratories) for the separation of components with some modifications. 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^{-1}. A SPD-20A UV-visible light detector (Shimadzu Corporation, Canby, OR) was set at 210 nm at a rate of 1 Hz to quantify malic, lactic, succinic, propionic and butyric acids. An RID-10A refractive index detector (Shimadzu Corporation) connected in series with the diode array detector was used to measure acetic acid, glucose, fructose and ethanol. External standardization of the detectors was carried out using at least five concentrations of the standard compounds.

Microbiological analysis of CJM and cucumber fermentations

Cover brine samples were aseptically collected as a function of time, serially diluted in 150 mmol l^{-1} NaCl solution and plated on Lactobacilli deMan Rogosa and Sharpe (MRS) agar (cat. 288130; Difco, Becton Dickinson and Co.) supplemented with 0·1% cycloheximide for the enumeration of presumptive LAB. An Autoplate 400 (Spiral Biotech, Norwood, MA) was used to inoculate MRS plates prior to anaerobic incubation at 30°C for 48 h. Colonies were enumerated using a Flash & Go Automated Colony counter (cat. 90006010; IUL Instruments, Barcelona, Spain).

Statistical analysis

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 dates were assessed at the $P < 0{\cdot}05$ level using analysis of variance. For all data sets, levels not connected by the same letter were significantly different.

Results

Screening for malic acid decarboxylase-deficient *L. plantarum*/*pentosus* cultures in commercial cucumber fermentations

There were 13 cultures unable to utilize malic acid that were isolated from cover brine samples collected from 478 commercial cucumber fermentations. These were identified as *Propionibacterium* sp. (5), *Lactobacillus namurensis* (3) and *Leuconostoc* sp. (5). No MDC-deficient *L. plantarum* or *L. pentosus* cultures were isolated.

Evaluation of allochthonous MDC-deficient cultures as candidates for starter cultures for cucumber fermentations brined with $CaCl_2$ instead of NaCl

All microbial counts presented in Table 3 correspond to the maximum levels of the allochthonous MDC-deficient cultures measured, which show no significant differences in CJM after 7 days of incubation. However, the fermentation conducted by the FS965 strain, a natural allochthonous MDC-deficient *L. plantarum* isolated from Cheddar cheese, had an ending pH 0·1–0·2 units higher ($3{\cdot}23 \pm 0{\cdot}01$). A final pH of $3{\cdot}1 \pm 0{\cdot}15$ was observed in all fermentations after 7 days and remained stable for at least 14 days. As expected, no changes in pH were observed in the uninoculated control (Table 3). Contrary to expectations, malic acid partially disappeared in the fermentations inoculated with *L. plantarum* LA990, LA1204 and FS963 by day 14 (Table 3), suggesting such cultures are not deficient in malic acid decarboxylation under the conditions tested here. Cultures able to decarboxylate malic acid showed more proficient growth than one of the allochthonous MDC-deficient *L. plantarum* strains (FS965). *Lactobacillus plantarum* FS965 presented

the lowest colony count on MRS plates at 7·11 ± 0·33, glucose and fructose utilization at 47·7 and 73·4% of the amount originally present, and lowest lactic acid production (Table 3).

Effect of cover brine acidification on the performance of *L. plantarum* starter culture in cucumber fermentations

Except for the uninoculated controls, no significant differences were observed at the end of fermentation pH among the cultures and acid treatments tested (3·3 ± 0·3). Counts of lactobacilli from MRS showed minimal differences by day 3 (8·6 ± 0·7 log of CFU per ml, Fig. 1). Although no variations were observed in substrates utilization and products formation, as a function of the acids utilized for pH adjustment (data not shown), the starter cultures behaved differently in terms of malic acid utilization. Table 4 shows that after 14 days of fermentation, the malic acid decarboxylating culture had exhausted malic acid (10·3 ± 0·4 mmol l^{-1}), while the MDC-deficient cultures utilized 67% or less. All starter cultures produced 64–104 mmol l^{-1} of lactic acid (Table 4), with the mutant strains producing less. This observation is in agreement with the fact that malic acid utilization aids in maintaining a higher pH as the result of the consumption of protons from the cytoplasm during the decarboxylation (Daeschel *et al.* 1984). Lower production of lactic acid corresponded to less carbohydrate utilization (Table 4). *Lactobacillus plantarum* LA0445 was able to utilize 77% of the glucose and 69% of the fructose naturally present in the cucumbers (31·8 ± 2·9 mmol l^{-1} glucose and 37·2 ± 3·1 mmol l^{-1} fructose), but did not complete the fermentation by day 14, presumably due to the fact that the pH had reached levels inhibitory for its growth and metabolic activity at 3·0 ± 0·1 (McDonald *et al.* 1993). In line with the expectations for facultative heterofermenting starter cultures, acetic acid was not produced as the result of fermentation. Of interest is the fact that numbers of lactobacilli reached undetectable levels by day 14 in jars inoculated with *L. plantarum* MU045, surviving long term only in the treatment in which acidification was performed with phosphoric acid (data not shown).

Evaluation of cucumber fermentations inoculated with mixed starter cultures of MDC-deficient strains and the heterofermentor *Lactobacillus brevis*

No significant differences in the end of fermentation pH were noted among the treatments with the various starter culture inocula, which dropped to 3·3 ± 0·1 after 21 days of incubation at 30°C. Malic acid was fully consumed in all treatments (data not shown). None of the fermentations were completed as evidenced by the 4·9 ± 0·6 and 1·4 ± 0·6 mmol l^{-1} residual glucose and fructose, respectively, and the production of 47·3 ± 6·7 mmol l^{-1} lactic acid, which was about 50–60% of the expected amounts (Table 5). The extend of lactic acid utilization in the presence of the mixed starter culture resembled that of the fermentation inoculated with *L. plantarum* FS965 to 10^5 CFU per ml, suggesting it was slower than those inoculated with *L. plantarum* FS965 to 10^7 CFU per ml (Table 5). Differences in the biochemistry of the fermentation were minimal between days 14 and 21, indicating that most of the metabolic activity occurred prior to day 14 (data not shown). The jars inoculated with *L. plantarum* FS965 to 10^7 CFU per ml had the least number of bloaters, followed by the one using a mixed starter culture (Table 5). Overall, the inoculated fermentation had a lower bloater index as compared to the uninoculated control (Table 5).

Discussion

Fermentations were performed in vacuum sealed jars to reduce the availability of oxygen, so that the production of CO_2 from tissue respiration and the metabolic activity of aerobic microbes would be minimal. The contribution of tissue respiration to the production of CO_2 is estimated at 7% in pasteurized unfermented cucumber jars (Fleming *et al.* 1973a). It was considered that limiting the sources of CO_2 enabled the study of the contribution of fermentation to gas formation, in particular if lactobacilli with deficiencies in malic acid decarboxylation were used as starter cultures.

Prior to this study, it was uncertain whether an MDC-deficient starter culture would dominate in a cucumber fermentation. McDonald *et al.* (1993) observed that lower NaCl levels and temperatures close to ambient reduce the lag and generation time of the MDC-deficient culture, *L. plantarum* MU045, which are longer than those observed for the parental MDC+ culture, *L. plantarum* LA0445. It was thus hypothesized that an increased lactic acid production by MDC-deficient cultures would occur in the absence of NaCl in cucumber fermentations brined with $CaCl_2$. It was additionally speculated that the use of autochthonous starter cultures, in particular if isolated from commercial cucumber fermentations, would be genetically predisposed to thrive in such habitat and better able to compete with the indigenous microbiota when inoculated in high numbers (Di Cagno *et al.* 2008, 2009; Pavunc *et al.* 2012). However, decarboxylation of malic acid is an energetically advantageous reaction, consuming one proton per molecule of acid, resulting in the neutralization of pH (Daeschel *et al.* 1984). Thus, it was understood that the lack of MDC in a starter culture could result in limited competitiveness.

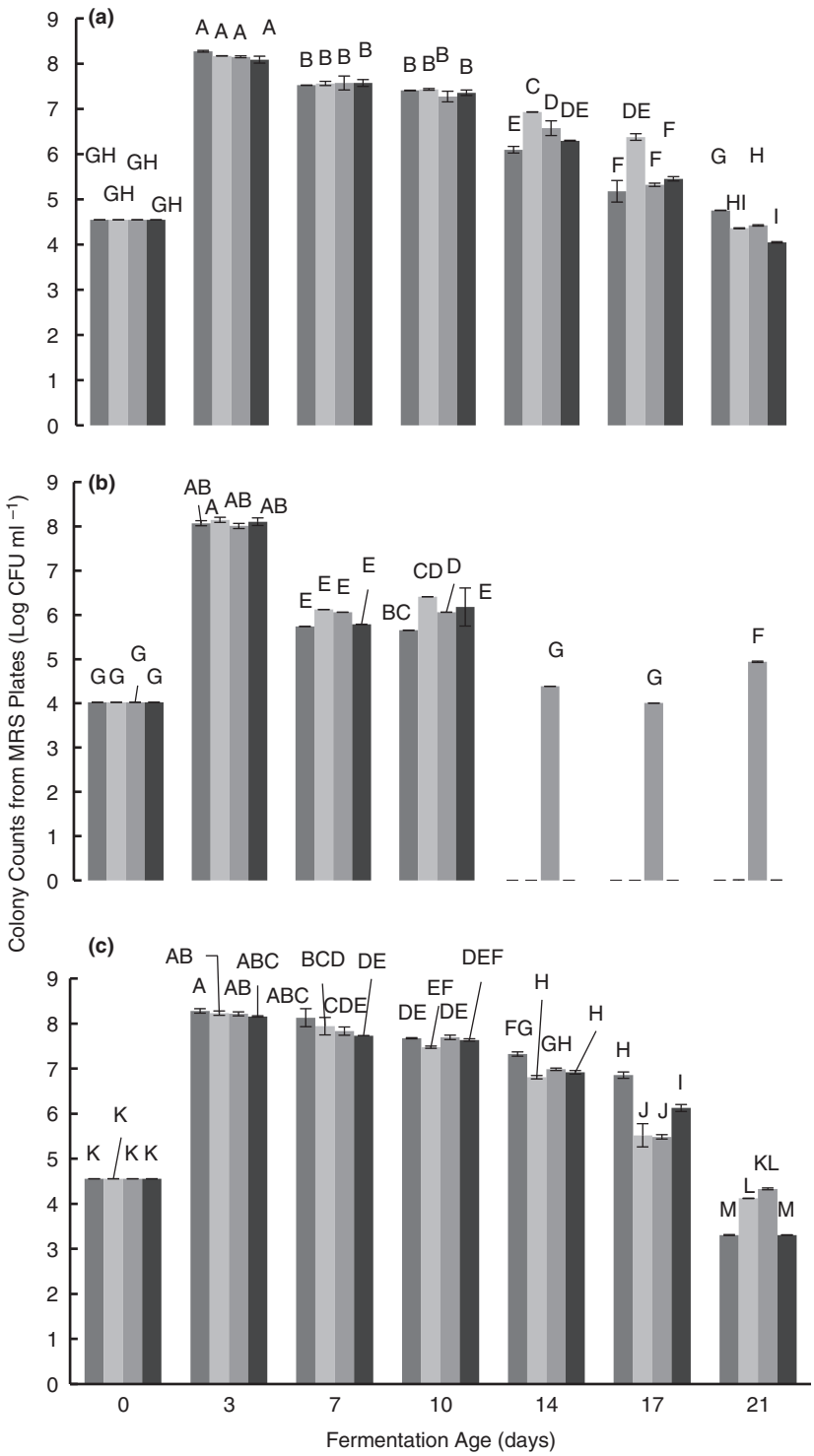

Figure 1 Colony counts from MRS plates in cover brine samples collected from cucumber fermentations brined with $CaCl_2$ and acidified with acetic, lactic or phosphoric acids. Panels a, b and c show colony counts for lactobacilli from MRS plates for cucumber fermentations inoculated with the MDC− cultures MU045 (a) and *Lactobacillus plantarum* FS965 (b) and the MDC+ *L. plantarum* LA0445 (c). Fermentations were acidified with acetic acid (■), lactic acid (■), phosphoric acid (■), or not acidified (■). Values shown represent technical duplicates of one trial with size 3A cucumbers. Levels not connected by the same letter are significantly different. [Colour figure can be viewed at wileyonlinelibrary.com]

Attempts to isolate MDC-deficient *L. plantarum* or *L. pentosus* strains from commercial cucumber fermentations were unsuccessful. Only 13 MDC-deficient bacteria were isolated from the 478 commercial cucumber fermentation tanks screened. The bacteria isolated included *Propionibacterium* sp., *Leuconostoc* sp. and *L. namurensis*. *Propionibacterium* produces propionic acid instead of lactic acid from sugars (Shu *et al.* 2013). *Lactobacillus namurensis* is a heterofermentative LAB, found in spoiled fermented cucumbers (Scheilinck *et al.* 2007, Medina *et al.* 2016), and *Leuconostoc* sp. produce CO_2 from sugars (Breidt *et al.* 2013). Thus, none of the cultures isolated represented suitable candidates for starter cultures that would robustly conduct a lactic acid homofermentation of cucumbers.

The MDC-deficient allochthonous cultures described in Table 2 were evaluated as potential starter cultures for low-salt cucumber fermentations given the unavailability of the autochthonous counterpart. The performance of the allochthonous starter cultures was compared to that of the MDC-deficient *L. plantarum* culture MU045, developed in our unit by chemical mutagenesis and its parental culture *L. plantarum* LA0445 (Daeschel *et al.* 1987). All cultures tested were inoculated to 10^6 CFU per ml, at least two logs of CFU per ml above the counts for the native lactobacilli population in the cucumbers used for experimentation, to favour their dominance in the system (Breidt and Fleming 1992). No significant improvement was observed when using MDC-deficient cultures instead of MDC+ *L. plantarum* in the completion of sugar utilization and the consequent lactic acid production. In line with previous reports for cucumber fermentations containing NaCl (McFeeters *et al.* 1982; McDonald *et al.* 1993), *L. plantarum* MU045 and FS965 grew slower as compared to the wild type in cucumber fermentations brined with $CaCl_2$, producing less lactic acid (Table 3). The deficient cultures also produced more acetic acid (20 mmol l^{-1}) as compared to the fermentation inoculated with the LA0445 parental culture (4 mmol l^{-1}) and did not fully utilized the sugars naturally present in the cucumbers, mostly glucose and fructose, in the absence of NaCl (Table 3). Together, these observations suggest that the inability of the deficient cultures to decarboxylate malic acid impairs the ability to compete in natural low-salt fermentations despite the high inoculation level.

Attempts to improve the performance of the MDC-deficient starter cultures in cucumber fermentations included acidification of the cover brines to reduce competition by the indigenous microbiota and promote its

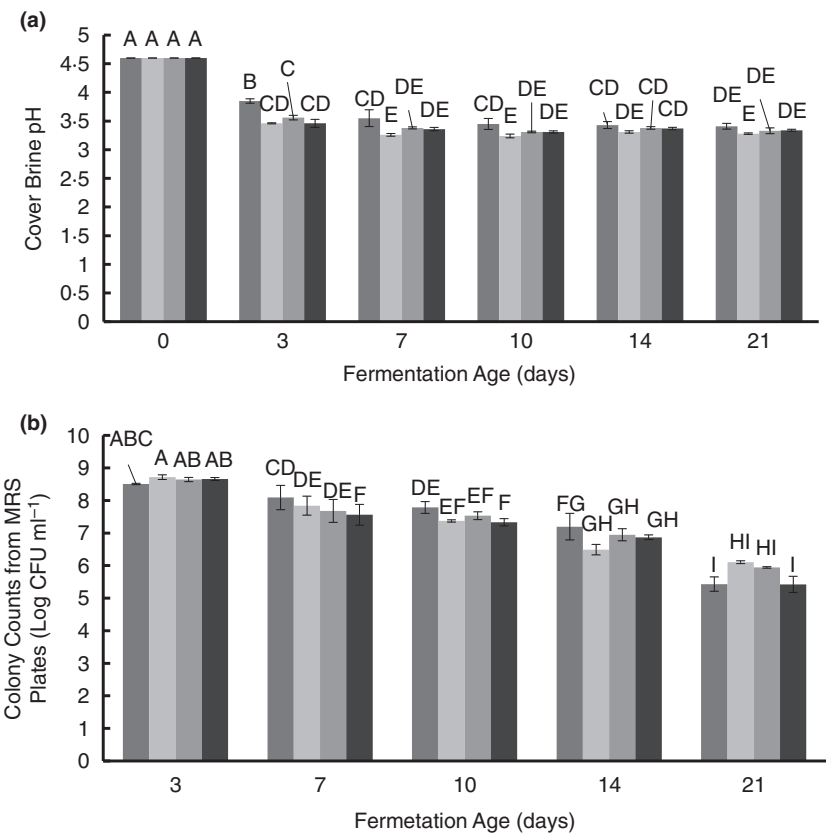

Figure 2 Colony counts from MRS plates and pH of cucumber fermentations brined with $CaCl_2$ and inoculated with mixed starter cultures. Panel (a) presents the means and standard deviations for the cover brine pH values. Panel (b) presents the means and standard deviations for colony counts from MRS plates. Fermentations inoculated with the MDC-deficient *Lactobacillus plantarum* FS965 to 7 (■) or 5 (■) log CFU per ml alone or in a combination with *L. plantarum* FS965 to 5 log CFU per ml with *Lactobacillus brevis* to 2 log CFU per ml (■). A noninoculated control (■) was also included in this experiment. Levels not connected by the same letter are significantly different.

prevalence (McDonald *et al.* 1991). It was also considered that acidification of the cover brine would facilitate the release of the gas upon production (Fleming *et al.* 1973b). No significant difference was observed on the performance of most of the starter cultures tested in the presence of various acids used to reduce the initial pH of the system to 4·6 (Fig. 1 and Table 3), a value still above of that inhibitory for growth of the deficient culture *L. plantarum* MU045 (Breidt and Fleming 1992).

The use of a mixed starter culture was considered to aid in increasing carbohydrate utilization in cucumber fermentations initiated at a relatively low pH (4·6). Mixed starter cultures of the MDC-deficient *L. plantarum* FS965 and *L. brevis*, a heterofermentative LAB, were considered to act in partnership to achieve the desired complete fermentation that was unachievable with the use of the deficient culture alone. *Lactobacillus brevis* was selected as a complementary starter culture given that it codominates in commercial fermentations along with the facultative heterofermentor (Pérez-Díaz *et al.* 2016). In making such selection, it was also considered that *L. brevis* tends to grow slower than *L. plantarum* in cucumber fermentations (Pérez-Díaz *et al.* 2016), which was thought to result in the deficient culture utilizing most of the sugars early in the process, while *L. brevis* would gain access to residual sugars converting a portion of them to acetic acid instead of lactic acid. Conversion of sugars to acetic acid instead of lactic acid was hypothesized to aid in achieving a higher end of fermentation pH, given that the former acid has a higher pKa (4·76) than the last at 3·86 (Featherstone and Rodgers 1981). It was also expected that the activity of the heterofermentative LAB, *L. brevis*, would result in a slightly higher production of the undesired CO_2. Even though minimal differences were observed in the colony counts of lactobacilli from MRS plates between days 3 and 21, despite the differences in inoculation levels (Fig. 2), more lactic acid was produced with higher inoculation levels (Table 5). The differences in lactic acid amounts produced may be reduced with longer incubation times for the vessels with the lower level of inocula. Colony morphologies observed on MRS plates only resembled that characteristic of *L. plantarum*, indicating that *L. brevis* was likely outcompeted and unable to finish the fermentation. Utilization of a *L. brevis* autochthonous strain, instead of the ATCC 14869 type strain used in this study, may result in enhanced proliferation during the fermentation. However, the data presented in Table 5 suggest that the type of starter culture is not as critical as the actual inoculation level to induce a faster fermentation with lower production of CO_2, likely from the indigenous microbiota. Dominance of the *L. plantarum* culture also seems to translate into a reduce bloater index under the conditions of the experiment in closed jars (Table 5). In other words, the inclusion of *L. brevis* as part of the mixed starter cultures was not better in terms of bloater index and fermentation chemistry, compared to higher inoculation level of the *L. plantarum* starter culture.

It is concluded that the utilization of an MDC-deficient starter culture for cucumber fermentations brined with $CaCl_2$ is not a critical factor to achieve a reduction in CO_2 production and/or bloater formation. The MDC-deficient cultures are ineffectively competing with the cucumber indigenous microbiota and have difficulties in completing a fermentation in the absence of NaCl, even if inoculated at levels as high as 10^7 CFU per ml. Suppression of microbial competition in cucumber fermentations brined with $CaCl_2$ by acidification to pH 4·6 does not represent an advantage for better performance of the MDC-deficient cultures. The use of a mixed starter culture of an MDC-deficient *L. plantarum* and *L. brevis* does not result in complete cucumber fermentations or the reduction of bloater index in cucumber fermentations brined with $CaCl_2$ in closed jars. The use of an MDC-deficient starter culture at high inoculation levels or of an MDC+ starter culture to lower inoculation levels in acidified cucumber fermentations brined with $CaCl_2$ represents a more effective strategy in the reduction of bloater index.

Acknowledgements

The authors thank Ms. Janet Hayes at the USDA-ARS, SAA Food Science Research Unit located in Raleigh, NC, for exceptional technical assistance and Ms. Sandra Parker for excellent administrative assistance. Ms. Yawen (Karen) Zhai was partially funded by Mount Olive Pickle Company, Mt. Olive, NC.

Conflict of Interest

No conflict of interest is to be declared.

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