



Growth of γ -Proteobacteria in Low Salt Cucumber Fermentation Is Prevented by Lactobacilli and the Cover Brine Ingredients

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ABSTRACT This study investigated the ability of γ -proteobacteria, indigenous to fresh cucumber, to grow in the expressed fruit juice (CJM) and fermentation. It was hypothesized that fresh cucumbers can support prolific growth of γ -proteobacteria but that the cover brine composition and acid production by the competing lactobacilli in the fermentation of the fruit act as inhibitory agents. The γ -proteobacteria proliferated in CJM with an average maximum growth rate (μ_{\max}) of 0.3895 ± 0.0929 and doubling time (T_d) of 1.885 ± 0.465 /h. A significant difference was found between the γ -proteobacteria μ_{\max} and T_d relative to *Lactiplantibacillus pentosus* LA0445 (0.2319 ± 0.019 ; 2.89/h) and *Levilactobacillus brevis* 7.2.43 (0.221 ± 0.015 ; 3.35/h) but not *Lactiplantibacillus plantarum* 3.2.8 (0.412 ± 0.119 ; 1.87/h). While inoculation level insignificantly altered the μ_{\max} and T_d of the bacteria tested; it impacted the length of lag and stationary phases for the lactobacilli. Unlike the lactobacilli, the γ -proteobacteria were inhibited in CJM supplemented with a low salt fermentation cover brine containing calcium chloride, acetic acid and potassium sorbate. The γ -proteobacteria, *P. agglomerans*, was unable to proliferate in cucumber fermentations brined with calcium chloride at a pH of 6.0 ± 0.1 and the population of *Enterobacteriaceae* was outcompeted by the lactobacilli within 36 h. Together these observations demonstrate that the prolific growth of γ -proteobacteria in CJM is not replicated in cucumber fermentation. While the γ -proteobacteria growth rate is faster than most lactobacilli in CJM, their growth in cucumber fermentation is prevented by the cover brine and the acid produced by the indigenous lactobacilli. Thus, the lactobacilli indigenous to cucumber and cover brine composition influence the safety and quality of fermented cucumbers.

IMPORTANCE While the abundance of specific γ -proteobacteria species varies among vegetable type, several harbor *Enterobacteriaceae* and *Pseudomonadaceae* that benefit the plant system. It is documented that such bacterial populations decrease in density early in vegetable fermentations. Consequently, it is assumed that they do not contribute to the quality of finished products. This study explored the viability of γ -proteobacteria in CJM, used as a model system, CJM supplemented with fermentation cover brine and cucumber fermentation, which are characterized by an extremely acidic endpoint pH (3.23 ± 0.17 ; $n = 391$). The data presented demonstrates that fresh cucumbers provide the nutrients needed by γ -proteobacteria to proliferate and reduce pH to 4.47 ± 0.12 . However, γ -proteobacteria are unable to proliferate in cucumber fermentation. Control of γ -proteobacteria in fermentations depends on the cover brine constituents and the indigenous competing lactobacilli. This knowledge is of importance when developing guidelines for the safe fermentation of vegetables, particularly with low salt.

KEYWORDS *Enterobacteriaceae*, fermentation, *Enterobacter*, lactobacilli, cucumber

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Knowledge of species level identity of the bacteria that are present in fresh vegetables has been augmented by the availability of high-throughput DNA sequencing technologies. It is currently understood that γ -proteobacteria are present in a variety of fresh vegetables among other microbes (1–9). The displacement or exacerbation of the abundance of species on fresh vegetables is often associated with decomposition, deterioration of quality, and improper post-harvest storage (1). The controlled imbalance of the microbial populations in fresh vegetables is called fermentation and is often dominated by indigenous microbes capable of producing and tolerating lactic acid, acetic acid and/or ethanol. Although it is known that *Enterobacteriaceae* and *Pseudomonadaceae* are particularly present on fresh and fermented vegetables (10–17) little is known about the long-term viability and functionalities in such habitat.

The population of viable γ -proteobacteria in fresh and fermented cucumbers is heavily represented by *Enterobacteriaceae*. Analysis of fresh cucumber homogenates using high-throughput sequencing technology revealed the presence of viable *Pantoea* in fresh cucumbers in a relative abundance of 9.25% (18). Viable *Pantoea*, *Citrobacter*, *Enterobacter*, and *Kluyvera* were detected in samples of a day-old cucumber fermentation. Plate counts for presumptive *Klebsiella* from 1-day-old fermentation cover brine samples reached 2.80 ± 0.36 log of CFU/mL in 30% of 9 commercial fermentation tanks of 40,000 L at pH 4.04 ± 0.15 . Additionally, total colony counts for presumptive *Enterobacteriaceae* present in the fermentation on days 1 and 7 were 3.74 ± 1.2 and 2.4 log CFU/mL, respectively. Colonies of enteric bacteria isolated from Violet, Red Bile agar supplemented with 1% glucose (VRBG) agar plates inoculated with fresh cucumber slurries and cucumber fermentation cover brine samples collected on day 1 and 3 were identified as *Pantoea*, *Citrobacter*, *Serratia*, *Providencia*, *Leclercia*, *Enterobacter* and *Kluyvera*. Other γ -proteobacteria found in fresh and fermenting cucumbers include *Ochrobactrum pseudogrignonense*, *Pseudomonas* sp., *Providencia rettgeri* and *Stenotrophomonas maltophilia*. However, such bacterial population consistently declines to undetectable levels in commercial cucumber fermentations on or before 10 days (18–20).

Limited knowledge exists regarding the proliferation or survival of *Enterobacteriaceae* and other γ -proteobacteria in vegetable fermentations. As for most microbes, it is generally understood that the presence of γ -proteobacteria in vegetable fermentations is modulated by temperature, water content, oxygen availability and salt concentration. Species of *Enterobacteriaceae* are known to produce enough carbon dioxide in cucumber fermentations to cause bloater defect (21–24). *Enterobacter cloacae* was isolated from gaseous commercial cucumber fermentations brined with 1 M (5.7%) to 3 M (17.4%) NaCl and found able to grow at 5 to 45°C, at a minimum pH of 4.25 (21). Evidently, γ -proteobacteria can influence the quality of fermented cucumbers and it is imperative to understand their growth characteristics in vegetable fermentations.

We aimed at characterizing the growth of γ -proteobacteria in cucumber juice medium (CJM), used as a model system for cucumber fermentations, and CJM supplemented with a low salt fermentation cover brine. Observations made from both media were compared to the growth and metabolic responses of lactobacilli that prevail in natural cucumber fermentations. Natural cucumber fermentations were inoculated with *Pantoea agglomerans*, a bacterium able to reach high cell densities in CJM, to observe persistence. Experimental CJM was initially prepared with juice expressed from size 1B, 2A/B and 3A/B fruits to identify an effect of its chemical composition on bacterial growth. It has been documented that the composition of pickling cucumbers varies with fruit size (25). Size 3 fruits (39 to 51 mm in diameter) contain less malic acid, more glucose and fructose, lower pH, less buffer capacity and less dry matter content than the smaller sizes 2 (27 to 38 mm in diameter) and 1 (<27 mm in diameter). The effect of varied inoculation levels on the growth of γ -proteobacteria and lactobacilli in CJM was also evaluated to understand potential variations in the densities of such populations as a function of their natural incidence in fresh cucumbers and potentials for bacterial competition. CJM was supplemented with a cover brine representative of low salt vegetable fermentations given that such bioconversion is known to support an

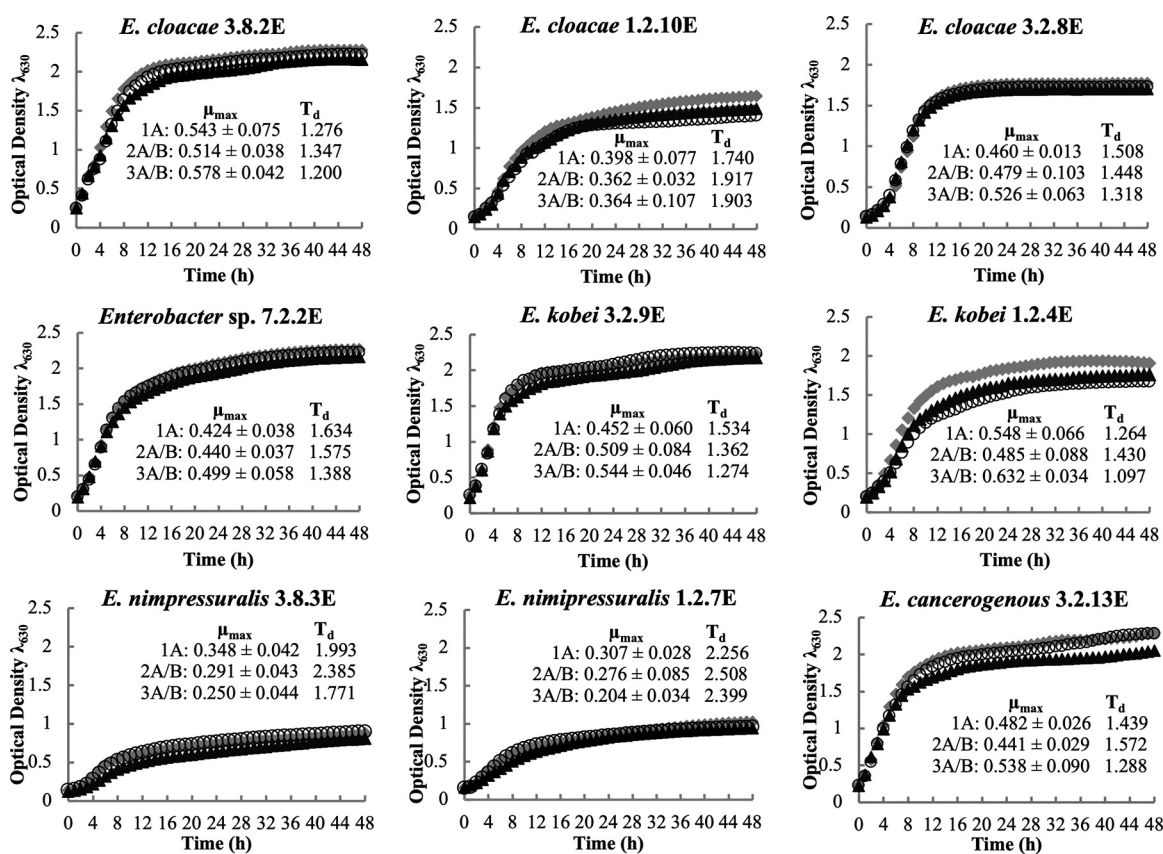


FIG 1 Growth of *Enterobacter* spp. in CJM prepared with juice expressed from size 1B (◆), 2A/B (●) and 3A/B (▲) fruits that had an initial pH of 5.82, 5.59, and 5.30, respectively. μ_{max} and T_d are shown within each panel by fruit size. The bacteria were inoculated into two independent lots of each CJM from independent pure cultures. No significant differences were calculated across CJM prepared with juice expressed from varied size cucumbers. Optical densities between 49 and 72 h remained at the same levels reported for 48 h.

accelerated acidification and decline of the population of *Enterobacteriaceae* (26, 27). We hypothesized that fresh cucumbers could support prolific growth of γ -proteobacteria but that the cover brine composition and acid production by the competing lactobacilli in the fermentation of the fruit act as inhibitory agents. The data suggest that most bacterial species can proliferate in CJM, the inoculation level of lactobacilli, but not of γ -proteobacteria, affects the length of lag and stationary phases of growth and that the cover brine constituents and certain lactobacilli can prevent the growth of most γ -proteobacteria in the bioconversion.

RESULTS

Growth of γ -proteobacteria in cucumber juice expressed from fruits of size 1, 2, and 3. Fig. 1 to 3 show there is insubstantial differences in growth rates and generation times of γ -proteobacteria in cucumber juice expressed from size 1A, 2A/B and 3A/B cucumbers. Although, *Enterobacteriaceae* grew in cucumber juice and produced enough gas to cause apparent effervescence (data not shown) they generally presented a maximum growth rate (μ_{max}) of 0.3948 ± 0.0967 and a generation time (T_d) of $1.875 \pm 0.505/h$, regardless of fruit size, where *Enterobacter* species were at the minimal end of the range with a μ_{max} and T_d of 0.441 ± 0.109 and $1.670 \pm 0.557/h$, respectively. *C. freundii* 3.2.3E was unique in presenting a biphasic growth curve, which was accompanied by a slimy culture supernatant resembling that characteristic of exopolysaccharide production (Fig. 2). Insignificant differences were observed among the growth curves obtained for *Enterobacteriaceae* compared to the other γ -proteobacteria tested, which presented an average μ_{max} of 0.3845 ± 0.0892 and T_d of $1.894 \pm 0.424/h$. Apparent differences were observed in the maximum cell densities reached by the

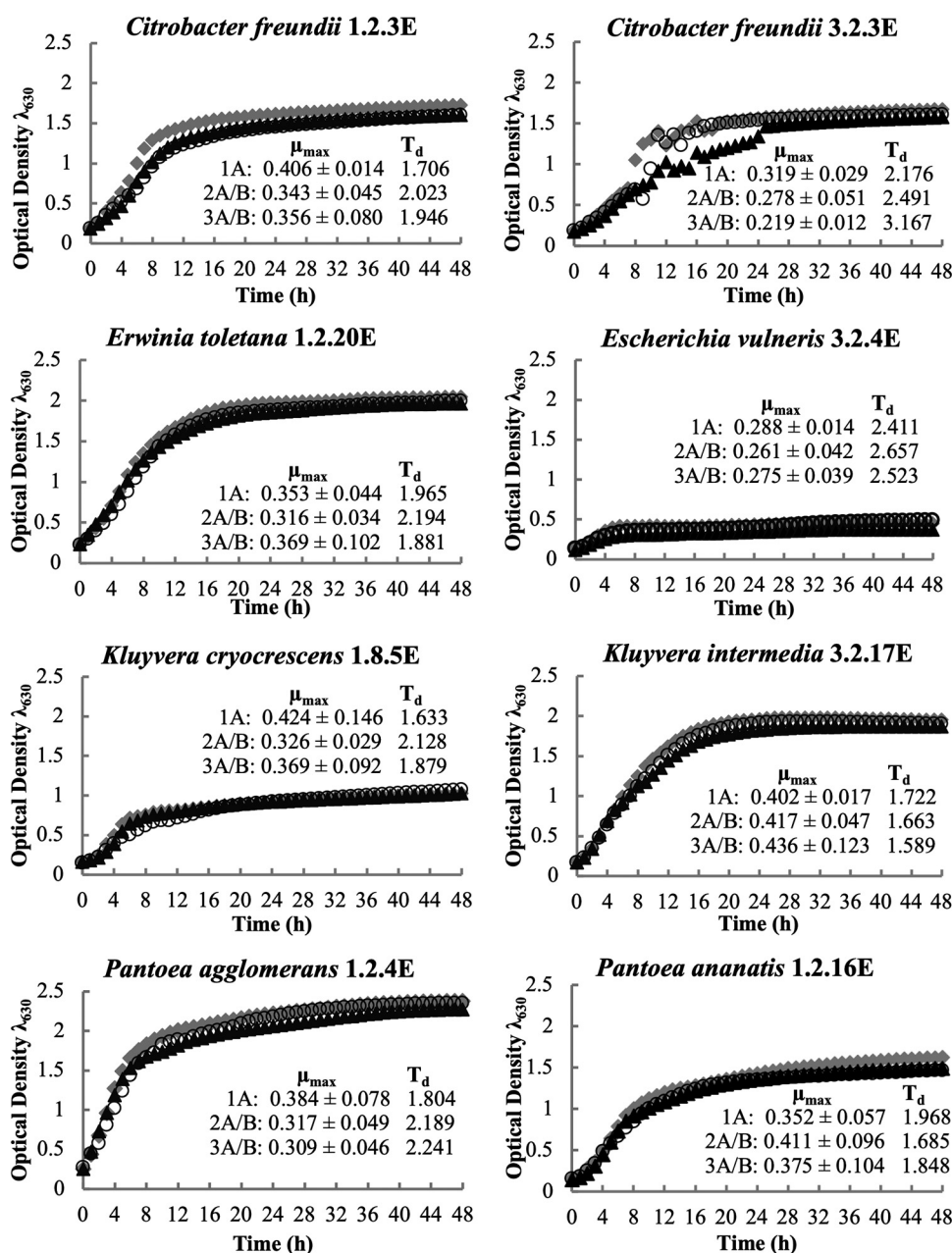


FIG 2 Growth of *Enterobacteriaceae* other than *Enterobacter* spp. in CJM prepared with juice expressed from size 1A (◆), 2A/B (●) and 3A/B (▲) fruits that had an initial pH of 5.82, 5.59, and 5.30, respectively. Maximum specific growth rate (μ_{max}) and generation time ($T_{d,max}$) are shown within each panel by fruit size. The bacteria were inoculated into two independent lots of each CJM from independent pure cultures. No significant differences were calculated across CJM prepared with juice expressed from varied size cucumbers. Optical densities between 49 and 72 h remained at the same levels reported for 48 h.

varied cultures with most optical densities at λ_{630nm} measuring between 1.00 and 2.20 and three outliers falling between 0.377 and 0.783 (Fig. 1 to 3).

The γ -proteobacteria could be separated into two main clusters based on endpoint pH of cultures in CJM prepared from fruits of varied sizes (Fig. 4). One cluster ($n = 19$) presented an endpoint pH of 4.47 ± 0.12 across CJM prepared with fruits of the three different sizes. It is estimated that such group of γ -proteobacteria removed about 50% of the intrinsic glucose, fructose, malic acid and succinic acid from the media. A fraction of the substrates utilized were converted to 13.93 ± 7.73 mM lactic acid and 32.60 ± 8.14 mM acetic acid and some cultures ($n = 6$) also produced ethanol

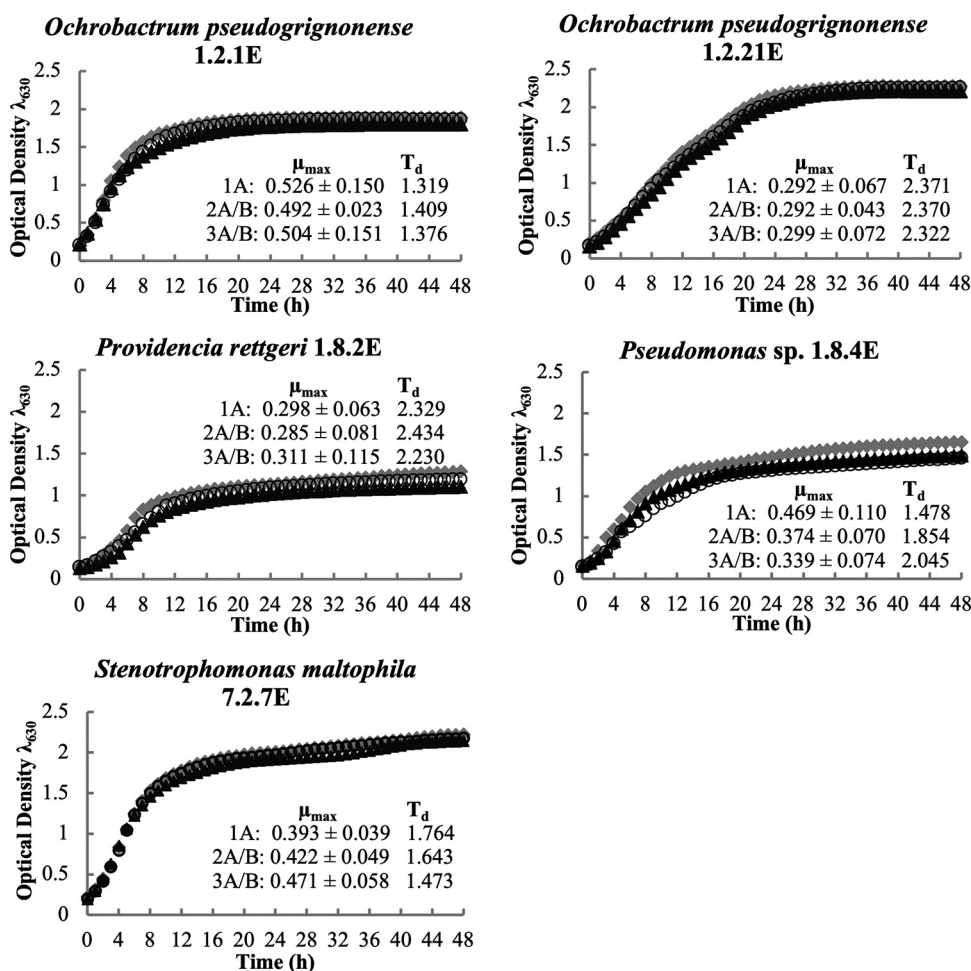


FIG 3 Growth of bacteria other than *Enterobacteriaceae* in CJM prepared with juice expressed from size 1B (◆), 2A/B (●) and 3A/B (▲) fruits that had an initial pH of 5.82, 5.59, and 5.30, respectively. μ_{\max} and T_d are shown within each panel by fruit size. The bacteria were inoculated into two independent lots of each CJM from independent pure cultures. No significant differences were calculated across CJM prepared with juice expressed from varied size cucumbers. Optical densities between 49 and 72 h remained at the same levels reported for 48 h.

(16.07 ± 1.59 mM). The second cluster had an average endpoint pH of 5.35 ± 0.19 across CJM prepared with fruits of varied sizes, a value that was near that of the not inoculated controls at 5.57 ± 0.30 (Fig. 4). This second cluster was composed by *E. cloacae* 3.2.8E and 3.8.2E, *L. adecarboxylata* 7.8.20E, *E. cancerogenus* 3.2.13E and *E. kobei* 3.2.9E (Fig. 4). It is estimated that these group of *Enterobacteriaceae* removed 70% of the intrinsic substrates which resulted in the formation of 29.07 ± 6.52 mM acetic acid. These group of five *Enterobacteriaceae* did not produce lactic acid or ethanol.

Effect of inoculation level on the growth of γ -proteobacteria in CJM. A subsample composed of five *Enterobacteriaceae* that grew to the highest cell densities in CJM derived from varied size fruits were chosen to evaluate the effect of inoculation level on growth. Statistically insignificant differences were observed among the growth curves and μ_{\max} of the chosen *Enterobacteriaceae* (Fig. 5). Monitoring bacterial growth by measuring optical density limits the ability to precisely determine the extent of lag phase; however, the data suggest that there was up to a 5 h difference in the beginning and ending of the logarithmic phase as a function of inoculation level.

Growth of certain lactobacilli in CJM prepared with juice expressed from cucumbers of varied sizes. No significant difference was calculated for growth curves and the deriving μ_{\max} and T_d obtained from *Lb. pentosus* LA0445 (0.2319 ± 0.019 ; 2.89/h), *Lb. plantarum* 3.2.8 (0.412 ± 0.119 ; 1.87/h) and *Lev. brevis* 7.2.43 (0.221 ± 0.015 ; 3.35/h) cultures in CJM as a function of cucumber size (Fig. 6). A significant difference

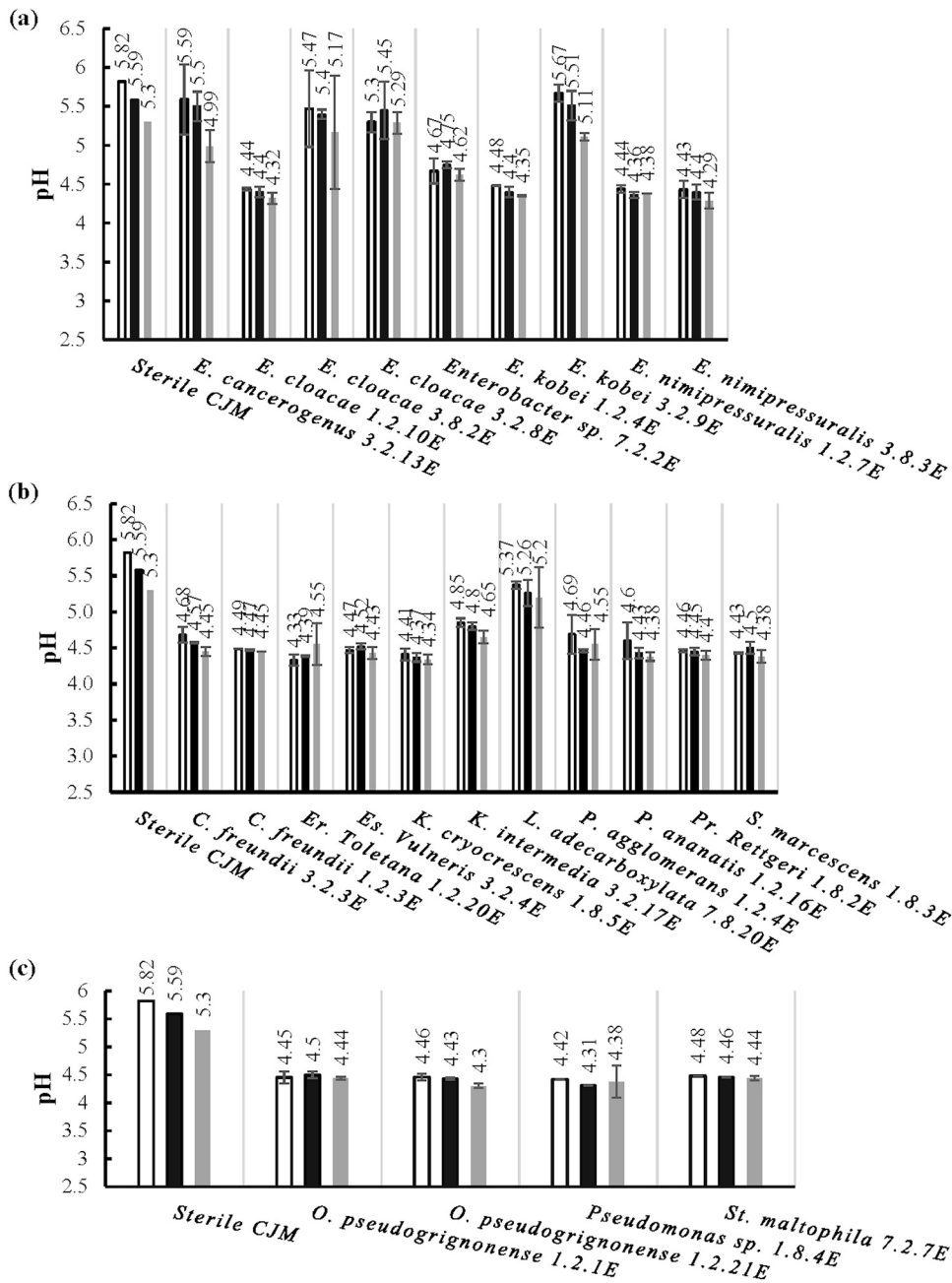


FIG 4 Endpoint pH of *Enterobacter* species (a), other *Enterobacteriaceae* (b) and other γ -proteobacteria (c) cultures in cucumber juice expressed from size 1 (\square), 2 (\blacksquare) and 3 (gray bars) fruits. Values shown are the average and standard deviation of independent triplicates.

was calculated for the growth curves corresponding to *Lb. pentosus* LA0445 compared to those of *Lb. plantarum* 3.2.8, but not to those of *Lev. brevis* 7.2.43. The μ_{max} and T_d of *Lb. pentosus* LA0445 was not significantly different from that of *Lev. brevis* 7.2.43, but it was different from that of *Lb. plantarum* 3.2.8 (Fig. 6). A significant difference was also calculated for the μ_{max} and T_d of γ -proteobacteria relative to *Lb. pentosus* LA0445 and *Lev. brevis* 7.2.43, but not to the robust starter culture *Lb. plantarum* 3.2.8.

Growth of certain lactobacilli in CJM as affected by inoculation level. Apparent differences in the beginning and ending of the logarithmic phase of the lactobacilli, *Lb. pentosus* LA0445, *Lb. plantarum* 3.2.8 and *Lev. brevis* 7.2.43, were observed in CJM inoculated at varied levels (Fig. 6). Higher inoculation levels induced an earlier start of

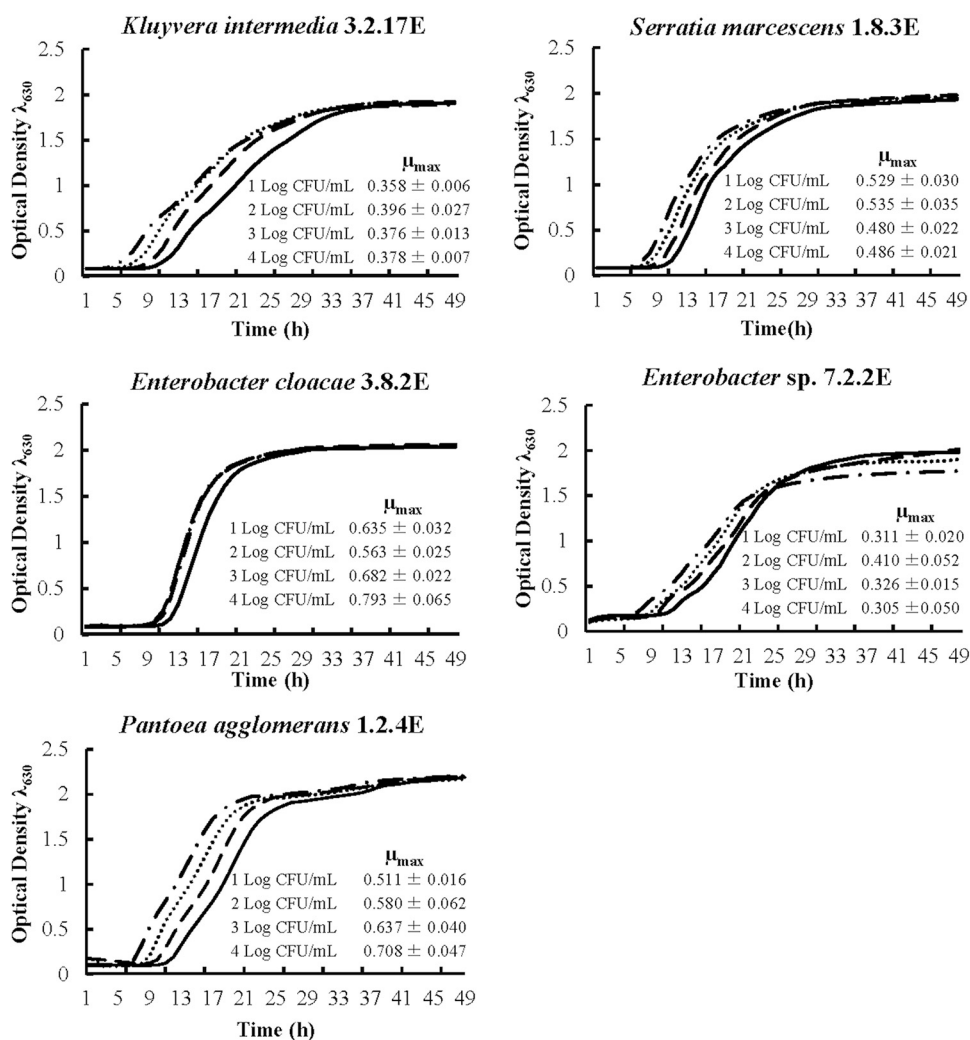


FIG 5 Growth of selected *Enterobacteriaceae* in CJM at 30°C in response to inoculation at 1 (—), 2 (---), 3 (···), and 4 (-.-) log CFU/mL. No significant differences were calculated across inoculation levels.

growth. A difference of up to 16 h could be observed between the start and end of the logarithmic phase in cultures inoculated at 1 log CFU/mL compared to 6 log CFU/mL (Fig. 6). The statistical analysis indicated no significant difference exist among growth curves representing *Lb. pentosus* LA0445 cultures initiated with the varied inoculation levels, except for the inoculation at 1 log CFU/mL relative to all other inoculation levels (Fig. 6). Growth of *Lb. pentosus* LA0445 from 1 log CFU/mL was also characterized by a reduced cell density in stationary phase (Fig. 6). For *Lb. plantarum* 3.2.8 the statistical test calculated a significant difference for inoculation to 1 and 2 log CFU/mL relative to 3, 4 and 5 log CFU/mL; while inoculation to 6 log CFU/mL was significantly different from inoculation at 1, 2, and 3 log CFU/mL (Fig. 6). A significant difference was calculated for *Lev. brevis* 7.2.43 growth curves inoculated at varied levels except for inoculation to 1 log CFU/mL relative to 2 log CFU/mL, and 5 log CFU/mL compared to 6 log CFU/mL (Fig. 6). No significant differences were calculated for the μ_{\max} .

Inhibition of *Enterobacteriaceae* in CJM by the fermentation cover brine components. The inoculation of a selected group of five *Enterobacteriaceae* individually in CJM supplemented with 6 mM potassium sorbate and 55 mM acetic acid, to adjust the initial pH to 5.2, resulted in the reduction in colony counts to below the limit of detection, no sugar utilization, and no acid production (Table 1). The five isolates selected for this experiment represent three genera and grew to high cell densities in the nonsupplemented CJM. On the contrary the inoculation of *Lb. plantarum* 3.2.8, *Lb.*

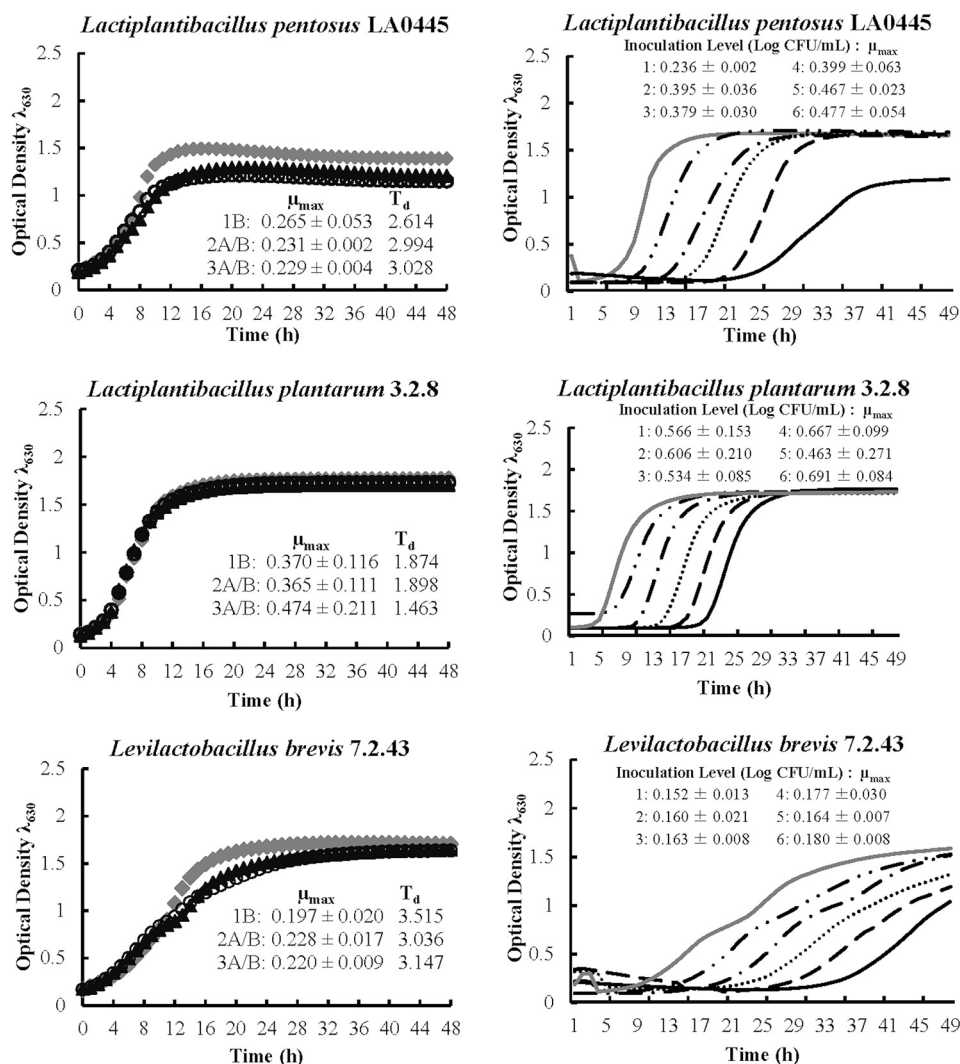


FIG 6 Left panels show the trends of optical density measured from lactobacilli cultures in CJM prepared with juice expressed from size 1B (◆), 2A/B (●) and 3A/B (▲) fruits that had an initial pH of 5.82, 5.59, and 5.30, respectively. No significant differences were calculated across CJM prepared with juice expressed from varied size cucumbers. μ_{max} and T_d are shown within each panel by fruit size. The panels to the right show trends in optical density for the *Enterobacteriaceae* cultures in CJM at 30°C in response to inoculation at 1 (—), 2 (- -), 3 (· · ·), 4 (- · -), 5 (- · -), and 6 (- · -) log CFU/mL. Statistical differences among inoculation level are described in the text. The bacteria were inoculated into two independent lots of each CJM from independent pure cultures.

pentosus LA0445 and *Lev. brevis* 7.2.43 in the same medium resulted in an increase in colony counts above 8.05 ± 0.18 log CFU/mL, the utilization of malic acid and most of the sugars, and the formation of lactic acid (Table 1). The heterofermentative bacterium, *Lev. brevis* 7.2.43 produced about 20 mM acetic acid and 47 mM lactic acid. The facultative heterofermentors, *Lb. plantarum* 3.2.8 and *Lb. pentosus* LA0445 produced more than 105 mM lactic acid.

Viability of *Enterobacteriaceae* in cucumber fermentations. Colony counts for presumptive *Enterobacteriaceae* were at 4.2 ± 0.5 log CFU/mL in cucumber fermentations after 18 h of incubation and declined after 36 h regardless of inoculation treatment (Fig. 7). The population of presumptive lactobacilli increased to 6.9 ± 0.3 log CFU/mL by 36 h in all the fermentations monitored. Colony counts for presumptive lactobacilli and yeasts and molds were not significantly different between treatments and the control. The pH of all treatments was at 5.9 to 6.0 and 4.5 to 5.1 after 18 and 36 h of incubation, respectively, likely as the result of mild acid production (Fig. 7). The fermentation biochemistry identified the utilization of about 47 mM sugars (glucose and

TABLE 1 Profile of CJM supplemented with 20.2 mM Ca (OH)₂, 6 mM potassium sorbate, 80 mM CaCl₂ and no NaCl at pH 5.2 ± 0.05 and inoculated with certain *Enterobacteriaceae* or lactobacilli to 4 log CFU/mL^a

Bacterial culture identification	Colony counts (log CFU/mL)	Substrate concn (mM)			Products concn (mM)		
		Glucose	Fructose	Malic acid	Lactic acid	Acetic acid	Ethanol
Not Inoculated Control	TFTC	29.64 ± 1.95	32.27 ± 1.50	9.34 ± 0.41	BDL	54.62 ± 1.49	BDL
<i>Enterobacteriaceae</i> (3.2.17E, 1.8.3E, 3.8.2E, 7.2.2E, 1.2.4E)	TFTC	29.47 ± 1.50	31.33 ± 1.32	8.49 ± 0.30	BDL	50.84 ± 1.48	BDL
<i>Levilactobacillus brevis</i> 7.2.43	8.23 ± 0.24	2.41 ± 0.90	2.67 ± 0.94	BDL	46.71 ± 2.77	71.25 ± 1.03	BDL
<i>Lactiplantibacillus plantarum</i> 3.2.8	7.88 ± 0.21	BDL	2.67 ± 1.01	BDL	114.35 ± 2.07	57.51 ± 1.92	BDL
<i>Lactiplantibacillus pentosus</i> LA0445	8.04 ± 0.15	3.28 ± 1.70	4.44 ± 0.52	BDL	108.68 ± 1.06	58.91 ± 1.06	BDL

^aTFTC, too few to count; BDL, below detection level.

fructose) with the production of 13 mM lactic and acetic acids (Fig. 7). Glucose and fructose were utilized to 7.09 ± 2.56 mM and 5.05 ± 2.26 mM, respectively, by 18 h of fermentation and less than half of the sugars were utilized after 36 h. No ethanol or succinic acid were produced.

DISCUSSION

It is concluded that cucumber juice has all the essential nutrients needed by γ -proteobacteria to proliferate to cell densities like those typically reached by fermentative lactobacilli. The chemical differences that characterize cucumber juices expressed from fruits of varied sizes (25) insignificantly affect growth of γ -proteobacteria as measured by optical density trends of the culture medium, μ_{\max} and T_d . The data collected evidence that the growth rate of γ -proteobacteria in CJM can occur at the same rate as that of the lactobacilli, *Lb. pentosus* LA0445 and *Lev. brevis* 7.2.43, which are indigenous to cucumber fermentations. This observation suggests that the growth rate of γ -proteobacteria is not a limiting factor if they were to compete with lactobacilli indigenous to cucumbers. However, the growth rate of γ -proteobacteria in CJM was significantly different from that of the robust starter culture *Lb. plantarum* 3.2.8, which is unique in the ability to produce exopolysaccharide and dominate in commercial fermentations compared to other *Lb. plantarum* strains (28). Thus, a robust starter culture such as *Lb. plantarum* 3.2.8 could outcompete the γ -proteobacteria as well as other lactobacilli in cucumber fermentations. Further studies are needed to understand the influence of microbial interactions on the persistence of γ -proteobacteria in cucumber fermentations, including acid production by the lactobacilli among others.

Inoculation level of lactobacilli in CJM affected the initiation of the logarithmic and stationary phases of growth (Fig. 6), which suggests that the natural levels of such bacteria in fresh cucumbers influences the initiation of acidification and confirms that the use of starter cultures can effectively accelerate the onset of the fermentation. The rate and initiation of acid production could extent or reduce the persistence of γ -proteobacteria in a fermentation as such bacteria stop growing at pH 4.47 ± 0.12 (Fig. 4). While the γ -proteobacteria reduced the CJM pH to 4.47 ± 0.12 or 5.35 ± 0.19 by producing lactic and/or acetic acid, their survival to the end of a cucumber fermentation (3.23 ± 0.17 , $n = 391$) is unlikely. Additionally, prevention of the growth or persistence of γ -proteobacteria in cucumber fermentations by adjusting the initial pH to 4.5 or less, as suggested by Zhai and Pérez-Díaz (29) seems a plausible approach for controlling such population.

Five out of the twenty-four γ -proteobacteria cultures included in this study maintained a pH of 5.35 ± 0.19 after incubation instead of the most observed end of fermentation pH of 4.47 ± 0.12 (Fig. 4). An apparent key difference among the five cultures that maintained a higher pH was the inability to produce lactic acid. However, inspection of the metabolic pathways published in the KEGG Genome Database for the 24 bacterial species included in this study suggest the presence of a functional Citric Acid Cycle and the metabolic potential to convert pyruvate to lactic acid, acetic acid, succinic acid and ethanol. Acetic acid could be converted to ethanol via acetyl-CoA and acetaldehyde or by generating acetyl-

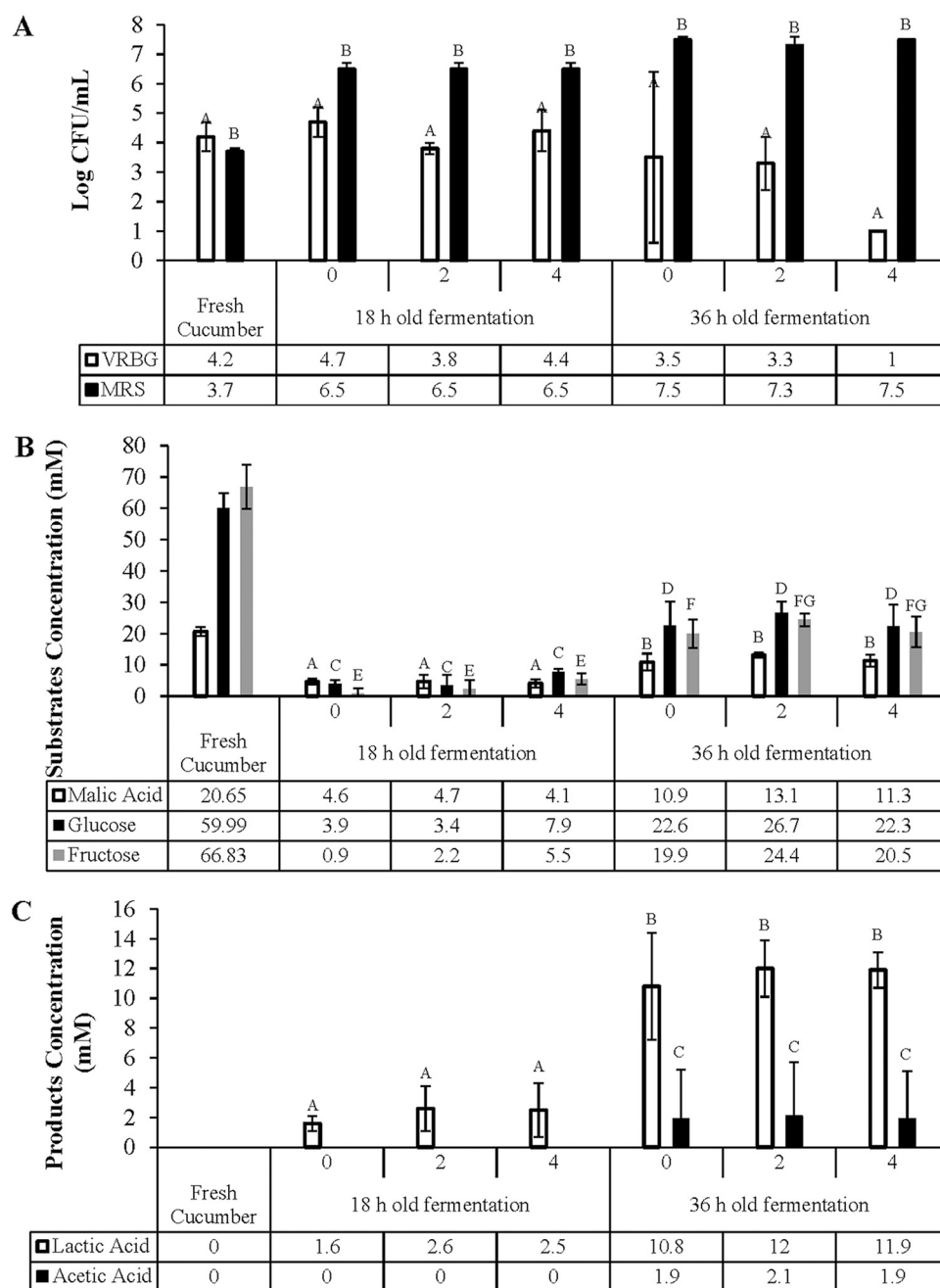


FIG 7 Colony counts for presumptive *Enterobacteriaceae* (□) and lactobacilli (■) from VRBG and MRS, respectively, from cucumber slurry samples collected from 18 h and 36 h old fermentations that had been inoculated with *Pantoea agglomerans* 1.2.4E (A). Concentrations of the fermentation substrates (B): malic acid (□), glucose (■) and fructose (gray bars); and the products formed (C) such as lactic acid (□) and acetic acid (■) measured from blended cucumbers collected at the same time points are also shown. The data presented represent the average and standard deviation of independent triplicates with three lots of size 2B (1.25 to 1.5 in diameter) cucumbers. Levels not connected by the same letter for a given variable are significantly different. No ethanol production was observed. Yeast and mold colony counts from YMA remained below detectable levels.

phosphate, and consequently ATP by substrate level phosphorylation. These observations suggest that production of acetic acid could be energetically advantageous for those γ -proteobacteria that grew to lower cell densities.

A key difference among the seven γ -proteobacteria that produced ethanol in CJM and the 13 species that did not is the presence of the coding gene for enzyme 1.2.1.3. Such enzyme is an aldehyde dehydrogenase that catalyzes the conversion of

acetaldehyde to ethanol with the regeneration of the cofactor NADH. *St. maltophilia* has an annotated putative aldehyde dehydrogenase (1.2.1.3), did not produce ethanol and is putatively unable to produce acetyl-phosphate. Consequently, the production of ethanol, which was undetected in the *St. maltophilia* CJM culture, may not be energetically advantageous for the species. Further studies are needed to fully understand the metabolic activities of γ -proteobacteria in cucumber fermentations.

A practice commonly used in the pickling industry to control for undesired microbial growth and obtain high quality fermented products is the supplementation of cover brines with acetic acid in the form of vinegar and potassium sorbate (19, 30). Thus, in this study experimental CJM was supplemented with acetic acid and potassium sorbate to observe the effect on the viability of γ -proteobacteria. In such experiment, calcium chloride was the only salt added in the cover brine as done for low salt cucumber fermentation (31). In addition to the antimicrobials and the calcium salt, the cover brine contained calcium hydroxide, which when combined with acetic acid acts as a buffer (32). The composition of the low salt cover brine was designed to control the initial fermentation pH to 5.2 ± 0.2 and still provide enough buffer for the bioconversion to proceed to completion (29). Such pH was additionally expected to enable growth of *Enterobacteriaceae* (33). Data show that the five *Enterobacteriaceae* individually inoculated in the CJM supplemented with the low salt cover brine were unable to maintain viability during incubation for 48 h (Table 1). The fact that the same conditions supported the growth of lactobacilli to 8.05 ± 0.18 log CFU/mL suggest that the *Enterobacteriaceae* significantly differ from fermentative bacteria in how they respond to acidic pH and a preservative such as potassium sorbate.

Enterobacteriaceae are known to tolerate sodium chloride and acidic pH. *Serratia marcescens* and an *Enterobacter* sp. were found to tolerate 1 M (6%) and 1.4 M (8%) sodium chloride, respectively, in Trypticase soy broth at a pH of 4.2 (34). Proliferation of *Enterobacteriaceae* from 6.6 ± 0.1 to 8.1 ± 0.1 log CFU/mL was observed in shredded cucumber fermentations brined with 0.9 M (5%) sodium chloride, as the only cover brine ingredient, at the suboptimal growth temperature of 23°C and a pH of about 6.5 (35). In such study, the *Enterobacteriaceae* population remained viable for up to 10 days, likely due to the delay in acid production to reduce the pH below 4.0. *Pseudomonas*, *Serratia*, *Pantoea*, *Erwinia*, *Citrobacter* and *Enterobacter*, among other bacteria were detected in fermentation cover brine samples, containing 0.9 M (5%) sodium chloride, after 6 months of storage. A reduction on the *Enterobacteriaceae* population was also observed by Świder and others (35) in parallel shredded cucumber fermentations brined with 90 and 270 mM (0.5 and 1.5%) sodium chloride as the sole cover brine ingredient. McDonald, Fleming and Daeschel (19) also detected viable *Enterobacteriaceae* in cucumber fermentations brined with 0.77 M (4.5%) sodium chloride for 3 days at 30°C. The colony counts for *Enterobacteriaceae* declined in such fermentations by about two log CFU/mL between days 3 and 5. Addition of 26.8 mM acetic acid to cucumber fermentation cover brines containing 0.77 M (4.5%) sodium chloride and the naturally produced lactic acid eradicated the natural *Enterobacteriaceae* populations in the batches within the first 5 days post-packing (19). Similar observations were reported by McMurtrie, Johanningsmeier, Price and Breidt (36). In such study the colony count for *Enterobacteriaceae* in the fermentation cover brines reached undetectable levels by day 3 in the presence of 25 mM acetic acid. Evidently, the salt and acid content in cucumber fermentation compromises the ability of *Enterobacteriaceae* to survive beyond 5 days in most cases.

It is speculated that the decline in the *Enterobacteriaceae* population in the CJM supplemented with a low salt cover brine was accelerated relative to the 3- and 5-day periods observed by others because of the concomitant presence of both acetic and sorbic acid in the medium. Thomas, Wimpenny and Davis (37) observed that *Escherichia coli* O157:H7 strain E32511 is sensitive to sorbic acid at varied acidic pH in BHI when incubated at temperatures between 20 and 35°C. Sorbic acid is a more effective antimicrobial compared to acetic acid at 30°C against *Escherichia coli* O157:H7 (38). About 6.9 mM sorbic acid can induce a 5 log-reduction of *E. coli* O157:H7 under conditions for which 393.6 mM acetic acid are needed for the same purpose. It is concluded that the supplementation of low salt

cucumber fermentation with 6.0 mM sorbic acid enhances safety by inducing a quicker decline of the γ -proteobacteria population, particularly *Enterobacteriaceae*.

P. agglomerans 1.2.4E, the γ -proteobacteria that reached the highest maximum cell density when inoculated in CJM was inoculated in laboratory scale fermentations brined with low salt and packed in closed jars to evaluate viability. The initial fermentation pH was maintained at 6.0 ± 0.2 , by eliminating acetic acid from the cover brine, to create favorable conditions for growth of *P. agglomerans* 1.2.4E. For the same reason, the preservative potassium sorbate, was eliminated from the cover brine formulation. Cucumber fermentations inoculated with *P. agglomerans* 1.2.4E to 2 or 4 log CFU/mL were not significantly changed compared to the not inoculated controls as evidenced by colony counts, the fermentation biochemistry and changes in pH (Fig. 7). By the 18 h mark glucose, fructose and malic acid were still equilibrating in the system and minimum amounts of lactic acid had formed (Fig. 7). Some acetic acid formed in all fermentations after 36 h of fermentation (Fig. 7), which could have derived from the metabolic activity of the inoculum, the natural *Enterobacteriaceae* or by an emerging community of heterofermentative lactobacilli. The colony counts from VRBG, representing the indigenous and inoculated *Enterobacteriaceae*, indicated that such population was viable past 18 h of fermentation while the fruits and the cover brine were equilibrating. During equilibration, the initial numbers of the indigenous microbial load, including *Enterobacteriaceae*, are likely diluted and community structures disrupted. The reduction of colony counts from VRBG by 36 h of incubation coincided with an increase in colony counts from MRS agar and a small increase in acid concentration to about 13 mM (Fig. 7). This observation evidences the superior ability of lactobacilli to establish themselves in a fermentation and the inability of *Enterobacteriaceae* to recuperate from the insult of the brining step. It is concluded that although *Enterobacteriaceae* persist in cucumber fermentations initially, the population is outcompeted by fermentative lactobacilli and die off within the first 36 h of the bioconversion in the absence of sodium chloride, acetic acid and potassium sorbate at 30°C. Data presented by others and discussed above agree with our conclusion and extends the survival window for up to 5 days. In another study by Zhai and Pérez-Díaz (24), it was observed that *Pantoea* is the only *Enterobacteriaceae* genus capable of surviving in low salt cucumber fermentations passed day 5 and that the use of a *Lb. pentosus* stater culture expands the spectrum to *Pseudomonas* sp. A low abundance of *Pantoea* and *Pseudomonas* species was also detected during the first week of fermentations brined with 100 mM calcium chloride and 6 mM potassium sorbate or 1.0 M sodium chloride alone (39). In such study *Citrobacter* was also detected in commercial cucumber fermentations brined with 100 mM calcium chloride and 6 mM potassium sorbate on day 6 but not beyond such time. Evidently, the natural production of lactic acid in cucumber fermentation, which occurs faster in low salt compared to fermentations brined with 1.0 M sodium chloride, adds a hurdle for the survival of γ -proteobacteria (39).

There is a historical instance where *Enterobacteriaceae*, particularly *Enterobacter* spp. were implicated in the spoilage of commercial fermentations characterized by the production of gas and increased incidence of bloater defect (21, 22). The observations made in this study evidence the limited ability of γ -proteobacteria to proliferate at the start of cucumber fermentations and the reduction of the population to below detection levels. However, it remains imperative to determine if surviving cells could impact the quality of products from the metabolic activities occurring early in the bioconversion and if the cells can persist in the system until conditions are permissive of growth during long term storage.

In summary, the diverse population of γ -proteobacteria indigenously present in cucumbers proliferates in the deriving juice to high cell densities regardless of the size of the fruits the juice is expressed from. The growth of γ -proteobacteria in CJM parallels that of most fermentative lactobacilli indigenous to cucumbers. Supplementation of CJM with a fermentation cover brine containing the preservative, potassium sorbate, and acetic acid negatively affects the ability of γ -proteobacteria to proliferate, unlike

that of lactobacilli. *Enterobacteriaceae* are unable to proliferate in cucumber fermentations brined without potassium sorbate and acetic acid with an initial pH of 6.0 ± 0.2 and are outcompeted by fermentative lactobacilli within the first 36 h of incubation. It is thus concluded that a cover brine formulation can be effectively used to control for undesired γ -proteobacteria in cucumber fermentations and that in its absence the safety of fermented products could be compromised. Additionally, it is relevant to secure a substantial number of viable lactobacilli during the early cucumber fermentation stage to ensure the control of undesired γ -proteobacteria.

MATERIALS AND METHODS

Preparation of cucumber juice medium. Fresh size 1B, 2A/B, and 3A/B cucumber fruits were procured from the local retail market (Raleigh, NC, USA) and separated into three size categories based on Lu, Fleming and McFeeters (25). Size 1, 2 and 3 cucumbers were equal or less than 2.69 cm, between 2.70 and 3.89 cm, and more than 3.9 cm in diameter, respectively. The A, B and C designation within sizes was defined by the length of the fruit. The size of the fruit was visually estimated during purchase and confirmed in the laboratory by cutting a cross-section from the thickest part of a fruit and measuring the diameter. A Juiceman, Jr. JM-1C (Salton, Beachwood, OH, USA) automatic juice extractor was used to prepare the CJM from individual lots of fresh cucumbers. The cucumber juices were stored in 1 L autoclavable glass bottles at -20°C until further processed. Three independent lots of cucumber of each size were used for preparing CJM. Prior to preparing the CJM, the juices were thawed and sieved with an 8" x 8" section of 100% cotton cheesecloth (grade number 90, 44 x 36 threads/inch, Cartridge Plus, Inc., Riva, MD, USA) to remove particulate. Portions of 500 mL of the strained juices were spun at 3,824 g for 30 to 60 min using a bucket rotor on an Eppendorf Centrifuge Model 5810 (Hamburg, Germany), and filter-sterilized using a 0.45- and 0.22-micron filtration units sequentially (Nalgene-Rapid Flow, Thermo Scientific, Santa Clara, CA, USA). The pH of each cucumber juice was measured using an Accumet Research 25 pH meter (Fisher Scientific, Carlsbad, CA, USA) equipped with a Gel-Filled Pencil-Thin pH Combination Electrode (Accumet, Fisher Scientific). The sterilized CJM was stored at 4°C until used.

Microbial cultures and preparation of inocula. Twenty-five γ -proteobacteria cultures isolated from days 1 and 3 of industrial cucumber fermentations were used, including 20 *Enterobacteriaceae*, two *Ochrobactrum pseudogrignonense* strains, a *Pseudomonas* sp., a *Providencia rettgeri*, and a *Stenotrophomonas maltophilia* (18). Specific strains used are described in Fig. 1 to 3. The cultures were transferred from frozen stocks to CJM. Independent triplicates of CJM cultures were incubated at 30°C under static conditions for 24 h. Cultures were pre-adapted in CJM for 24 h prior to experimentation. The inocula were suspended in a 0.85% NaCl solution prior to the inoculation of the experimental CJM, after centrifugation at 15,294 g for 10 min at 22°C (Eppendorf Centrifuge 5810R, Fisher Scientific, Fremont, CA, USA). The concentrations were estimated using McFarland Standards and confirmed by plating on Brain Heart Infusion (BHI) agar. Aseptically collected culture samples were serially diluted in 0.85% NaCl solution upon collection. *Enterobacteriaceae* were enumerated in BHI after incubating the plates at 30°C for 24 to 48 h. Spiral plating was done using an Eddy Jet 2W spiral plater (IUL Instruments, Barcelona, Spain) set for 50 μL depositions. Colonies were enumerated using a Flash & Go Automated Colony Counter (Neutec Group, Inc., Barcelona, Spain).

The lactobacilli strains used, *Lactiplantibacillus pentosus* LA0445, *Lactiplantibacillus plantarum* 3.2.8 and *Levilactobacillus brevis* 7.2.43, were isolated from commercial cucumber fermentations and chosen based on their prevalence in such habitat (40). The lactobacilli inocula were prepared as described above for the γ -proteobacteria except that BHI was substituted by Lactobacilli deMan, Rogosa and Sharpe (MRS) medium. The inoculation levels were controlled at 4 log CFU/mL unless otherwise stated.

Growth of *Enterobacteriaceae* and lactobacilli in CJM. A 96-well plate format was used to monitor bacterial growth by measuring absorbance at $\lambda_{630\text{nm}}$ using an ELx808 Absorbance Microplate Reader (BioTek, Winooski, VT, USA). The microplate reader uses a tungsten halogen light source and a photodiode detector to measure absorbance at preset time intervals, which was 1 h for this experiment. 20 μL of the inocula of an estimated concentration was added to 180 μL of CJM in a 200 μL well to achieve the desired inoculation level. The plate was incubated at 30°C for up to 72 h. The optical density at $\lambda_{630\text{nm}}$ was measured for each well every h, after shaking the plate for 10 sec, to obtain bacterial growth curves and determine the exponential and stationary phases of growth in CJM. Optical density data were plotted as a function of time and the curve fitted using a sigmoidal model in Microsoft Excel. The μ_{max} and T_d were calculated using the data corresponding to lag phase. Triplicate 96-well plates were prepared with three independent CJM lots and 3 independent inocula. Technical duplicates were run for each CJM lot.

pH measurements and analysis of metabolites by High Performance Liquid Chromatography (HPLC). Two hundred μL of each technical replicate of a given isolate were transferred from the 96-well plate into a sterile microcentrifuge tube. The combined culture samples were spun at 15,294 g for 10 min at 4°C (Brushless Microcentrifuge, Denville 260D, Denville Scientific, Inc., Holliston, MA, USA) to remove the cells. Supernatants were used to measure pH as described above and stored at -20°C for 24 h. The thawed supernatants were spun once again as described above and filtered sterilized using a 0.2-micron syringe filter (Pall Corporation Acrodiscs, Show Low, AZ, USA). Filtered samples were collected into HPLC glass vials (Agilent, Santa Clara, CA, USA) to determine the concentration of sugars, ethanol, and organic acids. The HPLC method described by McFeeters and Barish (41) was used with an Aminex 300 x 7.8 mm HPX-87H resin column (Bio-Rad Laboratories, Hercules, CA, USA). 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-visible detector (Shimadzu Corporation, Canby, OR, USA) 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 UV-visible 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 compared to the corresponding compounds on the standard curves at specific retention times using the LabSolutions Workstation (Shimadzu Corporation). Amounts reported for full strength CJM samples are considered estimates as evaporation occur during incubation of the 96-well plate reducing the total volume of the cultures and concentrating constituents.

Supplementation of CJM with cover brine. The cover brine contained 160.0 mM calcium chloride (CaCl_2) (Brenntag, Durham, NC, USA), 12.0 mM potassium sorbate (Sigma-Aldrich, St. Louis, MO, USA), 40.4 mM anhydrous calcium hydroxide $\text{Ca}(\text{OH})_2$ (Brenntag, Durham, NC, USA) and acetic acid in the form of vinegar (Fleischmann's Vinegar Company Inc., Cerritos, CA, USA) to adjust the pH to 5.20 ± 0.05 (29). The cover brine and CJM were mixed in a 50:50 (v:v) ratio diluting all constituents by 50%.

Cucumber fermentations inoculated with *Pantoea agglomerans* 1.2.4E. Three independent lots of fresh and washed cucumbers of about 1.9 Kg each, were fermented in closed and sterilized 3.8 L jars with a cover brine containing 25 mM CaCl_2 , 20.2 mM $\text{Ca}(\text{OH})_2$ and 2 mM allyl-isothiocyanate (AITC), at equilibrium. AITC was added in the cover brine to prevent the growth of yeasts indigenous to cucumber given that an inhibitory effect against bacteria indigenous to cucumbers is not known (42–45). The initial pH was adjusted to 6.0 ± 0.1 with hydrochloric acid (HCl) to exclude acetic acid from the medium given that some *Enterobacteriaceae* are sensitive to it (19). The amount of 3N HCl needed for pH adjustment were determined by titrating 200 mL of mixed samples containing 100 mL of blended cucumbers from the same experimental cucumber lot and 100 mL of the experimental cover brine. The amount of HCl to be added in the cover brine were extrapolated from the titration curve using its equation. The cucumbers and cover brine were packed in a 50:50 ratio (wt/wt). *Pantoea agglomerans* 1.2.4E was used for this experiment given that it reached the highest cell density when growing in CJM compared to all other γ -proteobacteria. *Pantoea agglomerans* 1.2.4E was inoculated in the jars after growing in CJM from frozen stocks and inoculated to 2 or 4 log of CFU/mL as two individual treatments to simulate the intrinsic levels in fresh cucumbers (18). The cucumbers packed in sterilized jars were closed with metal lug caps that had been submerged in boiling water for 15 sec to activate the plastisol and achieve a tight seal. The jars were incubated at 30°C and samples were collected after 18 and 36 h using aseptic techniques.

Chemical and microbiological analyses of fermentation samples. The cucumbers collected from jars at each sampling point were blended without the cover brine for 60 s at maximum speed using a Waring blender assembly (Waring Co., Torrington, CT, USA) and homogenized for 30 s using a Stomacher 400 (Tekmar Company). A homogenization bag equipped with a side filter of 250 μm porosity (Interscience Laboratories Inc.) was used to contain the blended cucumber slurries. Homogenate supernatants were used to measure pH and conduct HPLC analysis as described above. Homogenate samples were serially diluted in 0.85% NaCl solution upon collection using aseptic techniques for microbiological analysis. *Enterobacteriaceae* were enumerated in VRBG. All colony types were counted from VRBG plates. Colony counts for presumptive lactobacilli were determined by plating on 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. Spiral plating and colony enumeration were done as described above.

Statistical analysis. Significant differences among growth curves were determined by a Two Factor Anova Test with replication and a *post hoc* Bonferroni correction using Microsoft Excel. Significant difference among μ_{max} and T_d were determined using a Single Factor Anova with a T-test *post hoc* using Microsoft Excel. Significant difference among fermentation 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 and the interactions between treatments and sampling times were assessed. Statistically significant difference was determined at a P value ≤ 0.05 .

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We have no conflicts of interest to declare.

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