# Characterization of robust *Lactobacillus plantarum* and *Lactobacillus pentosus* starter cultures for environmentally friendly low-salt cucumber fermentations

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**Abstract:** Seven candidates for starter cultures for cucumber fermentations belonging to the *Lactobacillus pentosus* and *Lactobacillus plantarum* species were characterized based on physiological features desired for pickling. The isolates presented variable carbohydrate utilization profile on API® 50CHL test strips. The *L. pentosus* strains were unable to utilize D-xylose in MRS broth or the M medium. The lactobacilli were unable to produce histamine, tyramine, putrescine, and cadaverine in biogenic amine broth containing the necessary precursors. Production of D-lactic acid by the lactobacilli, detected enzymatically, was stimulated by growth in MRS broth as compared to cucumber juice medium (CJM). The lactobacilli utilized malic acid in the malate decarboxylase medium. Exopolyssacharide biosynthesis related genes were amplified from the lactobacilli. A sugar type-dependent-ropy phenotype was apparent for all the cultures tested in MRS and CJM. The genes associated with bacteriocin production were detected in the lactobacilli, but not the respective phenotypes. The antibiotic susceptibility profile of the lactobacilli mimics that of other *L. plantarum* starter cultures. It is concluded that the lactobacilli strains studied here are suitable starter cultures for cucumber fermentation.

**Keywords:** carbohydrate utilization, cucumber fermentation, exopolysaccharides, malic acid decarboxylation, starter culture

**Practical Application:** The availability of such starter cultures enables the implementation of low salt cucumber fermentations that can generate products with consistent biochemistry and microbiological profile.

## 1. INTRODUCTION

Utilization of starter cultures for food fermentation enables manufacturers to have some control on the growth of the indigenous microbiota, which often results in a consistent product with predictable characteristics (Holzapfel, 2002). Utilization of a starter cultures in vegetable fermentations enhances the production of lactic acid and organic volatiles, utilization of fermentable sugars, inhibition of undesirable bacteria such as *Enterobacteriaceae* and yeasts, increased bioavailability of vitamins, micronutrients, and essential amino acids, and firmness retention of finished products (Chang & Chang, 2010; Di Cagno et al., 2008).

Low salt cucumber fermentations represent microbiologically unstable systems that can benefit from the advantages associated with the utilization of starter cultures (Pérez-Díaz & McFeeters, 2011). Starter cultures designed for commercial cucumber fermentations that can meet Kosher requirements for vegetable products are scarcely available to date (Pérez-Díaz & McFeeters, 2011). An ideal starter culture for low salt cucumber fermentations would outcompete the indigenous fresh cucumber microbiota by rapidly acidifying the fruits, adapt to proliferate at temperatures ranging between 15 and 35 °C, preferably produce L-lactic acid and able to co-exist with other species/strains so that processors can effectively manage possible bacteriophage infections (Buckenhüskes, 1993; Daeschel & Fleming, 1984; Wouters, Grosu-Tudor, Zamfir, & De Vuyst, 2013). It is vital that the selected starter cultures convert most of the sugars to lactic acid only and are unable to decarboxylate malic acid to prevent bloater defect from the accumulation of carbon dioxide in the cucumber's mesocarp and endocarp (Daeschel, McFeeters, Fleming, Klaenhammer, & Sanozky, 1984; Etchells, Borg, & Bell, 1968). Homofermentation is also a desirable safety feature given that spoilage associated microbes can utilize acetic acid as an energy source and increase pH (Franco & Pérez-Díaz, 2012). Starter cultures of choice for cucumber fermentations should be Generally Recognized As Safe (GRAS), unable to produce biogenic amines and associated off-flavors (Di Cagno et al., 2008), unable to transmit antibiotic resistance genes (Clementi & Aquilanti, 2011), and ideally be able to produce antimicrobial compounds such as bacteriocins (De Vuyst, 2000; Rao et al., 2013).

While starter cultures are widely used by the dairy industry worldwide, this practice is less common in the commercial production of fermented vegetables. The majority of cucumber pickle processors worldwide currently rely on the indigenous microbiota and the application of cover brines with at least 5.6% sodium chloride (NaCl) to achieve high quality, stable, and safe fermentations. Therefore, starter cultures specifically selected for cucumber fermentations and low salt processing are not currently produced. In addition, the sources for alternative commercial lactic acid bacteria (LAB) starter cultures that are of vegetable fermentation origin are limited.

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This study characterizes six *L. pentosus* strains and one *L. plantarum* isolated from commercial cucumber fermentations (Pérez-Díaz et al., 2017, Anekella, 2020) based on the desirable physiological features for industrial starter cultures for cucumber fermentations. The six *L. pentosus* strains studied belong to RAPD-phylogenetic clusters B (*L. pentosus* 3.8.24), D (*L. pentosus* 3.8.24), and J (*L. pentosus* 1.2.13, 1.8.6, 1.8.9), which were associated with an ability to ferment cucumbers under various conditions of pH, temperature, and salt (Anekella, 2020). The *L. plantarum* strain 3.2.8 also tested expresses a ropy phenotype different from that generated by the *L. pentosus* strains (Anekella, 2020). *L. plantarum* 3.2.8 was isolated from a commercial cucumber fermentation, belongs to RAPD-phylogenetic cluster A and can ferment cucumber juice under various conditions, unlike 18 other *L. plantarum* isolated from the same habitat (Anekella, 2020). The physiological characteristics targeted in this study include carbohydrate utilization profile with emphasis on xylose utilization, ability to produce biogenic amines, exopolysaccharides and bacteriocins, antibiotic susceptibility profile, malic acid decarboxylation, and production of the D- and L-lactic acid isomers.

## 2. MATERIALS AND METHODS

### 2.1 Cultures, growth conditions, and DNA extraction

The cultures of LAB used in this study were obtained from the USDA-ARS, Food Science and Market Quality and Handling Research Unit, Culture Collection (Raleigh, NC, USA). The *L. pentosus* cultures 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, and 3.2.37, and *L. plantarum* 3.2.8 were isolated from commercial cucumber fermentations and selected among 243 other cultures for their ability to ferment cucumber juice under various conditions and for their RAPD-based genotype obtained as described by Anekella, 2020. The lactobacilli cultures used, including the *L. pentosus* listed above, *L. pentosus* LA0445, *L. plantarum* 3.2.8, WCSF1, ATCC14917 and 8041, *L. brevis* ATCC 14869 and ATCC 8287, *Leuconostoc mesenteroides* ATCC 8293, *Weissella paramesenteroides* ATCC 33313, and *Lactobacillus* 30a were transferred to deMan, Rogosa, and Sharpe plates (MRS; Cat No: 288130, BD Difco™, Ann Arbor, MI, USA) from frozen stocks, and incubated at 30 °C under aerobic conditions. *L. buchneri* ATCC4005, *L. johnsonii* NCK468, and *L. delbrueckii* NCK235 were transferred to MRS broth and incubated at 37 °C under anaerobiosis (Coy Anaerobic Chamber, Grass Lake, MI, USA). The *Candida etchelsii* Y095, *Issatchenkia occidentalis* Y0089, *Pichia manschurica* Y098, and *Zygosaccharomyces globiformis* Y0069, used as indicator yeasts due to their association with fermented cucumber spoilage, were propagated in Yeast and Mold agar (BD Difco™) (Bell & Etchells, 1952; Franco & Pérez-Díaz, 2012).

*L. delbrueckii* NCK235Genomic DNA was extracted from pure cultures using the MasterPure™ DNA purification kit (Cat No.: MCD85201, Epicentre, Madison, WI, USA) following the manufacturer's instructions. PCR reactions for gene amplification were performed using the PCR Master Mix (Cat No.: 166–5009EDU, BioRad, Hercules, CA, USA) using 10 μM stocks of the primers specified below (IDT Inc., San Diego, CA, USA), 1 μL of whole genomic DNA at 40 to 60 ng/μL. The PCR reaction volume was adjusted to 25 μL with nuclease free water (Cat No.: AM9937, Ambion®, Life Technologies, Grand Island, NY, USA). Magnesium chloride was added to the PCR mix as needed from a 10 mM stock (Cat No.: 42–303, Apex™, Genesee Scientific, San Diego, CA, USA). All the experiments were independently duplicated. All the nucleotide sequences obtained in this study were deposited in GenBank and accession IDs are provided below.

### 2.2 Carbohydrate utilization profile

An API® 50 CHL carbohydrate utilization kit (API®, BioMérieux, France) was used to evaluate the ability of the selected isolates to metabolize various carbon sources following the manufacturer's instructions. The isolates were grown overnight in MRS medium at 30 °C in 2 mL centrifuge tubes and spun at 2,655 × *g* for 3 min (Eppendorf Centrifuge Model 5810, Hamburg, Germany). Culture supernatants were decanted and cells were resuspended in phosphate buffer saline (PBS-pH 6.8). The cell suspensions used to inoculate the API® 50CHL strips were adjusted to an $OD_{600}$ of 0.1 (Thermo Spectronic Genesys20 Model 4001/4, Waltham, MA, USA). The strips were incubated aerobically for 48 hr at 30 °C and color changes were observed. The results were interpreted as per the manufacturer's guidelines and the sugar utilization pattern was submitted to the reference database APIWeb™ to obtain a matching profile and presumptive identification. All tests were independently duplicated.

### 2.3 D-Xylose utilization test and D-xylose isomerase (*xylA*) amplification

The D-xylose utilization assay was performed in MRS without sugar (MRS-N, Cat No.: L1021-06, US Biological, Salem, MA, USA) that was supplemented with 50 mM D-xylose (MRS-X) or 50 mM D-glucose (MRS-G) for the positive control. MRS medium without sugars (MRS-N) was used as negative control for growth. Changes in pH were recorded after 48 hr of incubation at 30 °C. *L. casei* ATCC 393 was used as negative control for its inability to utilize D-xylose (Posno et al., 1991). *L. pentosus* ATCC 8041 was used as the positive control. The cultures were also tested in the M-medium, specifically developed to observe the ability of LAB to utilize D-xylose (Chaillou, Pouwels, & Postma, 1999). Pure cultures were transferred to the M-medium supplemented with D-xylose (M-X), D-glucose (M-G), and no sugar (M-N; negative control) separately into 96-well plates and incubated in a plate reader at 37 °C (BioTek Instrument Inc., Winooski, VT, USA). Growth was monitored for 96 hr by optical density (600 nm). All tests were independently duplicated.

Genotypic detection of the xylose isomerase *xylA*, was performed with primers xylA1-F (5'-GACTGGCATG AAGGTACTATGG-3') and xylA1-R (5'-GCACCAACA CCACTTTGATAAG-3') with an expected amplicon size of 795 bp. Primers were designed using the PrimerQuest software (http://www.idtdna.com/primerquest/home/index) using the *L. pentosus* MD353 *xylA* sequence (Accession ID M57384) as the template. The PCR mix (Bio-Rad) was prepared as described above with the following amplification conditions: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, and a final extension cycle at 72 °C for 7 min PCR was performed in a Bio-Rad Real Time Thermal Cycler (Hercules, CA, USA).

### 2.4 Production of biogenic amines

The detection of putative genes encoding for enzymes involved in the production of biogenic amines was performed using multiplex and uniplex PCR as described by Coton and Coton (2005), de las Rivas, Marcobal, Carrascosa, and Muñoz (2006), and Marcobal, de las Rivas, Moreno-Arribas, and Muñoz (2005) using a minimum of 2 mM $MgCl_2$ for all the reactions. Genes *hdc, tdc, odc,*

and *ldc* encoding for histidine decarboxylase, tyrosine decarboxylase, ornithine decarboxylase, and lysine decarboxylase, respectively, were considered for this analysis. These enzymes are involved in biosynthesis of histamine, tyramine, putrescine, and cadaverine, respectively. *Lactobacillus* 30a was used as a positive control for the detection of *hdc, ldc*, and *odc*, and *L. brevis* ATCC 8287 for *tdc* detection (Coton & Coton, 2005; de las Rivas et al., 2006; Marcobal et al., 2005).

Phenotypic detection of biogenic amine production was performed using biogenic amine media plates as described by Landete, Pardo, and Ferrer (2007) containing single metabolic precursors. Four different sets of plates each with 1% of the following precursors were prepared: histidine-HCl, tyrosine, ornithine-HCl, or L-lysine (Sigma–Aldrich, St. Louis, MO, USA). Isolates of interest were adapted to the biogenic amine media by serially transferring them in the biogenic amine broth up to six generations and incubating at 30 °C for 24 hr between transfers. Cultures were then streaked on their respective biogenic amine agar plates, each containing a different precursor, and incubated for 48 hr at 30 °C. A color change from yellow to purple was designated as positive for biogenic amine production. All the tests were independently duplicated.

### 2.5 Production of D-/L-lactic acid

The concentrations of D- and L-lactic acid produced by the isolates in MRS and Cucumber Juice Media (CJM) supernatants were measured using the D-/L-lactic acid rapid assay kit (K/D-LATE, Megazyme, Wicklow, Ireland). CJM was prepared as described by Zhai and Pérez-Díaz (2020). Cultures were transferred from frozen stocks to MRS broth prior to inoculation of the experimental media and incubated at 30 °C overnight. The cultures were then transferred from MRS broth to the experimental MRS and CJM to a post-inoculation $OD_{600}$ of 0.1 and incubated for 48 and 72 hr, respectively, under static aerobic conditions. The D-and L-lactic acid concentrations were measured from the culture supernatants following the manufacturer's instructions. All the tests were independently duplicated and statistical analysis for significant difference was conducted using MS Excel 2010.

### 2.6 Malic acid decarboxylation

The ability of the isolates to decarboxylate malic acid was determined using the malic acid decarboxylating (MD) medium as described by Daeschel et al. (1984). Cultures were grown overnight in MRS broth at 30 °C, centrifuged in 15 mL tubes at 6,300 × *g* (Eppendorf Centrifuge Model 5810, Hamburg, Germany) for 5 min and the pellets were reconstituted in sterile saline solution (0.85% NaCl, w/v). Three microliters of the pure cultures were spotted on MD agar plates and incubated at 30 °C for 24 hr. A color change from blue to yellow was recorded as an indication of the absence of malic acid decarboxylase activity (Figure 1). The mutant strain *L. plantarum* MU0445 with no malic acid decarboxylase (*mdc*$^-$) activity was used as a positive control (Daeschel et al., 1984).

### 2.7 Exopolysaccharide (EPS) production activity

The ability of the isolates to produce EPS was evaluated using phenotypic and genotypic methods. Genotypic characterization included PCR amplification of genes involved in homo- and heteroexopolysaccharide production as described previously (Low et al., 1998; Mozzi et al., 2006; Palomba et al., 2012). The concentration of $MgCl_2$ was maintained at 2 mM

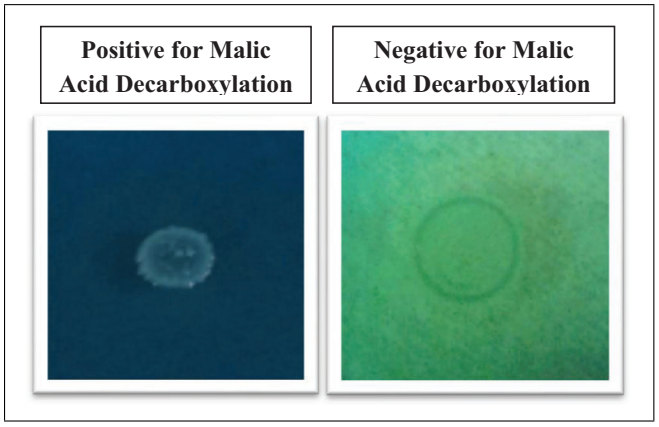


**Figure 1–MD agar plates inoculated with *Lactobacillus pentosus* 1.8.9 able to decarboxylate malic acid (left) and *Lactobacillus pentosus* LA0081 (MOP3-M6) unable to decarboxylate malic acid (right). Inability to decarboxylate malic acids induces a change in the color of the medium from blue to yellow.**

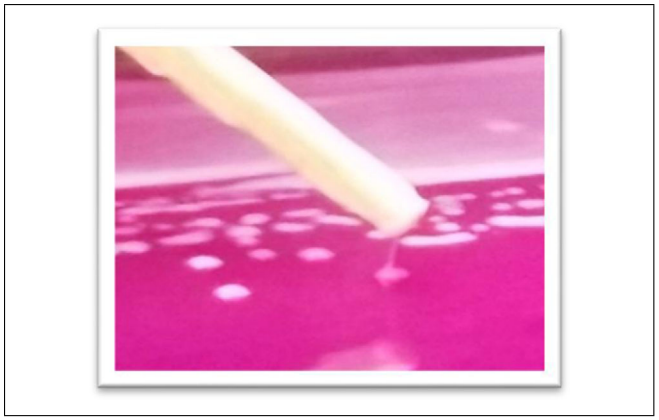

**Figure 2–Ropy phenotype observed for the exopolysaccharide producing culture *Lactobacillus plantarum* 3.2.8 on Cucumber Juice Agar supplemented with the ruthenium red dye.**

for all the PCR reactions. Two primer pairs were additionally designed using the EPS production associated genes in *L. pentosus* IG1 (NCBI locus tags: LPENT_00747 EPS biosynthesis protein (ebp1) and LPENT_00748 EPS chain length determinator Wwz (ecld1)) as templates. The following primer pairs were designed in this study as described above: *ebp1*-F (5'- CTGTTTACGTGGCATCTT GATTT-3') and *ebp1*-R (5'-CATGAGATGGTCCCGAGTTA TT-3') and *ecld1*-F (5'-GATGCGAACTATCCCGAACAT-3') and *ecld1*-R (5'-GTCCT ACTGGTTGATGCTGATT-3') with expected amplicon sizes of 411 and 439 bp, respectively. PCR mix was prepared as described earlier with 2.5 mM $MgCl_2$ for these two primer pairs using the conditions described for *xylA* above with an annealing temperature of 50 °C instead of 52 °C. The sequences were deposited in NCBI Genbank and accession numbers were obtained.

EPS production phenotypes were detected on MRS agar plates supplemented with 0.008% ruthenium red (CAS No.: R2751; Sigm–Aldrich) and the following sugars individually: 111 mM glucose, 111 mM sucrose, 111 mM fructose or an equimolar ratio of glucose and fructose (55 mM each). The production of ropy EPS forms a barrier around the colonies impeding the absorption of ruthenium red by the cells. Thus, colonies of EPS producing microbes appear white on the pinkish-red plates (Stingele, Neeser, & Mollet, 1996) (Figure 2). Conversely, colonies formed by non-EPS producers appear translucent or pinkish on plates.

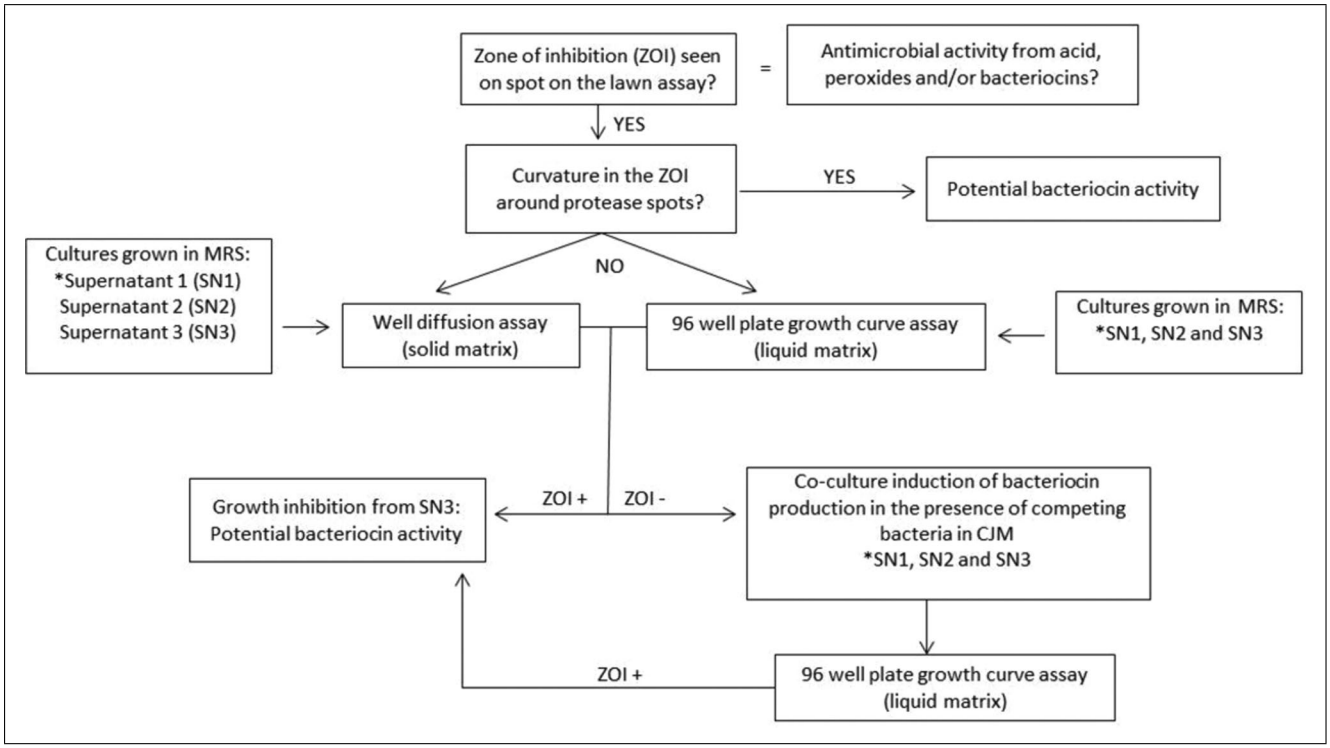


**Figure 3–Flow chart of the experimental approach followed to evaluate the bacteriocin activity of the lactobacilli. *SN1: Un-supplemented supernatant; SN2: supernatant supplemented with NaOH to pH 7.0, and SN3: supernatant supplemented with Catalase**

*L. rhamnosus* ATCC 53103 was used as a positive control for EPS production (Lebeer, Claes, Verhoeven, Vanderleyden, & De Keersmaecker, 2011).

Cucumber juice plates were also used to evaluate an EPS production phenotype, to mimic the fermentation matrix. Cucumber juice plates were prepared by blending fresh cucumbers (procured from a local pickle manufacturer) in a commercial juice maker (Warring Commercial, Stamford, CT, USA) and frozen at –20 °C. The cucumber slurry was thawed right before preparation of the medium and filtered through cheesecloth to remove particulates. The sieved juice was centrifuged at 2,060 × *g* (Eppendorf Centrifuge Model 5810) for 30 min and the supernatant filtered sterilized using a 0.22-micron filtration unit (Rapid Flow™-Nalgene®, Thermo Scientific, Waltham, MA, USA). Agar solution (45 g/L) was autoclaved separately, tempered to approximately 50 °C and added to the cucumber juice at a 3:2 (v/v) ratio of cucumber juice: agar solution. Ruthenium red was sterile filtered and added to the medium to 0.008% just before pouring the plates. All the tests were independently duplicated.

### 2.8 Antimicrobial and bacteriocin activity assays

Figure 3 describes the workflow for the phenotypic analysis of antimicrobial activity. The presence of an antimicrobial activity from acid, peroxides, and/or bacteriocins was evaluated using the lawn-on-the-spot test as described by Dobson, Sanozky-Dawes, and Klaenhammer (2007) and Moraes et al. (2010). Four microliters of overnight cultures were spotted on MRS agar plates and incubated for 24 hr at 30 °C. Ten microliters of overnight indicator bacteria or yeast were added to 10 mL of Lactobacilli MRS or Yeast and Mold (YM) soft agar (0.7%), respectively, prior to the overlay. The indicator species used in this assay were heterofermentative LAB including *L. brevis* ATCC 14869, *Leuconostoc mesenteroides* ATCC 8293, *Weissella paramesenteroides* ATCC 33313, the fermented cucumbers spoilage bacterium *L. buchneri* ATCC 4005, and the yeasts *Candida etchelsii* Y095, *Issatchenkia occidentalis* Y089, *Pichia manschurica* Y098 and *Zygosaccharomyces globiformis* Y069 (USDA-ARS culture collection). The indicator yeasts used have been associated with fermented cucumber spoilage (Bell & Etchells, 1952; Franco & Pérez-Díaz, 2012). *L. johnsonii* NCK468 was used as a positive control for bacteriocin production against the sensitive indicator *L. delbrueckii* NCK235 (courtesy of Dr. Todd Klaenhammer, North Carolina State University, Raleigh, NC, USA). Once the cultures producing an inhibition around the spots were identified, the same assay was performed subsequently with 2 μL of the following enzymes and chemical reagents spotted adjacent to the growth spots. The following enzymes secured from Sigma–Aldrich were used to detect the proteinaceous nature of the antimicrobial activity: proteinase K, α-chymotrypsin, papain, pepsin, and trypsin TPCK. Catalase (Sigma–Aldrich) was used to detect a peroxide-like antimicrobial. Five molar NaOH was used to neutralize the cultures and detect an acid-like activity. Enzymes were reconstituted in 1 mg/mL in phosphate buffer (pH 7). The spotted enzymes and reagents were allowed to dry on plates for 20 min prior to overlaying with soft agar. The soft agar was allowed to solidify for 20 min at ambient temperature and plates were incubated at 30 °C for 48 hr. Zones of inhibition (ZOI) were measured in mm. A curvature around ZOI was expected where the enzymes or reagents effective against the antimicrobial substance were added (Figure 4). A ZOI > 1 mm were considered positive (+). The experiment was conducted in duplicate.

Subsequently, well diffusion assay and 96-well plate growth assays were performed to characterize bacteriocin like antimicrobial activity from the cultures of interest, to confirm the observations made from the lawn-on-the-spot assay and also to study the effect of growth matrix (liquid *vs*. solid matrix; Corsetti, Settani, & Van

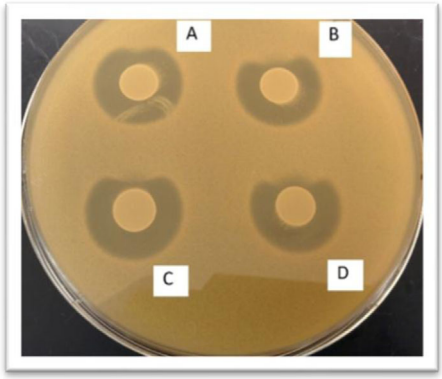


**Figure 4–Antimicrobial activity of the positive control *Lactobacillus johnsonii* NCK 468 against *Lactobacillus delbrueckii* NCK235. The curvatures around the zone of inhibition were formed due to the localized addition of the following enzymes: (A) Papain, (B) Pepsin, (C) Proteinase K, and (D) $\alpha$-Chymotrypsin**

Sinderen, 2004; Nieto-Lozano, Reguera-Useros, Peláez-Martıńez, & Hardisson de la Torre, 2002). MRS culture supernatants were obtained after spinning the overnight grown cultures in 50 mL tubes at 6,300 × *g* for 15 min (Eppendorf Centrifuge Model 5810, Hamburg, Germany). Culture supernatants were filter sterilized using 0.22 μm PTFE syringe filters (Sigma–Aldrich), aliquoted into three equal volumes and treated as follows: (a) supernatant without any treatment—SN1, (b) supernatant neutralized to pH 7 using 5 N NaOH (Sigma–Aldrich) to eliminate the effect of acids—SN2, and (c) supernatant neutralized to pH 7 and treated with catalase (Sigma-Aldrich) (stock concentration of 5 mg/mL) to a concentration of 1 mg/mL and incubated at 30 °C for 1 hr to eliminate the effect of both acid and peroxides—SN3. The same bacterial indicator strains described above for the lawn-on-the-spot assay were used as indicators and added to 10 mL Lactobacilli MRS soft agar (0.7% agar) to a concentration of $10^4$ cells/mL. The soft agar layer was overlaid on MRS hard agar (1.5% agar) and allowed to solidify for 20 min. Wells were made on the plate using the wider end of sterile Pasteur pipettes and each well was filled with 30 μL of the three supernatants described above, separately. The plates were left at room temperature for 2 hr for the supernatant to radially diffuse through the soft agar. Zones of inhibition were measured around the well after an incubation of 48 hr at 30 °C. All the experiments were performed in duplicate.

Growth curve of the indicator organisms (described above) were obtained using Lactobacilli MRS broth supplemented with SN1, SN2, and SN3 to detect any antimicrobial compounds produced by the cultures of interest and evaluate the effect of the supernatants on a possible bacteriocin-like activity. Growth was monitored in the presence of the three supernatants in a 96-well plate using a BioTek ELX808 plate reader (BioTek Instrument Inc., Winooski, VT, USA). The indicator cells were inoculated in half-fold diluted MRS medium (0.5× MRS) and mixed with the three different supernatants at a ratio of 1:1 (v/v) and 1:10 (v/v) separately with a slight modification as described by Patel, Prajapati, Holst, and Ljungh (2014). Cells were transferred to 0.5× diluted MRS broth with no supernatants as positive controls. Bacterial growth was monitored for 48 hr.

Co-culture induction of bacteriocin production was studied on the selected cultures in the presence of competing LAB that are commonly present during commercial cucumber fermentation (Rojo-Bezares et al., 2007). The cultures of interest were inoculated in CJM to a post-inoculation $OD_{600}$ of 0.01 ($10^4$ CFU/mL) with each competitor to an $OD_{600}$ of 0.001 ($10^3$ CFU/mL). They were incubated for 24 hr at 30 °C and three different supernatants were extracted as described above. The 96-well plate method was used to determine the antimicrobial potential of these supernatant as described above. All the experiments were performed in independent duplicates and statistical analyses for significant difference were conducted on MS Excel 2010.

Genotypic characterization of bacteriocin production was done by PCR based detection of genes associated with plantaricin and pentocin biosynthesis. The primer pair O-48/O-105 was used to detect class IIa bacteriocin coding genes as described by Yi, Zhang, Tuo, Han, and Du (2010). Class IIb plantaricin associated genes were amplified using primers developed by Cho, Huch, Hanak, Holzapfel, and Franz (2010), Diep, Håvarstein, and Nes (1996), Doulgeraki, Paraskevopoulos, Nychas, and Panagou (2013), and Rojo-Bezares et al. (2007). Additionally, the primer pair bip1-F (5'- CCTGACCAATCGGGGGATTT-3') and bip1-R (5'- AAGTGCAACCGGCCCTTAAT-3') was designed in this study as described above and targeted the bacteriocin immunity protein from *L. pentosus* IG1. The bip1-F and bip1-R primer pair was expected to produce an amplicon of 146 bp. PCR amplification was performed as described above using 2.5 mM $MgCl_2$ using 51 °C as annealing temperature and an extension step of 30 s instead of 1 min.

### 2.9 Antibiotic susceptibility profile

The antibiotic susceptibility profiles of the seven isolates of interest were obtained by exposure of the cells to ampicillin, chloramphenicol, clindamycin, gentamicin, erythromycin, and tetracycline at concentrations between 0.016 and 256 μg/mL. The experiment was performed following the European Food Safety Authority (EFSA) standards and published methods by Flórez et al. (2006) on *Lactobacillus* Susceptibility Medium (LSM) inoculated to 8 Log CFU/mL (European Food Safety Authority, 2012). The Minimum Inhibitory Concentration (MIC, μg/mL) were defined using the E-test strips (Bio-Mérieux, France). The results of the E-test strips were interpreted following the manufacturer's guidelines.

## 3. RESULTS AND DISCUSSION

### 3.1 Carbohydrate utilization

Table 1 shows that the *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1,8.9, 3.8.24, and 3.2.27 were unable to utilize L-rhamnose, D-turanose, arabinose, ribose, and xylose in the API® 50CHL strips and utilized cellobiose, gentiobiose, trehalose, glucose, and fructose that are of relevance in cucumber fermentations (Ucar et al., 2020a and 2020b). The characterization of the *L. pentosus* isolates, including the ATCC type strain was atypical using the miniaturized biochemical strips API® 50CHL (Boyd, Antonio, & Hillier, 2005; Ghotbi, Soleimanian-Zad, & Sheikh-Zeinoddin, 2011). However, the characterization of *L. plantarum* 3.2.8 and corresponding control culture *L. plantarum* ATCC 14917 aligned with the genetic identification.

While the *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, and 3.2.37 isolated from commercial cucumber fermentations were capable of utilizing glycerol, they did not metabolize D-xylose in the API® 50CHL strip test (Table 1), in MRS broth supplemented with D-xylose (data not shown), or in the M medium (data not shown) specifically developed to evaluate D-xylose utilization (Chaillou et al., 1999). The average absorbance at 600 nm from the

**Table 1–Sugar utilization profiles of the lactobacilli as defined by the API® 50CHL test strips. Red colored boxes represent negative results and the green colored boxes represent a positive result for the utilization of a given substrate. The black box encapsulates the data obtained for three strains that belong to the same RAPD-phylogenetic cluster (J).**


**Table 2–Accession numbers in the National Center for Biotechnology Information (NCBI) database for the nucleotide sequences obtained from the amplicons derived from the lactobacilli extracted DNA using primers targeting genes associated with bacteriocin production and xylose utilization.**

| | Accession Numbers for the Partial Gene Sequences Obtained | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Isolate ID** | ***xylA*** | ***plnD*** | ***plnE*** | ***plnEF*** | ***plnG*** | ***bip1*** | ***ebp1*** | ***ecld1*** |
| 1.2.11 | NA* | NA | KT281855 | NA | KT281846 | KT215180 | NA | KT215171 |
| 1.2.13 | NA | NA | NA | NA | KT281847 | KT215186 | KT215168 | KT215172 |
| 1.8.6 | NA | NA | NA | NA | KT281848 | KT215181 | KT215169 | KT215173 |
| 1.8.9 | NA | NA | NA | NA | KT281849 | KT215185 | NA | KT215174 |
| 3.8.24 | NA | NA | NA | NA | KT281850 | KT215182 | NA | KT215175 |
| 3.2.37 | NA | NA | KT281856 | NA | KT281851 | KT215187 | NA | KT215176 |
| LA0445 | KT124139 | NA | KT281857 | NA | KT281852 | KT215183 | KT215170 | KT215177 |
| 3.2.8 | NA | KT281844 | KT281858 | KT281860 | KT281853 | NA | NA | KT215178 |
| *L. plantarum* WCFS1 | NA | KT281845 | KT281859 | KT281861 | KT281854 | KT215184 | NA | KT215179 |
| *L. pentosus* ATCC 8041 | KT124140 | ND† | ND | ND | ND | ND | ND | ND |

*NA: no PCR amplification was obtained
†ND: not determined



*L. pentosus* cultures in MRS-G was 1.2 ± 0.2; while no changes in absorbance were measured from the cultures in MRS-X and MRS-N. *xylA* amplicons were not obtained when genomic DNA extracted from the *L. pentosus* of interest was used as the template for PCR, even though the expected amplicon was obtained when using genomic DNA extracted from *L. pentosus* LA0445, which was isolated from a commercial cucumber fermentation in 1988 (Fleming, McFeeters, Daeschel, Humphries, & Thompson, 1988) (Table 2).

## 3.2 Biogenic amine production

Phenotypically, none of the cultures studied here (1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, 3.2.37, and 3.2.8) showed an indication of biogenic amines production in culture media containing biogenic amine precursors, even after six serial transfers in the inducing medium prior to examination. There were no color changes from yellow to purple in the agar plates containing the biogenic amine precursors. The positive control for histamine, putrescine, and cadaverine production changed the color of the medium to purple and tyramine plates had a clear halo around the colonies (Figure 5). Primers associated with biogenic amine production, specifically designed to amplify putative decarboxylase homolog genes from various lactobacilli, did not produce amplicons when using

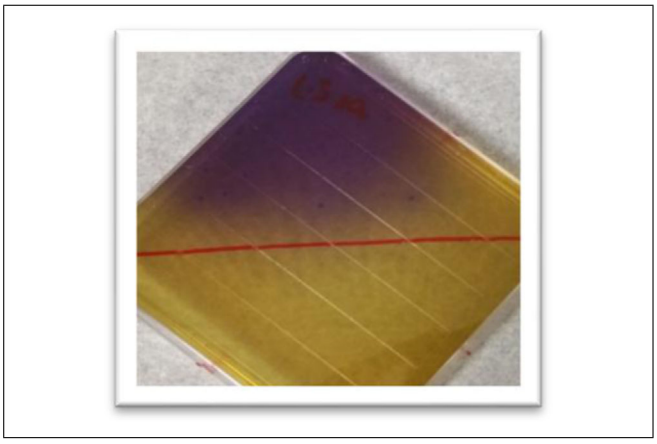

**Figure 5–Pictogram of histamine production by Lactobacillus 30a, used a positive control, on the Biogenic Amine Agar. The color of the medium changed from yellow to purple upon inoculation and incubation**

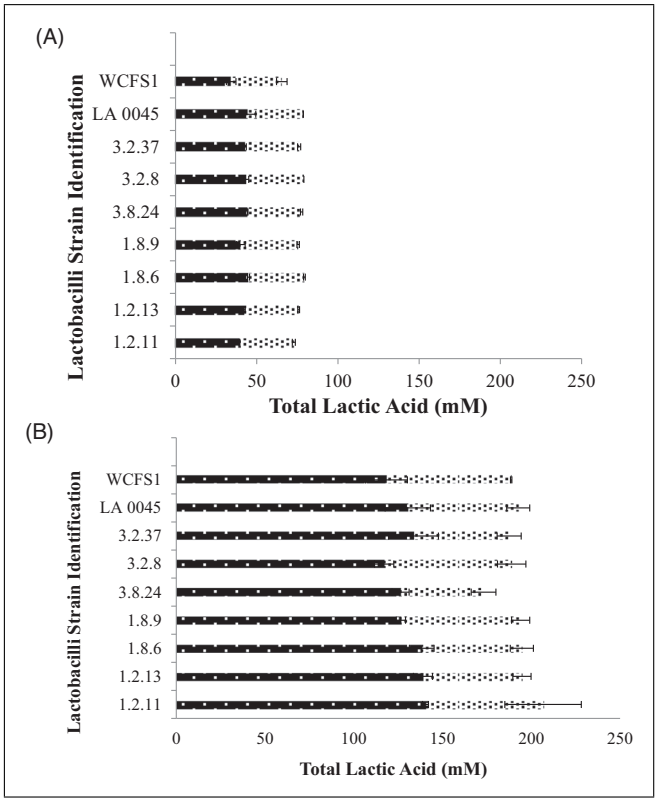


**Figure 6–Milimolar (mM) concentrations of the D- and L-lactic acid isomers produced by the lactobacilli. Panel (a) presents the production of D- (black and dotted bars) and L- (white and dotted bars) lactic acid by the lactobacilli in Cucumber Juice Medium. Panel (b) presents the production of D- (black and dotted bars) and L- (white and dotted bars) lactic acid by the lactobacilli in MRS broth.**

genomic DNA extracted from the cultures of interest as templates. The positive control produced the expected change on the culture plate (Figure 5) and produced the expected PCR amplicon.

### 3.3 Production of D- and/or L-lactic acid

D-Lactic acid was produced by the seven lactobacilli strains (1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, 3.2.37, and 3.2.8) to 41.9 ± 3.4 mM and 129.9 ± 8.9 mM in CJM and MRS broth, respectively, after reaching stationary phase (Figure 6). L-Lactic acid was produced by the lactobacilli to 33.7 ± 1.3 mM and 61.2 ± 5.7 mM CJM and MRS, respectively (Figure 6).

### 3.4 Malic acid decarboxylation

No color changes were observed in the MD medium to yellow, suggesting that the *L. plantarum* 3.2.8 and *L. pentosus* cultures 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, and 3.2.37 possess malic acid decarboxylating activity. The positive control, *L. plantarum* MU0445, produced a color change to yellow in the MD medium (Breidt & Fleming, 1992; Figure 2).

### 3.5 Exopolysaccharide production

*L. pentosus* 1.8.6, 1.8.9, 3.8.24, and 3.2.37, *L. plantarum* 3.2.8, and the positive control presented a ropy EPS production phenotype in lactobacilli MRS plates supplemented with ruthenium red, but not *L. pentosus* 1.2.11 and 1.2.13 (Table 3). The ability to produce EPS in lactobacilli MRS agar plates was dependent on the type of sugar (Table 3). However, these two cultures that did not produce EPS on MRS plates with various sugars, formed slightly white colored colonies on cucumber juice plates supplemented with ruthenium red dye, suggesting a moderate EPS production in the comparatively less nutrient rich medium. Amplicons for *ecld*, associated with EPS chain length determinator, were detected in the seven cultures (1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, 3.2.37, and 3.2.8) tested (Table 2). Amplicons for *ebp1*, coding for the EPS biosynthesis protein, were only obtained from *L. pentosus* 1.2.13, 1.8.6, and LA0445 (Table 2).

### 3.6 Production of bacteriocins

No amplicons were produced from PCR reactions containing primer pair O-48 and O-105 and DNA from each of the seven lactobacilli (1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, 3.2.3, and 3.2.8) individually. Such primer pair targeted the conserved region of class IIa bacteriocins. However, amplicons were obtained for members of the *pln* operon including *plnD, E, E/F*, and *G* from *L. plantarum* 3.2.8. *plnG* and *bip1* were amplified from all the *L. pentosus* isolates indicating that they potentially produce class IIb bacteriocins (Table 2). *L. plantarum* WCFS1, used as positive control, produced amplicons for all the genes tested (Table 2). *L. pentosus* LA0445, used to increase diversity among the *L. pentosus* strains studied, was isolated more than two decades ago from commercial cucumber fermentations and presented responses similar to the other strains.

Co-culture induction of bacteriocin production by the isolates in the presence of the competing LAB such as *L. brevis* ATCC 14869, *Leuconostoc mesenteroides* ATCC 8293, *Weissella paramesenteroides* ATCC 33313, and the fermented cucumbers spoilage bacterium *L. buchneri* ATCC 4005 in CJM resulted in the display of insignificant production of antimicrobial activity (Table 4). A proteinaceous antimicrobial effect against the competing yeasts *Candida etchelsii* Y095, *Issatchenkia occidentalis* Y0089, *Pichia manschurica* Y098, and *Zygosaccharomyces globiformis* Y0069 was not observed (data not shown). Similar growth rate constants were observed from cultures inoculated in untreated supernatants as compared to cultures growing in the presence of treated supernatants. Growth rate constants from SN1 (untreated supernatant) were significantly lower ($p < .05$) from both the neutralized supernatants (SN2 and SN3) with or without catalase indicating that the minimal antimicrobial activity observed is only due to acid production.

### 3.7 Antibiotic susceptibility profile

*L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, and 3.2.37 and the *L. plantarum* 3.2.8, presented an MIC range below the cut-off values proposed by the EFSA against ampicillin, chloramphenicol, gentamicin, and tetracycline (Table 5). *L. plantarum* ATCC 14917 that was used for quality control, displayed MIC values lower than the cut-off values described in the guidelines for ampicillin, gentamicin, and tetracycline (Table 5). Strains 1.2.13, 1.8.6, 1.8.9, 3.8.24, and ATCC 14917 displayed an intermediate resistance against erythromycin at 2 μg/mL, which is 1 μg/mL above the cut-off value. Isolates 1.8.6, 1.8.9, 3.8.24, and 3.2.37 showed an intermediate resistance against clindamycin as well with a cut-off value of > 2 μg $mL^{-1}$ (Table 5).

### 3.8 Discussion

Seven lactobacilli strains isolated from a commercial cucumber fermentation were evaluated for their potential to serve as starter culture. The strains presented heterogenous carbohydrate utilization profiles across RAPD-phylogenetic clusters and few differences within a cluster. *L. pentosus* LA0445 that was isolated

**Table 3–Exopolysaccharide production phenotypes presented by the lactobacilli on MRS supplemented with ruthenium red and various sugars and cucumber juice medium supplemented with the red dye.**

| | Sugar Source | | | | |
|---|---|---|---|---|---|
| Isolates | Glucose | Fructose | Sucrose | Glucose and Fructose | Cucumber Juice |
| *Lactobacillus pentosus* | | | | | |
| 1.2.11 | −[!] | − | − | − | + |
| 1.2.13 | − | − | − | − | + |
| 1.8.6 | +[*] | − | + | + | + |
| 1.8.9 | + | − | − | + | + |
| 3.8.24 | − | − | − | − | + |
| 3.2.37 | − | − | − | − | + |
| LA0445 | + | − | + | + | + |
| *Lactobacillus plantarum* | | | | | |
| 3.2.8 | + | + | + | + | + |
| WCFS1 | + | + | + | + | + |
| *Lactobacillus rhamnosus* | | | | | |
| ATCC 53103 | + | + | + | + | + |

[!](−) Indicates formation of pink colored colonies on plates suggesting lack of EPS production.
[*](+) Indicates the observation of a ropy EPS phenotype.

**Table 4–Growth rate ($h^{-1}$) of the indicator bacteria in the presence of the lactobacilli cultures supernatants supplemented with nothing (SN1), NaOH to pH 7.0 (SN2), or Catalase to 1 mg/mL (SN3).**

| | *L. brevis* | | | *L. buchneri* | | | *L. mesenteroides* | | | *W. paramesenteroides* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolates | SN1[a] | SN2[b] | SN3[b] | SN1[a] | SN2[b] | SN3[c] | SN1[a] | SN2[b] | SN3[b] | SN1[a] | SN2[b] | SN3[b] |
| 1.2.11 | 0.03 ± 0.02 | 0.08 ± 0.01 | 0.07 ± 0.02 | 0.02 | 0.08 | 0.1 | 0.03 ± 0.01 | 0.09 | 0.07 ± 0.01 | NG | 0.13 | 0.17 ± 0.01 |
| 1.2.13 | NG | 0.08 ± 0.01 | 0.09 ± 0.03 | 0.02 | 0.08 | 0.1 | 0.02 ± 0.01 | 0.14 | 0.17 | NG | 0.12 ± 0.01 | 0.17 |
| 1.8.6 | 0.05 ± 0.03 | 0.09 ± 0.01 | 0.11 ± 0.02 | 0.02 | 0.08 | 0.09 | 0.04 | 0.1 ± 0.01 | 0.11 ± 0.05 | NG | 0.13 ± 0.01 | 0.17 |
| 1.8.9 | NG | 0.1 ± 0.04 | 0.07 ± 0.02 | 0.02 | 0.08 | 0.11 | 0.02 | 0.11 ± 0.01 | 0.11 ± 0.02 | NG | 0.13 | 0.17 ± 0.01 |
| 3.8.24 | NG | 0.13 | 0.16 | 0.03 | 0.08 | 0.1 ± 0.01 | 0.02 | 0.11 ± 0.01 | 0.15 ± 0.01 | NG | 0.06 | 0.09 ± 0.01 |
| 3.2.8 | NG | 0.15 | 0.17 | 0.02 ± 0.01 | 0.08 | 0.1 | NG | 0.12 ± 0.01 | 0.15 | NG | 0.05 ± 0.01 | 0.09 |
| 3.2.37 | NG | 0.14 ± 0.01 | 0.17 | 0.02 | 0.08 | 0.1 | NG | 0.11 ± 0.01 | 0.15 ± 0.01 | NG | 0.04 | 0.09 ± 0.01 |
| LA0445 | NG | 0.13 ± 0.01 | 0.17 | 0.02 | 0.08 | 0.1 ± 0.01 | NG | 0.11 | 0.14 ± 0.01 | NG | 0.05 ± 0.01 | 0.07 |
| WCFS1 | NG | 0.13 | 0.16 | 0.03 | 0.08 | 0.1 | NG | 0.1 | 0.14 | NG | 0.05 ± 0.02 | 0.08 ± 0.01 |

Specific growth rate of the indicators in the supernatants marked with the same letters (a, b or c) indicate that there is no significant difference among the two. Specific growth rate of the indicators in the supernatants marked with different letters (a, b, or c) indicate that there is a statistical difference between the two.



from cucumber fermentation more than 20 years ago for its acid resistance (Fleming et al., 1988), metabolized carbohydrates in a pattern different than all the other *L. pentosus* including 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, 3.2.8, and 3.2.37 and obtained from commercial cucumber fermentations in 2012 (Pérez-Díaz et al., 2017). These observations suggest metabolic diversity even among strains of the same origin. Possession of a mosaic of carbohydrate utilization gene cassettes is one of the remarkable features of *L. plantarum* (Siezen et al., 2010; Tanganurat, Quinquis, Leelawatcharamas, & Bolotin, 2009). A similar trend can be expected for *L. pentosus*, a close relative of *L. plantarum* (Anukam et al., 2013). *L. plantarum* has been considered as a "natural metabolic engineer" for its ability to adapt to various environments and utilize various sugars (Siezen & van Hylckama Vlieg, 2011). Discrepancies in carbohydrate metabolism can also be possibly due to the absence of related genes or loss of plasmids encoding unusual carbohydrate metabolism associated genes as a result of evolution and adaptation to specific niches (Siezen & van Hylckama Vlieg, 2011). The difficulties in characterizing *L. pentosus* using API 50 CHL test strips and the inability of such species to utilize glycerol have been previously reported (Bringel, Curk, & Hubert, 1996; Ghotbi et al., 2011; Yu et al., 2012).

Reinstating *L. pentosus* as a species divergent from *L. plantarum*, was partially based on the ability of *L. pentosus* to differentially utilize pentoses such as D-xylose and glycerol (Zanoni, Farrow, Phillips, & Collins, 1987). However, past studies indicate that some *L. pentosus* are unable to utilize D-xylose and some *L. plantarum* can utilize both D-xylose and glycerol (Bringel et al., 1996; Ghotbi et al., 2011; Shoemaker, 2004; Tamminen et al., 2004). D-Xylose is present in fresh cucumbers at 0.4 to 0.6 mg/g of mesocarp tissue and to a higher concentration in the carpel, depending on the size of the cucumbers (McFeeters & Lovdal, 1987; Miller, 1989). However, it is not the primary fermentation substrate, which may explain the lack of D-xylose utilization ability by the *L. pentosus* tested. Acidification of fermentation cover brines and cucumbers from the metabolism of the primary sugars, glucose and fructose,

**Table 5–Minimum inhibitory concentrations (MIC μg/mL) of the tested antibiotics against the lactobacilli. MIC values above the cut-off value are highlighted in bold indicating intermediate-resistance.**

| | MIC (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| **Isolates** | **Ampicillin** | **Chloramphenicol** | **Clindamycin** | **Erythromycin** | **Gentamicin** | **Tetracycline** |
| 1.2.11 | 2 | 7 | 2 | 1 | 10 | 16 |
| 1.2.13 | 1 | 6 | 2 | **2** | 12 | 10 |
| 1.8.6 | 1 | 7 | **4*** | **2** | 6 | 18 |
| 1.8.9 | 2 | 7 | **4** | **2** | 7 | 16 |
| 3.8.24 | 1 | 8 | **4** | **2** | 14 | 28 |
| 3.2.37 | 1 | 8 | **3** | 1 | 7 | 20 |
| LA0445 | 2 | 5 | 0 | 1 | 3 | 14 |
| 3.2.8 | 1 | 6 | 1 | 1 | 5 | 16 |
| ATCC14917 | 1 | 8 | 2 | **2** | 11 | 6 |
| ATCC8041 | 2 | 8 | 1 | 1 | 4 | 11 |
| Cut-off value | 2 | 8 | 2 | 1 | 16 | 32 |

may also be preventing the catabolism of D-xylose by *L. pentosus*. Expression of the *xyl* operon is induced by xylose and repressed by the presence of glucose and other sugars such as ribose and arabinose (Lokman et al., 1997). Lack of xylose utilization by the *L. pentosus* isolated from cucumber fermentations may be due to loss of a plasmid potentially encoding the *xyl* operon (Lokman et al., 1997; Posno et al., 1991).

Cucumbers contain amino acids that can potentially serve as biogenic amine precursors. None of the isolates in this study presented a biogenic amines production phenotype. Additionally, the corresponding decarboxylase coding genes were not amplified. These observations suggest that utilization of the *L. plantarum* and *L. pentosus* isolates as starter cultures is unlikely to result in the formation of biogenic amines. The current practice of spontaneous wild fermentation has higher chances of biogenic amine formation due to the mixed composition of microorganisms present in the environment and the cucumbers (Buckenhüskes, 1993; EFSA, 2011). So, addition of these non-biogenic forming starter cultures can potentially outcompete the biogenic amine producers from the natural microbiota.

There was a substantial difference in the ratio at which D- and L-lactic acid isomers were produced in the two media tested (Figure 1). The higher D-lactic acid production observed in MRS medium may be associated with active metabolism and fast replication in the presence of excess glucose (Goffin et al., 2005; Kandler, 1983). CJM contains moderate amounts of glucose and fructose (~60 mM) in solution (McFeeters & Lovdal, 1987), which may be translating into a reduced metabolic activity and growth rate as compared to the nutrient rich MRS medium. Lower production of D-lactic acid in CJM also explains lower production of this isomer in cucumber fermentation and thus beneficial minimal carry over in finished products.

Malic acid decarboxylation by lactobacilli in cucumber fermentation is one of the main causes of the formation of hollow cavities inside the fruits, better known as bloater defect (Fleming & Pharr, 1980). The isolates in this study were able to decarboxylate malic acid. Thus, utilization of these isolates in cucumber fermentations will require application of air or nitrogen purging at regular intervals to promote dissipation of the $CO_2$ produced during the bioconversion to the surface of the cover brines. Research efforts directed to identify naturally occurring non-decarboxylating variants of *L. plantarum* or *L. pentosus* have been unfruitful (Zhai, Pérez-Díaz, Diaz, Lombardi, & Connelly, 2018).

Formation of biofilms through firm anchoring of EPS to solid surfaces aids bacteria to aggregate, proliferate, and thus predominate in fermentations (Patel, Majumder, & Goyal, 2012). Exopolysaccharides have also been shown to help in cell survival during food processing (Nwodo, Green, & Okoh, 2012). Production of EPS in CJM but not in MRS, by the isolates of interest highlights the influence of the natural habitat in the induction of such phenotype (Table 3). The putative gene encoding for *ebp1* was not found in any of the isolates tested suggesting that such open reading frame is probably not conserved among the species. Genes coding for glycosyltransferase that are needed to produce homoexopolysaccharides, were not amplified from these isolates, indicating that they primarily produce heteroexopolysaccharides (De Vuyst & Degeest, 1999; Palomba et al., 2012).

Bacteriocins confer an ecological advantage because they are often produced under stress and competition as a survival strategy, depending on the growth matrix (Ivey, Massel, & Phister, 2013). The presence of plantaricin (the bacteriocin from *L. plantarum*) associated genes in the genomes of the targeted microbes was studied. The results suggest that the isolates possess class IIb bacteriocins associated with the genes *plnE, plnG*, and *bip1* (Table 2). In addition, the *L. plantarum* cultures seem to have *plnD, pln E/F* (Table 2). The genes on the *pln* locus are highly variable among species (Diep, Straume, Kjos, Torres, & Nes, 2009). Differences in the nucleotide sequences of the amplicons derived from the lactobacilli were detectable at the strain level. Although a genotypic potential for bacteriocin production was evident, no bacteriocin like activity was detected in the CJM, even after co-culture induction with competing bacteria. Co-culture induction and subsequent growth measurement in the presence of SN2 and SN3 did not inhibit the indicators showing no bacteriocin like activity. The antimicrobial activity by the supernatants observed against the competing LAB seems to be only from acid production (Table 4). The antibacterial effect of bacteriocins is dependent on the strain, growth environment, and presence of other agents (Cintas, Casaus, Herranz, Nes, & Hernandez, 2001). Thus, it is not surprising for bacteriocin production to go undetected under conditions similar to those present in cucumber fermentations.

Even though antibiotic resistance started as a clinical problem, it is now widespread in non-clinical situations especially in the food fermentation and starter culture industries (Kastner et al., 2006). Currently, the food chain is attributed as one of the predominant routes for antibiotic gene dissemination between pathogens and opportunistic bacteria in humans. Lactobacilli are susceptible

**Table 6–Data summary table for the characterization of seven candidate cultures for starter cultures for cucumber fermentations.**

| Isolate ID | Utilization of sugars present in cucumbers: Xylose | Utilization of sugars present in cucumbers: Glucose, fructose, and trehalose | D-Lactic acid production in CJM | Ropy phenotype in CJM | Antibiotic resistance profile | Bacteriocin production | Malic acid decarboxylation | Biogenic amines production |
|---|---|---|---|---|---|---|---|---|
| *Lactobacillus pentosus* | | | | | | | | |
| 1.2.11 | − | + | 54% | + | Acceptable | − | + | − |
| 1.2.13 | − | + | 56% | + | Borderline for Erithromycin | − | + | − |
| 1.8.6 | − | + | 53% | + | Borderline for Erithromycin & Clindamycin | − | + | − |
| 1.8.9 | − | + | 53% | + | Borderline for Erithromycin & Clindamycin | − | + | − |
| 3.8.24 | − | + | 57% | + | Borderline for Erithromycin & Clindamycin | − | + | − |
| 3.2.37 | − | + | 56% | + | Borderline for Clindamycin | − | + | − |
| LA0445 | − | + | 57% | + | Acceptable | − | + | − |
| *Lactobacillus plantarum* | | | | | | | | |
| 3.2.8 | − | + | 55% | + | Acceptable | − | + | − |

to protein and cell wall biosynthesis inhibiting antibiotics, which are those that were tested here. *L. pentosus* LA0445 showed a very low MIC of 0.032 μg/mL against clindamycin, while all the other isolates showed higher MIC. The resistance against clindamycin for some isolates is slightly above the EFSA proposed cut-off value of 2 μg/mL (Table 5). Although this observation represents a relevant difference worth of further study, it is not a cause for concern. Various cut-off values have been proposed for *L. plantarum* against clindamycin including 32 μg/mL by Danielsen and Wind (2003), 12 μg/mL by Flórez et al. (2006), and 4 μg/mL by Ammor, Belén Flórez, and Mayo (2007). Even within the *L. plantarum* cluster the MIC range for a given antibiotic is widely distributed, for example, the MIC for clindamycin was reported between 0.03 and 16 μg/mL (Danielsen & Wind, 2003; Klare et al., 2007). A broad range of MIC for various antibiotics by *L. pentosus* strains isolated from olive fermentation have also been reported (0.002 to 2 μg/mL for ampicillin, chloramphenicol, clindamycin, and 0.002 to 16 μg/mL for erythromycin and tetracycline; Muñoz et al., 2014). The test medium (broth *vs*. agar) also has an impact on the MIC values that explains variable values compared to other reports (Klare et al., 2007). Thus, it is concluded that the isolates studied here can be used as starter cultures for commercial cucumber fermentation, as their MIC values are within the proposed cut-off values or the commonly reported MICs.

Table 6 summarizes the data obtained, by documenting the responses of *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, and 3.2.37 and *L. plantarum* 3.2.8 to test for key physiological features that are of relevance for cucumber fermentation. It is concluded that the lactobacilli tested show a variable carbohydrate utilization pattern, the *L. pentosus* isolates do not ferment D-xylose, none of the seven strains (1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24, 3.2.37, and 3.2.8) produce biogenic amines, and all the lactobacilli tested decarboxylate malic acid and produce a variable ratio of lactic acid isomers and EPS, depending on the growth medium. No phenotypic trait of bacteriocin production was observed from *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24 and 3.2.37 or the *L. plantarum* 3.2.8, even though they possessed the associated genes, and the MIC values for antibiotics are within the cut-off values. Thus, the features observed in the seven isolates studied here, including *L. plantarum* 3.2.8 and *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24 and 3.2.37 should not preclude their utilization as starter cultures in commercial fermentation.

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## AUTHOR CONTRIBUTIONS

K. Anekella contributed to the experimental design, conducted experimentation, collected data, interpreted the results, and drafted the manuscript. I. M. Pérez-Díaz defined the scientific approach, contributed to the experimental design, and edited the manuscript.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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