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Reduction of D-lactate content in sauerkraut using starter cultures of recombinant *Leuconostoc mesenteroides* expressing the *ldhL* gene

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The D-form of lactate, which causes metabolic stress upon excessive dietary intake, is mainly produced by *Leuconostoc* sp., the predominant species in sauerkraut. To shift the metabolic flux of D-lactate from pyruvate to L-lactate, we expressed the L-lactate dehydrogenase (*ldhL*) gene in *Leuconostoc mesenteroides* ATCC 8293. The *ldhL* gene from *Lactobacillus plantarum* was introduced into *L. mesenteroides* using the shuttle vectors pLeuCM and pLeuCM42. To elevate the expression level of *ldhL* in *L. mesenteroides*, the nucleotides for pyruvate kinase promoter were fused to *ldhL* and cloned into above vectors to construct pLC18pkL and pLC42pkL. As results, introduction of pLC42pkL in *L. mesenteroides* significantly improved both L-LDH activity and L-lactate productivity during fermentation, decreasing the D-/L-lactate ratio. When used as a starter culture for sauerkraut fermentation, recombinant *L. mesenteroides* harboring pLC42pkL increased L-lactate concentration and decreased D-lactate concentration compared to the wild type strain. We newly developed a recombinant *L. mesenteroides* which has high L-lactate dehydrogenase activity and applied this strain to minimize the harmful effect of D-lactate during the sauerkraut fermentation. To the best of our knowledge, we demonstrate for the first time the effective use of recombinant *Leuconostoc* sp. for quality improvement of fermented foods.

[**Key words:** *Leuconostoc*; D-/L-Lactate; D-/L-Lactate dehydrogenase; Lactic acid bacteria; Sauerkraut]

It has been reported that after the consumption of certain foods, D-lactate can accumulate in the blood of patients suffering from short-bowel syndrome and intestinal failure, leading to a manifestation of D-lactic acidosis and encephalopathy (1–4). Furthermore, newborn infants may fail to completely metabolize D-lactate that is ingested or produced by intestinal microorganisms due to liver immaturity (1). When D- and L-lactate contents were analyzed in various fermented foods, notably the average D-lactate content was significantly high (88.97 mM) in commercial sauerkraut products (5).

Sauerkraut fermentation is the result of spontaneous lactic acid production by lactic acid bacteria (LAB) in the presence of salt at optimal temperatures and anaerobic conditions. The commercial fermentation of sauerkraut results from resident LAB on cut and salted cabbage and, therefore, has the potential for high variability in quality due to variation in the naturally occurring microflora. Early predominance of heterofermentative LAB was considered to be essential in the production of good-quality sauerkraut (6). Among them, *Leuconostoc* is the major bacterial genus present from the initial to the middle stages of fermentation (7). Due to a lack of L-lactate dehydrogenase (L-LDH), these species predominantly convert pyruvate to D-lactate using D-lactate dehydrogenase (D-LDH).

In this study, we attempted to construct an L-lactate-producing *Leuconostoc* strain and used it as a starter culture for sauerkraut fermentation. For this, the *ldhL* gene from *Lactobacillus plantarum* was cloned and expressed in *Leuconostoc mesenteroides* using two different vectors, pLeuCM and pLeuCM42, which use rolling circle and theta-type replication mechanisms, respectively. In order to improve L-LDH activity sufficiently to alter the D-/L-lactate ratio, we employed the strong pyruvate kinase promoter for the expression of *ldh*L; the pyruvate kinase was identified as one of the most highly expressed proteins in *L. mesenteroides* ATCC 8293 from two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) analyses of cell lysates (8). Finally, the characteristics of the transformant strains were investigated in *in vitro* tests and their effect as starter cultures during sauerkraut fermentation was analyzed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. The plasmids pLeuCM (9) and pLeuCM42 (10) were used for cloning and expression of the *ldhL* gene from *L. plantarum* KCTC 3104. *Escherichia coli* DH5α, grown in Luria–Bertani broth at 37°C with shaking, was used for intermediate manipulation of plasmids. *L. plantarum* and *L. mesenteroides* ATCC 8293 were cultured in MRS medium (Difco

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strain/plasmid/oligonucleotide	Genotype/relevant features	Reference
Strain		
Lactobacillus plantarum KCTC 3104	Wild-type	
Leuconostoc mesenteroides ATCC 8293	Wild-type	
Escherichia coli DH5α	Cloning host; F′ φ80dlacZΔM15 endA1 recA1 hsdR17(r_K^-,m_K^+)supE44-thi-1gyrA96 relA1Δ(lacZYA-argF)U169 deoR λ^- gal-phoA	24
Plasmid		
pLeuCM	Amp^r, Cm^r; *E. coli-Leuconostoc* shuttle vector	9
pLeuCM42	Amp^r, Cm^r; *E. coli-Leuconostoc* shuttle vector	9
pLC18lld	Amp^r, Cm^r; pLeuCM derivative containing ldhL gene	This study
pLC42lld	Amp^r, Cm^r; pLeuCM42 derivative containing ldhL gene	This study
pLC18pkL	Amp^r, Cm^r; pLeuCM derivative containing ldhL gene with pyruvate kinase promoter	This study
pLC42pkL	Amp^r, Cm^r; pLeuCM42 derivative containing ldhL gene with pyruvate kinase promoter	This study
Oligonucleotide		
LpldhLF	5′-AATTGGATCCTGACGTGTCTGGGCATAT-3′	
LpldhLR	5′-AATTGGATCCCTAAGGTACCCACAAGCA-3′	
775-F	5′-AATTGAATTCTTGATCAAGAATTTTGGA-3′	
PKL-F	5′-AGGAGCTATTTAACCTTGTCAAGCATGCCA-3′	
LPK-R	5′-TGGCATGCTTGACAAGGTTAAATAGCTCCT -3′	
LpL-F	5′- AATTGAATTCCGTGTCTGGGCATATTG -3′	
LpL-R	5′- AATTGAATTCACATTTAATCGTATGAAATG-3′	

Laboratories; Detroit, MI, USA) at 37°C and 30°C, respectively. Chloramphenicol (50 μg/mL) and ampicillin (50 μg/mL) were used for *E. coli* selection, and chloramphenicol (10 μg/mL) was used for *L. mesenteroides* selection.

Construction of recombinant plasmids The *ldhL* gene (GenBank accession no. X70926) containing its original constitutive promoter region was amplified from genomic DNA of *L. plantarum* by PCR using the primer sets LpldhLF and LpldhLR or LpL-F and LpL-R. The purified PCR products were digested with either *Bam*HI for ligation into *Bam*HI-digested pLeuCM or *Eco*RI for ligation into *Eco*RI-digested pLeuCM42, resulting in pLC18lld (11) and pLC42lld, respectively. To replace the promoter region, the pyruvate kinase promoter (GenBank accession no. YP_818257.1) was amplified from genomic DNA of *L. mesenteroides* ATCC 8293 by PCR using primers 775-F and LPK-R. Promoterless *ldhL* gene was amplified using primers PKL-F and LpL-R. Since LPK-R and PKL-F share complementary sequences, the two resulting fragments were hybridized and the overlapping product was re-amplified using primers 775-F and LpL-R. This fragment, containing the *ldhL* gene with the pyruvate kinase promoter, was digested with *Eco*RI, and ligated into pLeuCM and pLeuCM42, resulting in pLC18pkL and pLC42pkL, respectively. All recombinant plasmids were transformed into *E. coli* DH5α cells and the transformants were selected on LB medium containing ampicillin. The recombinant plasmids were then transformed into *L. mesenteroides* ATCC 8293 by electroporation, as previously described (12). Transformation was performed using a Gene-Pulser unit combined with a Pulse Controller (Bio-Rad; Richmond, CA, USA).

Preparation of sauerkraut Sauerkraut was prepared as described by Pederson and Albury (6); a fresh cabbage (5 kg) was trimmed of its outer leaves, shredded to 3-mm-thick pieces, and salt was added to a final concentration of 2% (wt/wt) NaCl in 10 L glass jars. The jars were covered with flexible plastic film and weighted down with a heavy brick on top of the film. After inoculation with starter cultures (10^7 colony-forming units (CFU)/mL) of *L. mesenteroides* ATCC 8293 or *L. mesenteroides* ATCC 8293 (pLC42pkL), sauerkrauts were incubated at 18°C for 20 days.

Microbial analysis The bacterial growth in sauerkraut was measured by analysis of colony formation (CFU/mL) using the culture-pouring method. Brines from sauerkraut sample were serially diluted (10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}) with sterile physiological saline (0.85% NaCl) and spiral-plated onto PES (phenylethyl alcohol containing 2% (w/v) sucrose) for *L. mesenteroides*, PESC (PES containing 5 μg/mL of chloramphenicol) for *L. mesenteroides* harboring pLC42pkL, Lactobacillus Selection medium (LBS; Difco Laboratories; Detroit, MI, USA) for lactobacilli, and MRS for total LAB. PES and PESC plates were incubated at 30°C for 48 h. LBS and MRS plates were incubated anaerobically in gas pack jars at 37°C for 48 h.

LDH activity assays To quantify the intracellular activities of D-LDH and L-LDH, cells were washed with a solution containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. To lyse the cells, washed cells (5-fold concentrated) were incubated in the same buffer containing 0.5 mg/mL lysozyme and 50 mg/mL mutanolysin for 20 min at 37°C. To measure D- and L-LDH activities, the supernatant was incubated in 100 mM Tris–HCl (pH 8.0) solution with 15 mM NAD^+ and 100 mM of either D-lactate or L-lactate, respectively. To measure total LDH activity, the supernatant was incubated in 100 mM potassium buffer (pH 7.5) containing 20 mM pyruvate and 0.15 mM NADH. Reaction mixtures were incubated at 37°C and absorbance was monitored at 340 nm. Protein concentration was measured using a Thermo BCA protein assay kit (Pierce Biotechnology Inc.; Rockford, IL, USA). One unit of activity corresponded to the oxidation of 1 μmol NADH per minute.

Analysis of organic acids Quantification of organic acids was performed using a high-performance liquid chromatography (HPLC) system, consisting of a YOUNG-LIN M930 solvent delivery pump (Younglin; Seoul, Korea) with a YOUNG-LIN M720 absorbance detector and Aminex HPX-87 ion exclusion column (7.8 mm ID × 300 mm, Bio-Rad, USA). The mobile phase flow rate (0.004 N H_2SO_4) was 0.6 mL/min and UV absorbance was monitored at 250 nm. Analyses of D- and L-lactate contents in MRS medium as well as sauerkraut were carried out by following the method of Yoon et al. (5). In brief, lactate isomers were measured using an 8.0 × 50-mm Shodex ORpak CRX853 coupled with a CRX-G column (Showa Denko; Tokyo, Japan) on the same HPLC system with 1 mM $CuSO_4$ in H_2O (1 mL/min) as the eluent.

RESULTS AND DISCUSSION

Cell growth and pH changes of *L. mesenteroides* harboring four different recombinant plasmids Four recombinant plasmids were constructed and they were transformed into *L. mesenteroides* ATCC 8293. The constructed vectors, pLC18lld and pLC42lld, contained *ldhL* gene from *L. plantarum* with its original promoter. While, pLC18pkL and pLC42pkL contained *ldhL* gene fused with native promoter of the pyruvate kinase gene. The growth curves of transformants carrying pLC18lld, pLC42lld, pLC18pkL, and pLC42pkL showed similar growth patterns to the host strain, growing rapidly for the initial 12 h of cell cultivation to reach maximum cell mass (OD_{660nm} 2.5) and a pH of 4.3, which was maintained until 24 h of cultivation (data not shown).

Activities of D-LDH and L-LDH in the host strain and the transformants The enzyme activities of D-LDH and L-LDH of the host strain and its transformants were measured throughout the 48 h culture period. As results, L-LDH activities of the host and its transformants were very different. In the cell lysate of the host strain, L-LDH activity was hardly detectable, elaborating below 0.06 units/mg protein throughout the culture period. In contrast, the highest activity was observed in the transformant harboring pLC42pkL, in which levels increased rapidly to reach a maximum of 0.43 units/mg after 20 h, while the activities of the other transformants were 4–10 fold lower (Fig. 1A). In case of the transformant harboring pLC42pkL, the *ldhL* gene was expressed under the control of the constitutive pyruvate kinase promoter in a theta-type replication vector. The results implicate that both of these factors including the promoter types and the vector-replication modes contribute to the increased L-LDH activity observed. In contrast, no significant differences in D-LDH activities were observed between the host and its transformants (Fig. 1B).