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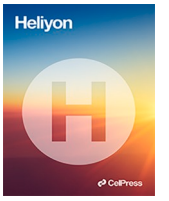

Research article

Transcriptional analysis of the molecular mechanism underlying the response of *Lactiplantibacillus plantarum* to lactic acid stress conditions

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ABSTRACT

Lactic acid bacteria (LAB) present various benefits to humans; they play key roles in the fermentation of food and as probiotics. Acidic conditions are common to both LAB in the intestinal tract as well as fermented foods. *Lactiplantibacillus plantarum* is a facultative homofermentative bacterium, and lactic acid is the end metabolite of glycolysis. To characterize how *L. plantarum* responds to lactic acid, we investigated its transcriptome following treatment with hydrochloride (HCl) or DL-lactic acid at an early stage of growth. Bacterial growth was more attenuated in the presence of lactic acid than in the presence of HCl at the same pH range. Bacterial transcriptome analysis showed that the expression of 67 genes was significantly altered (log2FC $>$ 2 or $<$ 2). A total of 31 genes were up- or downregulated under both conditions: 19 genes in the presence of HCl and 17 genes in the presence of DL-lactic acid. The fatty acid synthesis-related genes were upregulated in both acidic conditions, whereas the lactate racemization-related gene (*lar*) was only upregulated following treatment with DL-lactic acid. In particular, *lar* expression increased following L-lactic acid treatment but did not increase following HCl or D-lactic acid treatment. Expression of *lar* and production of D-lactic acid were investigated with malic and acetic acid; the results revealed a higher expression of *lar* and production of D-lactic acid in the presence of malic acid than that in the presence of acetic acid.

1. Introduction

Kimchi is a representative Korean fermented food consisting of kimchi cabbage, radish, red pepper powder, garlic, ginger, and onion.

Organic acids contribute to the quality and taste of kimchi by creating the typical sour taste and preventing the growth of undesired bacteria by lowering the pH of kimchi [1]. Diverse organic acids, such as lactic acid, malic acid, acetic acid, oxalic acid, citric acid, and succinic acid, have been identified in kimchi. The organic acid content is known to vary with storage temperature and bacterial composition of kimchi. For example, higher temperatures alter the organic acid content more rapidly, and the addition of *Leuconostoc mesenteroides* starter increases lactic acid and acetic acid levels, suggesting the role of LAB in organic acid production [2,3]. Lactic acid is the most abundant organic acid in kimchi, and its content is significantly increased during fermentation (~50 fold), reaching

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5000–9000 mg/kg [2,4].

Lactic acid production in kimchi is closely related to the presence of hetero- and homofermentative LAB strains. The increase in lactic acid content has been found to be consistent with the emergence of *Lactobacillus* [2,4–6].

Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*) is a facultative homofermentative bacterium and is widely used in the production of lactic acid, the end product of carbohydrate fermentation [7]. It has shown potential as a probiotic for acid tolerance and provides diverse health benefits, such as alleviation of inflammatory bowel disease, management of gastrointestinal disorders, prevention of diarrhea, and lowering of cholesterol [7,8].

Lactiplantibacillus plantarum is known to cause over-acidification of kimchi, and it accounts for 80% of the LAB isolated from over-acidified kimchi [9]. It produces DL-lactic acid [10]. The microbe has been identified in kimchi, dairy products, vegetables, meat, silage, wine, and gastrointestinal products, suggesting its ability to adapt to diverse environmental niches [8,11]. This acidic condition affects bacterial adaptation by regulating gene expression, and more interestingly, bacterial genes are differentially regulated by exposure to organic or inorganic acids. A diverse range of bacteria, such as *Listeria monocytogenes*, *Cronobacter sakazakii*, *Staphylococcus aureus*, and *Escherichia coli,* show specific mechanisms of action, in addition to sharing many mechanisms induced by organic acids [12–14]. Organic and inorganic acids show different modes of action while penetrating the cytoplasmic membrane because of the dissociation difference [15]. Therefore, DL-lactic acid could influence gene regulation differently than HCl.

In this study, we characterized the response of *L. plantarum* to lactic acid by analyzing its transcriptome. *L. plantarum* growth and metabolite production in the presence of DL-lactic acid were compared with those in the presence of HCl.

2. Materials and methods

2.1. Bacterial strain and culture conditions

In this experiment, *L. plantarum* WiKim18, which was previously isolated from kimchi [11], was cultured at 30 °C for 16 h in de Man, Rogosa, and Sharpe (MRS) media (Miller, Becton Dickinson, and Co., Sparks, MD, USA). The bacterial culture was harvested by centrifugation at 5000×*g* for 20 min and diluted in MRS medium at an absorbance of 0.05 at 600 nm. To monitor the growth effect in the different pH environments, MRS medium was prepared with the addition of DL-lactic acid (Fisher Scientific, Loughborough, UK), HCl (Daegjung, Gyeonggi-do, Korea), acetic acid (Fluka, Buchs, Switzerland), malic acid (Daegjung, Gyeonggi-do, Korea), D-lactic acid (TCI, Shanghai, China), and L-lactic acid (Sigma-Aldrich, St. Louis, USA). The effect of various organic acids on the growth of *L. plantarum* was measured at 600 nm using a microplate reader (Tecan, Mannedorf, Zurich, Switzerland).

The bacteria were cultivated in MRS media. The pH was adjusted by adding HCl (18.0 mM; pH 5.5, 34.0 mM; pH 5.0 and 55.0 mM; pH 4.5), DL-Lactic acid (24.2 mM; pH 5.5, 48.4 mM; pH 5.0 and 84.6 mM; pH 4.5), acetic acid (1.4 mM; pH 5.5, 3.6 mM; pH 5.0 and 10.4 mM; pH 4.5), malic acid (11.0 mM; pH 5.5, 27.0 mM; pH 5.0 and 52.9 mM; pH 4.5), D-lactic acid (25.4 mM; pH 5.5, 53.1 mM; pH 5.0 and 100.2 mM; pH 4.5) and L-lactic acid (20.9 mM; pH 5.5, 43.0 mM; pH 5.0 and 80.9 mM; pH 4.5).

2.2. RNA preparation and transcriptome analysis

To evaluate the global gene expression of *L. plantarum* under acidic conditions (DL-lactic acid pH5.5 and HCl 5.5), the bacterial culture was harvested by centrifugation at 5000×*g* for 20 min, diluted with MRS medium after adjusting the pH with organic acids to an absorbance of 0.05 at 600 nm, and further incubated at 30 °C for 4 h. Total RNA was extracted using RNeasy Mini kits (Qiagen, USA) and DNaseI (Invitrogen, Waltham, USA) according to the manufacturer's instructions. The depletion of remaining DNA was validated using qRT-PCR before library preparation. To construct the sequencing libraries, 1 μg of total RNA was used. Libraries for Illumina sequencing were prepared using the TruSeq RNA Library Prep kit (Illumina, USA) following the manufacturer's protocol. Transcriptome analysis was performed at Macrogen Inc. (Seoul, Korea) using Illumina Hiseq 2000 (Illumina, Inc., San Diego, CA, USA) with paired-end (2 × 100 bp) sequencing. Raw reads from the sequencer were used to remove low-quality and adapter sequences, and the processed reads were aligned to the reference genome sequence, *L. plantarum* WCFS1 (GCF_000203855.3, *L. plantarum* WCFS1), using HISAT [16]. The known transcripts were assembled using StringTie v2.1.3b [17,18]. The expression abundance of transcripts and genes was calculated as read count or fragments per kilobase of exon per million fragments mapped (FPKM) value per sample.

The relative transcript abundance was measured in FPKM reads. The Non-supervised Orthologous Groups (eggNOG), Gene Ontology Biological Process (GOBP), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to analyze functionally related groups and metabolic pathways. Differentially expressed genes (DEGs) and clusters were calculated using edgeR and t-SNE in the R package. Transcriptome data were normalized to z-scores, and hierarchical clustering was performed. Gene ontology (GO) and KEGG terms and metabolic pathways were enriched with significantly up- or downregulated genes in the cluster (enrichment with ENSG option with $q < 0.05$ with FDR).

2.3. Quantitative RT-PCR

Gene expression levels were measured using quantitative real-time polymerase chain reaction (qRT-PCR). The bacteria were cultured for another 4 h in MRS medium, and the pH was adjusted with DL-lactic acid, D-lactic acid, L-lactic acid, and HCl. RNA (1 μg) was reverse-transcribed, cDNA was generated, and real-time polymerase chain reaction (qRT-PCR) was performed using TB Green Premix Ex Taq II (TaKaRa, Shiga, Japan). Relative expression levels were calculated and normalized to that of the 16S rRNA gene. Gene expression was quantified using RT-PCR. The relative expression was calculated using the ΔΔCT method. Primers were designed based

on the nucleotide sequences of *L. plantarum* in the NCBI database (GCF_000203855.3, *L. plantarum* WCFS1) (Supplementary Table 1).

2.4. Quantification of metabolites

To quantify the glucose content, the bacterial culture supernatant extract was analyzed using a TripleTOF 5600 plus instrument (SCIEX, Framingham, MA, USA) coupled with an Acquity UPLC system (Waters, Milford, MA, USA). Glucose was quantified in negative

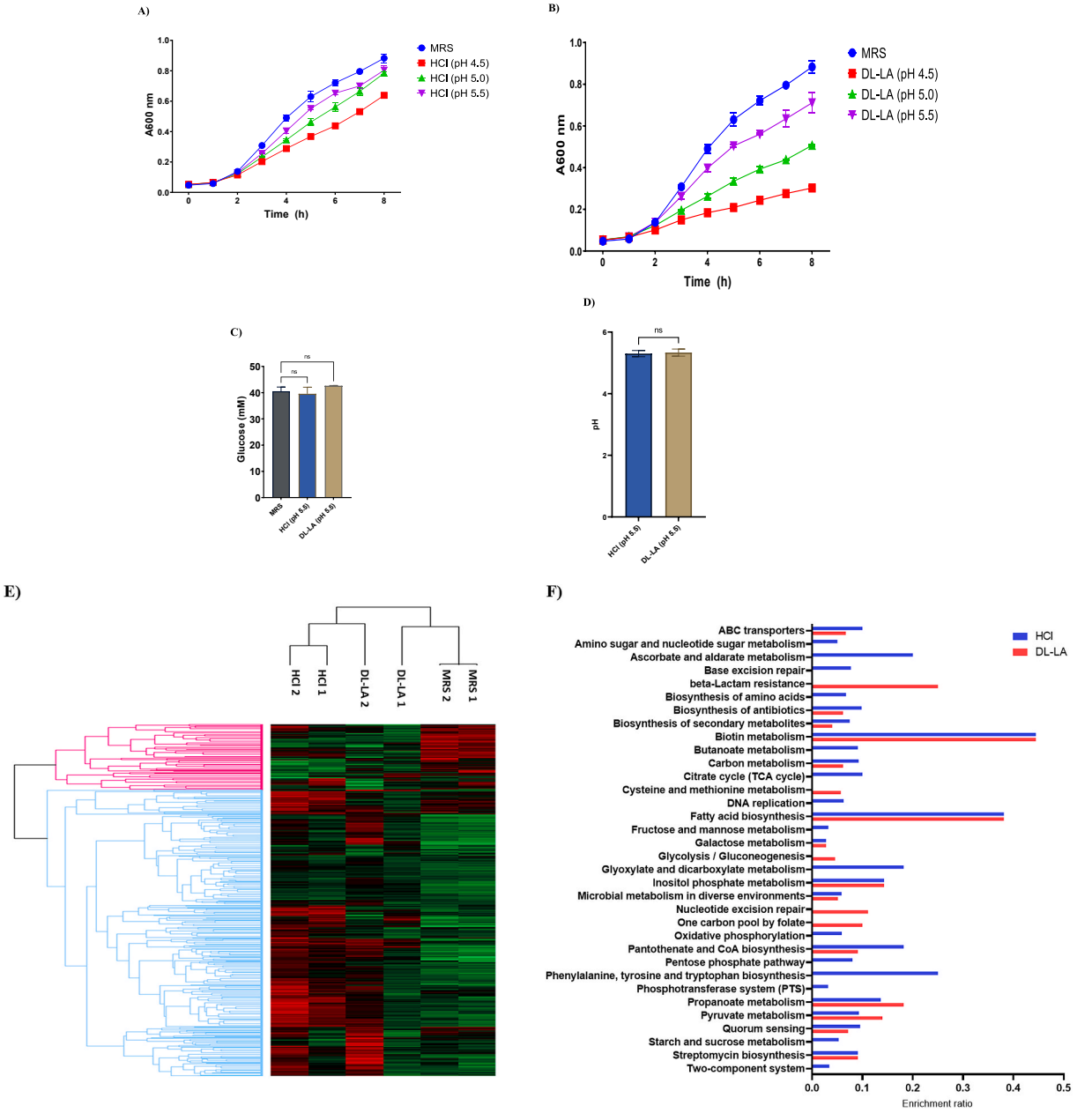

Fig. 1. Growth of L. *plantarum* and transcriptional profiles under HCl and lactic acid treatment. The bacteria were cultured at 30 °C in MRS media in which pH was adjusted with HCl or lactic acid, and bacterial growth was monitored spectrophotometrically at 600 nm. A) HCl, B) lactic acid ●: MRS control, ▼ pH 5.5, ▲: pH 5.0, ■: pH 4.5. C) Glucose content after 4 h of bacterial culture was measured by LC-MS. Significance was calculated by comparison with MRS as the control and represented as NS; non significant. D) pH after 4 h of bacterial culture. E) Transcriptome data were visualized by a heatmap using the z-score value of the transcriptome. F) Enrichment and KEGG pathway analyses were performed to understand the functions of the differentially expressed genes (DEGs). The enrichment was performed using KOBAS (http://kobas.cbi.pku.edu.cn/genelist/). Red: upregulated genes in the DL -LA (logFC>1), blue: upregulated in HCl (log2FC > 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

multiple reaction monitoring (MRM) mode with spray voltage of −4.5 kV, scan rate of 10 spectra/sec, collision energy of −30 eV, source temperature of 300 °C, and mass range of m/z 50–1000. MRM MS generated the following transitions: glucose m/z 180 > 89, salicin ((internal standard), m/z 285 > 123. A reversed-phase column (Acquity UPLC BEH C18 column 2.1 × 100 mm, 1.7 μm particle size; Waters) was used to separate the compounds. The mobile phase consisted of distilled water (solvent A) and acetonitrile (solvent B), containing 10 mM ammonium acetate, at a flow rate of 0.3 ml/min. The metabolite peak areas obtained were normalized to those of the internal standard using the SCIEX OS software (SCIEX, Framingham, MA, USA).

The D-/L-lactic acid contents were measured using a D-/L-lactic acid assay kit (Megazyme, Bray, Ireland) following the manufacturer's protocol, and the produced NADH was measured at an absorbance of 340 nm.

2.5. Statistical analysis

Data are presented as mean ± standard deviation. Two-way analysis of variance was performed using GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA), and Tukey's multiple comparison test to compare means.

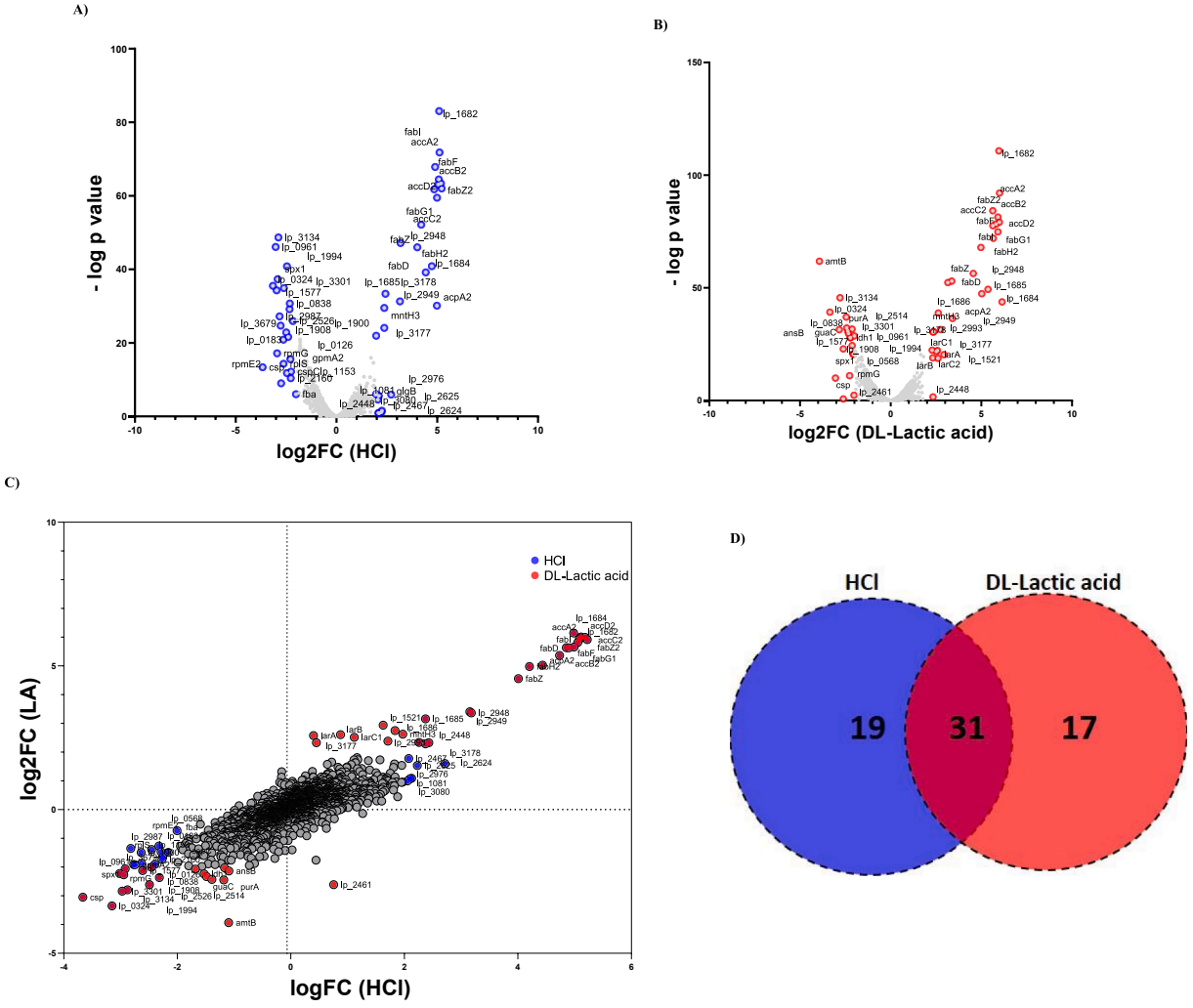

Fig. 2. Global comparison of transcriptional profiles and DEGs under HCl and lactic acid treatment. Global gene expression was analyzed using a scatterplot of statistical significance (p-value) versus gene expression fold changes following treatment with A) HCl and B) lactic acid. C) Global comparison of gene expression fold changes in the transcriptome (HCl vs. lactic acid). Blue dots and red dots represent up- or downregulated genes (log2FC > 2 or < 2) following HCl or lactic acid treatment, respectively. D) Venn diagram showing the comparison of genes under different acid stresses (log2FC > 2 or < 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Major transcriptional changes in *Lactobacillus plantarum* cultured in MRS medium (pH 5.5 adjusted with HCl or DL-lactic acid).

Gene name	(HCl-control)			(Lactic acid-control)			Product	ENSG ID	eggNOG ID
	log2FC	p-value	q (FDR)	log2FC	p-value	q (FDR)			
lp_1684	4.98	7.90E-31	8.12E-29	6.14	1.77E-44	2.53E-42	membrane protein	lp_1684	COG1811
accA2	5.11	1.59E-72	2.04E-69	6.01	8.24E-93	1.06E-89	acetyl-CoA carboxyl transferase	lp_1680	COG0825
accD2	5.19	4.50E-64	2.31E-61	5.99	7.62E-80	3.92E-77	acetyl-CoA carboxylase	lp_1679	COG0777
lp_1682	5.10	9.29E-84	2.39E-80	5.97	1.58E-111	4.05E-108	phosphopantetheinyl transferase	lp_1682	COG2091
fabZ2	5.22	1.03E-62	3.77E-60	5.92	1.46E-75	4.70E-73	hydroxymyristoyl-ACP dehydratase	lp_1677	COG0764
fabI	5.08	3.56E-65	2.29E-62	5.92	4.07E-82	2.61E-79	acyl-carrier proteinreductase	lp_1681	COG0623
accC2	5.06	8.15E-64	3.49E-61	5.81	5.19E-79	2.22E-76	acetyl-CoA carboxylase	lp_1678	COG0439
accB2	4.99	3.46E-60	9.89E-58	5.66	1.07E-72	3.06E-70	acetyl-CoA carboxylase	lp_1676	COG0511
fabG1	4.85	1.73E-62	5.57E-60	5.64	2.75E-78	1.01E-75	3-oxoacyl-ACP reductase	lp_1674	COG1028
fabF	4.90	1.40E-68	1.20E-65	5.63	6.29E-85	5.39E-82	3-oxoacyl-ACP synthase	lp_1675	COG0304
fabD	4.73	1.41E-41	2.41E-39	5.37	4.42E-50	7.58E-48	acyl-carrier protein S-malonyltransferase	lp_1673	COG0331
acpA2	4.43	6.81E-40	1.03E-37	5.02	4.02E-48	6.46E-46	acyl carrier protein	lp_1672	COG0236
fabH2	4.21	7.48E-53	1.92E-50	4.98	1.17E-68	3.01E-66	3-oxoacyl-ACP synthase	lp_1671	COG0332
fabZ	4.01	1.00E-46	1.84E-44	4.56	4.41E-57	9.45E-55	hydroxyacyl-acyl carrier protein	lp_1670	COG0764
lp_2949	3.15	5.23E-32	5.84E-30	3.40	3.61E-37	4.21E-35	membrane protein	lp_2949	COG4640
lp_2948	3.18	6.89E-48	1.48E-45	3.36	1.02E-53	2.02E-51	hypothetical protein	lp_2948	COG4640
lp_1685	2.37	3.12E-30	3.09E-28	3.16	4.45E-53	8.16E-51	LysR family transcriptional regulator	lp_1685	COG0583
lp_1521	1.63	3.47E-06	4.70E-05	2.94	4.50E-21	2.57E-19	alcohol dehydrogenase	lp_1521	COG0604
lp_1686	1.84	2.82E-15	1.39E-13	2.75	3.12E-32	3.20E-30	acyl-CoA hydrolase	lp_1686	COG1607
mntH3	1.97	1.18E-22	8.90E-21	2.62	1.45E-39	1.86E-37	manganese transport protein	lp_2992	COG1914
larB	0.88	0.0216803	0.0672928	2.61	1.81E-19	8.94E-18	lactate racemization operon protein	lp_0105	COG1691
larA	0.40	0.281845	0.453563	2.57	6.77E-23	4.70E-21	lactate racemization operon protein	lp_0104	COG3875
larC1	1.12	0.000485	0.0031477	2.52	1.65E-22	1.09E-20	lactate racemization operon protein	lp_0106	COG1641
lp_2993	1.71	1.22E-16	6.55E-15	2.38	3.08E-31	2.82E-29	nucleotide-binding protein	lp_2993	COG0589
lp_2448	2.26	0.0285048	0.0826832	2.34	0.0237935	0.0773064	prophage P2a protein regulator	lp_2448	COG1396
lp_3178	2.43	4.62E-34	5.39E-32	2.33	4.31E-31	3.82E-29	extracellular protein	lp_3178	ENOG502DPC1
larC2	0.45	0.148891	0.291432	2.33	1.11E-19	5.83E-18	lactate racemization operon protein	lp_0107	COG1641
lp_3177	2.37	8.54E-25	6.86E-23	2.29	4.70E-23	3.35E-21	membrane protein	lp_3177	ENOG502E61F
lp_2467	2.08	0.123991	0.256568	1.78	0.215001	0.386514	prophage P2b protein terminase	lp_2467	COG3747
lp_2624	2.71	1.18E-06	1.83E-05	1.60	0.0192774	0.0657068	hypothetical protein	lp_2624	ENOG5029PD5
lp_2625	2.23	0.0719777	0.169865	1.53	0.272011	0.450721	hypothetical protein	lp_2625	ENOG502AXGE
lp_3080	2.13	0.126467	0.258979	1.09	0.504812	0.671862	membrane protein	lp_3080	COG4858
cscD	2.10	2.73E-06	3.85E-05	1.07	0.0467974	0.128768	cell surface protein	lp_2976	ENOG5029QI7
lp_1081	2.05	2.66E-05	0.0002631	1.00	0.0914476	0.20984	hypothetical protein	lp_1081	COG3391
lp_0183	−2.33	7.29E-30	6.94E-28	0.52	0.498192	0.667339	membrane protein	lp_0183	COG4095
fba	−2.01	9.50E-07	1.52E-05	−0.73	0.0649089	0.164189	fructose-bisphosphate aldolase	lp_0330	COG0191
lp_2987	−2.82	6.34E-28	5.82E-26	−1.35	3.22E-08	5.99E-07	hypothetical protein	lp_2987	ENOG5029PH8
lp_1153	−2.45	1.59E-12	6.19E-11	−1.39	3.29E-05	0.0003143	TetR family transcriptional regulator	lp_1153	COG1309
rpmE2	−2.29	2.62E-16	1.37E-14	−1.47	6.65E-08	1.15E-06	50S ribosomal protein L31	lp_0512	COG0254
lp_2526	−2.16	1.16E-26	9.93E-25	−1.49	4.32E-14	1.61E-12	GNAT family acetyltransferase	lp_2526	COG0454
lp_1900	−2.64	1.35E-21	9.35E-20	−1.50	1.59E-08	3.07E-07	membrane protein	lp_1900	ENOG502DGWH
cspC	−2.26	6.84E-13	2.79E-11	−1.66	7.39E-08	1.27E-06	cold shock protein CspC	lp_0997	COG1278
lp_2160	−2.27	4.18E-11	1.30E-09	−1.74	2.65E-07	3.95E-06	hypothetical protein	lp_2160	ENOG5029PG6
rplS	−2.63	4.97E-15	2.36E-13	−1.87	1.07E-08	2.12E-07	50S ribosomal protein L19	lp_1640	COG0335
lp_0126	−2.40	2.46E-22	1.81E-20	−1.90	3.79E-15	1.50E-13	stress-responsive transcription regulator	lp_0126	COG1983
cscB	−2.77	2.09E-25	1.73E-23	−1.91	6.99E-14	2.50E-12	cell surface protein	lp_3679	ENOG502BM49
gpmA2	−2.75	9.82E-10	2.77E-08	−1.92	1.05E-05	0.0001136	phosphoglycerate mutase family protein	lp_3170	COG0588
lp_1994	−2.45	1.51E-41	2.43E-39	−2.01	1.98E-29	1.64E-27	hypothetical protein	lp_1994	ENOG5029P3S
lp_2488f	−1.17	0.0524792	0.131721	−2.02	0.0043471	0.0205745	hypothetical protein	lp_2488f	ENOG5029QK0
spx1	−2.92	4.65E-38	6.64E-36	−2.06	3.30E-21	1.97E-19	arsenate reductase activity	lp_0836	COG1393
lp_0568	−1.68	4.60E-15	2.23E-13	−2.07	2.56E-21	1.57E-19	hypothetical protein	lp_0568	ENOG502E7AN
lp_1577	−2.61	1.29E-35	1.65E-33	−2.12	4.15E-25	3.14E-23	hypothetical protein	lp_1577	ENOG502C91 M
ansB	−1.09	2.76E-10	8.07E-09	−2.13	1.98E-32	2.12E-30	aspartate ammonia-lyase	lp_2830	COG1027
lp_0961	−3.02	8.01E-47	1.58E-44	−2.22	1.45E-28	1.13E-26	transposase	lp_0961	COG3415
ldh1	−1.53	5.43E-15	2.54E-13	−2.24	1.06E-28	8.48E-27	L-lactate dehydrogenase	lp_0537	COG0039

(*continued on next page*)

Table 1 (*continued*)

Gene name	(HCl-control)			(Lactic acid-control)			Product	ENSG ID	eggNOG ID
	log2FC	p-value	q (FDR)	log2FC	p-value	q (FDR)			
rpmG	−2.94	7.27E-18	4.06E-16	−2.27	9.48E-12	2.77E-10	50S ribosomal protein L33 type 1	lp_1569	COG0267
lp_2514	−1.48	1.05E-15	5.41E-14	−2.33	1.58E-30	1.36E-28	carbohydrate transporter	lp_2514	COG0477
lp_0838	−2.32	2.03E-31	2.17E-29	−2.36	1.33E-31	1.27E-29	membrane protein	lp_0838	COG4767
guaC	−1.39	6.47E-14	2.82E-12	−2.43	6.87E-33	7.68E-31	GMP reductase	lp_3271	COG0516
purA	−1.18	4.76E-12	1.72E-10	−2.45	9.63E-38	1.18E-35	adenylosuccinate synthase	lp_3270	COG0104
lp_1908	−2.49	1.45E-23	1.13E-21	−2.61	1.26E-23	9.24E-22	membrane protein	lp_1908	COG3307
lp_2461	0.76	0.58759	0.732687	−2.61	0.191277	0.356476	prophage P2b protein 20	lp_2461	ENOG5029Q57
lp_3134	−2.88	2.10E-49	4.90E-47	−2.79	2.42E-46	3.66E-44	extracellular protein	lp_3134	COG5294
lp_3301	−2.97	5.07E-35	6.20E-33	−2.84	3.52E-32	3.48E-30	bacteriocin transport protein	lp_3301	COG1286
csp	−3.66	4.38E-14	1.94E-12	−3.05	1.10E-10	2.90E-09	cold shock protein	lp_1160	COG1278
lp_0324	−3.15	2.95E-36	3.99E-34	−3.35	5.89E-40	7.97E-38	hypothetical protein	lp_0324	ENOG5029PNG
amtB	−1.10	6.36E-08	1.34E-06	−3.93	1.56E-62	3.65E-60	ammonium transport protein	lp_0349	COG0004

DEGs were analyzed using edgeR in the R package. Categorically annotated Gene Ontology Biological Process (GOBP) and eggNOG functional category (http://eggnog5.embl.de/#/app/home).

3. Results

3.1. Bacterial growth and transcriptome analysis

To evaluate the effect of lactic acid on the growth of *L. plantarum*, we cultured the bacteria in MRS media with pH adjusted using HCl or lactic acid and monitored the growth spectrophotometrically. Bacterial growth was inhibited by the addition of HCl and DL-lactic acid. DL-lactic acid treatment inhibited bacterial growth to a greater extent than HCl treatment did, at the same pH (Fig. 1A and B).

To investigate the molecular mechanisms involved under lactic acid conditions, we performed transcriptomic analysis using RNA-seq technology. To avoid transcriptome differences in bacterial growth, we isolated the RNA of bacteria present in DL-lactic acid or HCl-adjusted MRS media (pH 5.5) after a 4 h culture. Bacterial growth was found to be similar, and there was no significant difference ($p > 0.05$) in glucose content after 4 h incubation between the different media (Fig. 1C). The pH after 4h was similar (Fig. 1D).

Transcriptome data analysis was performed using FPKM values of the processed data. The expression value was normalized to a z-score and hierarchical clustering was performed and classified into two categories as up-regulated genes and down-regulated genes (Fig. 1E).

DEGs were calculated using the "edgeR" package in R (Supplementary Table 2). Categorical enrichment analysis was performed using KOBAS for KEGG pathway functional enrichment analyses of the DEGs [19]. The results of the gene enrichment showed that the biotin synthesis, fatty acid biosynthesis, and ABC transporter were positively affected in acid-stress conditions; several amino acid and carbohydrate metabolisms were differently effected (Fig. 1F).

Highly up- or downregulated genes were compared (Fig. 2A–D). DEG analysis showed that 67 genes were expressed >2 or < −2 in log2FC in the HCl or DL-lactic acid treatment (Table 1).

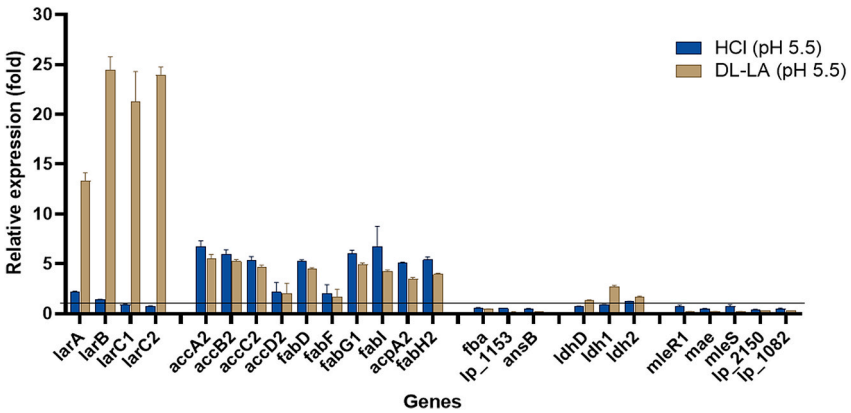

Fig. 3. qRT-PCR analysis of gene expression profiles. Genes expression were quantified using real-time polymerase chain reaction (RT-PCR). The relative expression was calculated using the ΔΔCT method. The red line represents a value of 1 for the relative expression (fold). All expression data were normalized to 16S rRNA. Results are expressed as mean of fold change compared with HCl conditions (SD as error bars, n = 3). Comparison with sustained stimulation by two-way ANOVA: *, $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Both acidic conditions increased the expression of acetyl-CoA carboxylase, acyl carrier proteins (lp_1680, lp_1679, lp_1676, lp_1678), oxoacyl-ACP reductase (lp_1674), oxoacyl-ACP synthase (lp_1675, lp_1671), acyl-CoA hydrolase (lp_1686), and membrane proteins (lp_3177, lp_2949, lp_1684). Interestingly, the expression of lactate racemization-related genes (lp_0105, lp_0104, lp_0106, and lp_1017) increased only following DL-lactic acid treatment (Table 1).

The results of the transcriptome analysis were validated using qRT-PCR. Gene expression data were similar to those of the transcriptome analysis. *larA* expression increased by 2.2 ± 0.1 following HCl treatment and to 13.3 ± 0.8 following lactic acid treatment. The lar genes related to lactate racemization, *larB*, *larC1*, and *larC2*, showed significantly increased expression in the DL-lactic acid-treated group only. Expression of acetyl-CoA synthase-related genes (*accA2*, *accB2*, *accC2*, *accD2*, *fabD*, *fabF*, *fabG1*, *fabI*, *acpA2*, and *fabH2*) increased under both acidic conditions.

We further quantified the genes related to lactic acid metabolism: lactate dehydrogenase-encoding genes (*ldhD*, *ldh1*, and *ldh2*) and malolactic acid fermentation-related genes (*mleR1*, *mae*, *melS*, malate-lactate hydroxyisocapronate dehydrogenase (lp_2150), and malate-lactate dehydrogenase (lp_1082)). *ldh* expression decreased under both acidic conditions, but qRT-PCR showed a 1.3-to 2.7-fold increase under DL-lactic acid treatment. Malolactic acid fermentation-related gene expression was decreased under both acidic conditions, according to both transcriptome and qRT-PCR data (Fig. 3).

The RNA-seq data discussed in this paper have been deposited in NCBI's Gene Expression Omnibus [20] and are accessible through the GEO Series accession number GSE211635 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211635).

3.2. Effect of diverse organic acids on D-lactic acid production

We further investigated the effects of organic acids on the expression of *lar* genes and D-lactic acid production by *L. plantarum*. The effects of isomeric lactic acid on bacterial growth and gene expression were also quantified. Bacterial growth was inhibited by D- and L-lactic acid treatment, and growth was inhibited to a larger extent than that following HCl treatment. However, no differences were observed between D- and L-lactic acid treatments in terms of bacterial growth inhibition (Fig. 4A and B).

The expression of *lar* genes was quantified by qRT-PCR and a distinct expression profile was shown among the D-lactic acid and L-lactic acid treatments. L-lactic acid significantly increased the expression of *lar* genes. *larA* expression was increased by 14.5 ± 1.0 fold

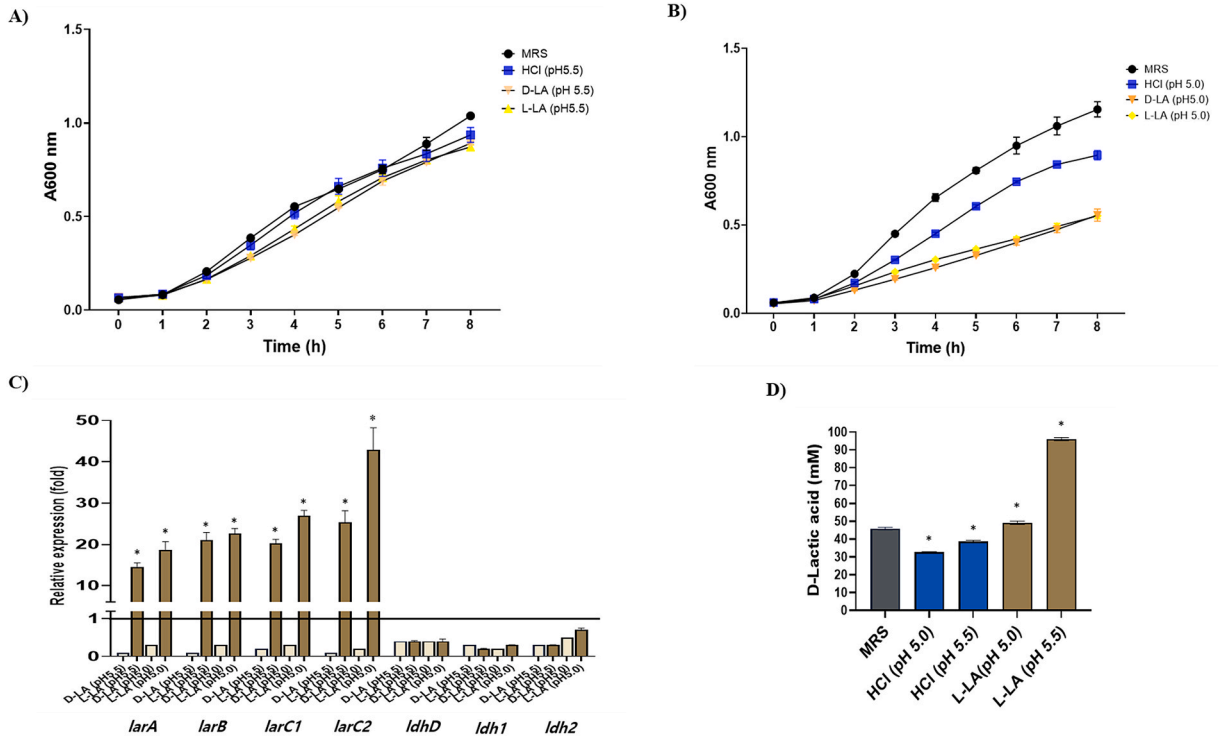

Fig. 4. *L. plantarum* growth and gene expression under D-lactic acid and L-lactic acid treatment. The bacteria were cultured at 30 °C in MRS media in which pH was adjusted to A) pH 5.5 and B) pH 5.0 with D - or L -lactic acid and bacterial growth was monitored spectrophotometrically at 600 nm ●: MRS (control), ■: HCl, ▼: D-lactic acid ▲: L-lactic acid. C) qRT-PCR analysis of *lar, ldh* expressions following D-lactic acid (D-LA) or L-lactic acid (L-LA) treatment with pH 5.5 and 5.0. The red line represents a value of 1 in relative expression (fold). Bar heights (error bars) represent means (SDs) from three independent repeats of real-time PCR. Significance was calculated by comparison with D-LA as the control and represented as *$p <$ 0.05. D) D-lactic acid production following HCl and L-lactic acid (L-LA) treatment with pH 5.0 and 5.5 conditions. LC/MS/MS Data are the means ± S.D. (n = 3). *, $p < 0.05$ compared with the control (MRS) group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

by L-lactic acid (pH 5.5) and 18.6 ± 2.0 fold by L-lactic acid (pH 5.0) treatment. However, D-lactic acid treatment did not induce the expression of *lar* genes (Fig. 4C).

We then compared D-lactic acid production from the cultures with pH adjusted using HCl or L-lactic acid. As shown in Fig. 4D, L-lactic acid treatment significantly increased the D-lactic acid content ($p < 0.05$). L-lactic acid treatment increased the D-lactic acid content to 49.1 ± 0.9 mM at pH 5.0 and 96.0 ± 0.8 mM at pH 5.5, and HCl treatment increased the D-lactic acid content to 32.7 ± 0.2 mM at pH 5.0 and 38.7 ± 0.6 mM at pH 5.5. *ldh* gene expression did not increase with the addition of L- or D-lactic acid to MRS media (Fig. 4C).

We also investigated the effects of other organic acids abundantly found in kimchi on bacterial growth and gene expression. We prepared MRS medium with malic and acetic acid. Both the organic acids inhibited the growth of *L. plantarum*, which was more attenuated by the addition of acetic acid than by the addition of malic acid at the same pH (Fig. 5A and B). The expression of *lar* and *ldh* genes were compared using qRT-PCR. The expression of *lar* was significantly higher following malic acid treatment than that following acetic acid treatment. *larA* expression was increased by 1.4 ± 0.1-fold under acetic acid treatment but increased by 26.6 ± 2.5-fold under malic acid treatment (Fig. 5C). *ldh* expression did not change significantly with acetic acid or malic acid treatment. However, *ldh* showed differential expression patterns in response to acetic acid and malic acid treatments. *ldhD* and *ldh2* expression was increased by 1.6 ± 0.1- and 2.1 ± 0.2-fold following treatment with acetic acid and malic acid, respectively, but *ldh1* expression was decreased by 0.6 ± 0.1- and 0.7 ± 0.1-fold following treatment with acetic acid and malic acid, respectively. The D-lactic acid content following malic acid treatment (31.9 ± 0.8 mM) was higher than that following acetic acid treatment (26.0 ± 0.1 mM), which was consistent with the results of *lar* gene expression following acetic acid and malic acid treatment.

4. Discussion

Lactiplantibacillus plantarum is a type of homo-fermentative LAB and can metabolize hexose and pentose to lactic acid [21]. *L. plantarum* is generally recognized as safe and is widely used in probiotics, food additives, and industrial lactic acid production [8,

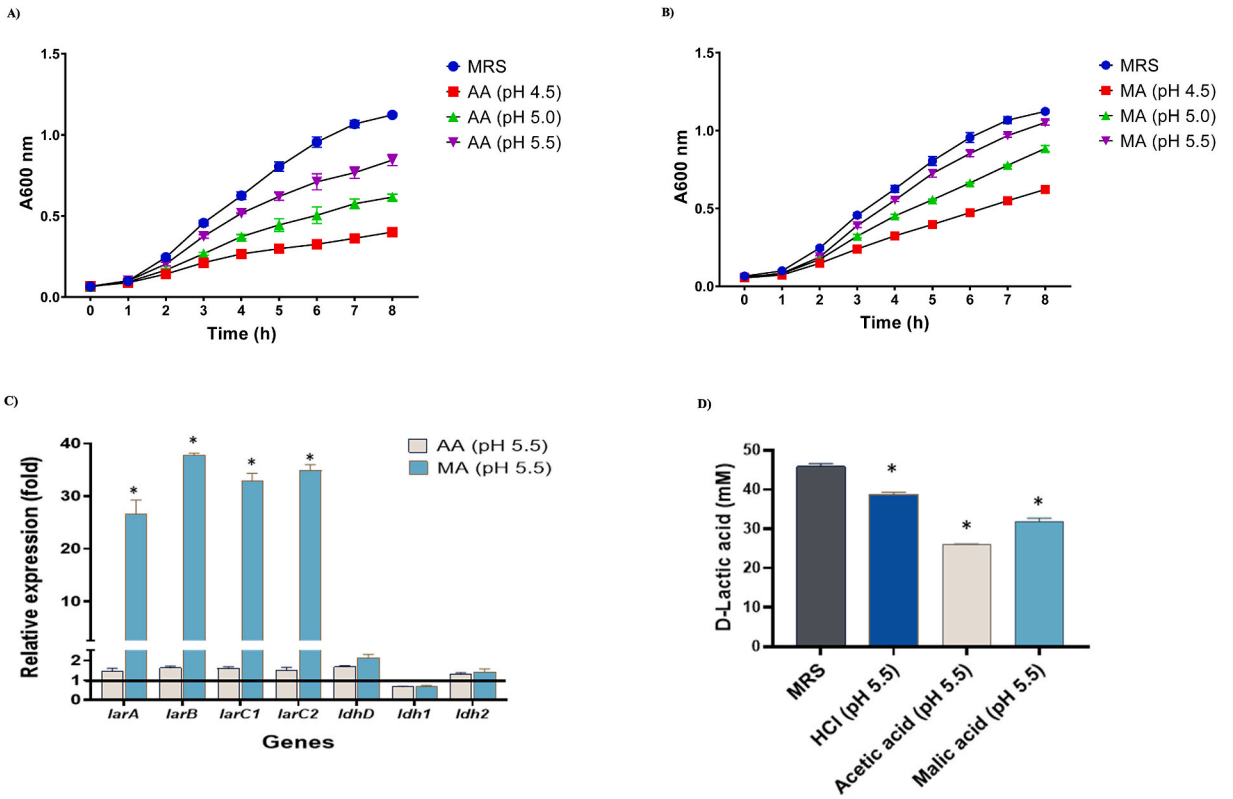

Fig. 5. *L. plantarum* growth and gene expression under acetic acid and malic acid treatment. The bacteria were cultured at 30 °C in MRS medium with pH adjusted using A) acetic acid and B) malic acid and bacterial growth was monitored spectrophotometrically at 600 nm ●: MRS control, ■: pH 4.5, ▲: pH 5.0, ▼ pH 5.5. C) qRT-PCR analysis of *lar, ldh* expressions after acetic acid (AA) or malic acid (MA) treatment with pH 5.5 condition. The red line represents a value of 1 for relative fold expression. Error bars represent the standard deviation of three biological replicates. Significance was calculated by comparison with AA as the control and represented as $*p < 0.05$. D) D-lactic acid production in MRS at pH 5.5 adjusted with HCl, AA, and MA. LC/MS/MS data represent the three experimental samples analyzed. Significance was calculated by comparison with MRS as the control and represented as $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

22]. Overall, *L. plantarum* is more resistant to lactic acid than other LAB [23].

In this study, we investigated the molecular mechanisms underlying lactic acid production by comparing the bacterial transcriptome under HCl and lactic acid conditions. We observed that *L. plantarum* growth was inhibited more by DL-lactic acid treatment than by HCl treatment at the same pH (pH 4.5–5.5) (Fig. 1). This result is consistent with previous findings on the toxic effects of organic acids on bacteria [24]. Non-dissociated forms of organic acids diffuse across the bacterial cell membrane and differentially inhibit bacterial growth [13,15,25].

In this study, enrichment showed that the biotin metabolism, fatty acid biosynthesis, and ABC transporter function was effected under both acidic conditions (Fig. 1F). Biotin is an essential cofactor for diverse metabolic functions including fatty acid biosynthesis [26]. Our previous study also showed that the ABC transporter and fatty acid biosynthesis function was affected by lactic acid treatment [27]. Previous studies have shown the response of genes to the acidic stress conditions. Transcriptome analysis of *L. plantarum* ATCC 14917 in acidic pH showed upregulated carbohydrate metabolism and fatty acid synthesis genes [28]. *Lactobacillus casei* ATCC 334 in the acidic condition (with HCl) showed that the carbohydrate function was up-regulated and the fatty acid content changed [29].

To investigate the genes involved in lactic acid stress, we performed transcriptional analysis and compared the DEGs between HCl and DL-lactic acid treatment conditions at the same pH (pH 5.5) (Fig. 2A–C). The gene expression profile showed that 67 genes were significantly altered (FC $>$ 2 or $<$ 2) after treatment with HCl or DL-lactic acid. A total of 31 genes were up- or downregulated under both conditions, and 17 genes showed altered expression only following lactic acid treatment (Fig. 2D). Fatty acid metabolism-related genes, such as those encoding acetyl-CoA carboxylase and oxoacyl ACP reductase/synthase acyl carrier proteins, were highly upregulated under both acidic conditions and lactate racemization genes were only upregulated under DL-lactic acid conditions (Table 1 and Fig. 3).

This also suggested that the composition of the cell membrane improves the resistance of the cells to acid stress [30]. A study on lactic acid stress on *L. plantarum* L2 showed DEGs in the metabolic function and enriched protein processing of phosphates and ABC transporters [30]. *L. plantarum* WCFS1 transcriptional analysis under acidic conditions (with lactic acid) showed increased carbohydrate metabolism and cell surface proteins [31]. *L. plantarum* ZDY2013 transcriptome analysis under acidic conditions (with HCl) showed the down-regulation of transporter function. *Lactobacillus rhamnosus* LGG under acidic conditions (pH 4.5) also showed the up-regulation of transporter and down-regulation of carbohydrate metabolism genes. However, previous studies have not discussed the details of the acidic condition. Therefore, it remains unclear whether the transcriptional changes are due to acidity, organic acid, or inorganic acid stress.

In this study, we found that several membrane-associated proteins were up- or down-regulated under both acidic conditions. Membrane proteins (lp_1684, lp_3080, lp_2949, lp_3178, lp_3177, lp_0309, lp_1946), amino acid transporter (lp_3278), and manganese ABC transporter (lp_2992) were up-regulated, and cold shock protein (lp_1160, lp_0997), carbohydrate proton transporter (lp_1792), and ammonium transporter (lp_0349) were down-regulated under both acidic conditions (Supplementary Table 2). It is reported that cell membrane and lipid physiology were related to the stress and modulation of integrity fluidity and lipid composition, which act as protection against acids [32]. Our results are consistent with previous findings, and suggest that both organic and inorganic stress conditions can impact membrane function.

The genes differently expressed at DL-lactic acid treatment and HCl may be more relevant within fermentation environments. Interestingly, the expression of lactate racemization genes increased only under treatment with DL-lactic acid (Table 1 and Fig. 3). This trend may be explained by the ability of *Lactobacillus* species to produce and accumulate the L (+)-isomer, which results in the conversion of lactic acid into the D (−)-isomer until equilibrium is reached and a racemic mixture is formed [7].

A previous study showed that the D-/L-lactic acid content was similar at the initial stage of fermentation, but the D-lactic acid content was significantly higher than the L -lactic acid content during the fermentation [33]. This study supports that L-lactic acid production enhance *lar* gene expression, and this contributes to the increase in D-lactic acid content in kimchi.

The *lar* gene is abundant in *Lactobacillus* spp. For example, *larA* is found in 25% of *Lactobacillaceae* species and 9% of *Leuconostocaceae* species [34]. *L. plantarum lar* is composed of genes that encode lactate racemase (*larA*), cofactor biosynthetic enzymes (*larB*, *larC* and *larE*), lactic acid channel (*larD*), transcriptional regulator (*LarR*), and ATP-binding cassette (ABC) transporter systems, with two adjacent gene clusters transcribed in opposite directions [34]. Gene expression was regulated by the transcriptional regulator *LarR*. L-lactic acid triggers the binding and multimerization of *LarR,* and D-lactic acid may act to prevent the L-lactic acid binding [35].

L. plantarum uses *lar* to produce D-lactic acid at a low growth rate. Under conditions of zero growth, the D-*ldh*-encoding gene in *L. plantarum* is downregulated, whereas *lar* genes are upregulated [36]. *lar* is also important for cell wall biosynthesis, as it need D-lactic acid [36,37].

In this study, we also observed similar gene expression, whereby upregulation of *lar* expression was only increased by L-lactic acid treatment (Fig. 4). A previous study suggested that *larR* binds to D-lactic acid and prevents the binding of L-lactic acid [38]. A study on the *ldhL* deletion mutant showed that an excess amount of L-lactic acid increases the expression of the acetyl-CoA carboxylase gene (*accA-accD*). Acetyl-CoA is an important element of fatty acid synthesis and intermediate in the acetic acid to lactic acid bioconversion in *L. plantarum* [39]. However, the *larR* box was not identified upstream of *Acc* [35]. In this study, we found that *acc* gene expression increased with HCl and DL-lactic acid treatment, suggesting that the increase in *acc* gene expression was not related to the lactic acid regulon, which is regulated by *larR*. However, the *larR* box was found upstream of genes not related to lactate or carbon metabolism, suggesting that lactic acid could act as a global gene regulator in *L. plantarum* [35].

Organic acid production is a common property among LAB, and *L. plantarum* lactate homeostasis is important for survival and adaptation in fermentative environments. During kimchi fermentation, malic acid, acetic acid, and lactic acid were the most abundant organic acids, and malic acid content was significantly reduced at Week 1, whereas lactic and acetic acid contents increased, during

fermentation [2,40].

Malic acid and acetic acid are the most abundant organic acids in kimchi. Furthermore, *L. plantarum*, which converts malic acid to lactic acid, was used for malolactic acid fermentation. *Lactiplantibacillus plantarum* also converts lactic acid to acetic acid [41–43]. Therefore, we investigated the bacterial growth and *lar* gene expression of *L. plantarum* treated with malic acid and acetic acid. Both organic acids inhibited bacterial growth (Fig. 5). The *lar* gene expression and D-lactic acid production were higher following malic acid treatment than that following acetic acid treatment (Fig. 5C and D). This suggests that *lar* may be involved in the malolactic acid fermentation pathway via lactic acid conversion. However, the production of D-lactic acid following treatment with malic acid was lower than that following HCl treatment, suggesting that a sufficient amount of L-lactic acid is essential for the expression of the *lar* gene as well as conversion to D-lactic acid. L-lactic acid (endogenous compound) and D-lactic acid are harmful enantiomers and are related to D-lactic acidosis [44]. Understanding the factors that influence the production of L and D lactic acid by *L. plantarum* can help optimize the fermentation process for improved product quality and safety. In this study, we found that an excess amount of lactic acid induced the expression of *lar* and played an important role in the conversion of isomeric lactic acid. Interestingly, *ldh* expression was not significantly altered following organic acid or HCl treatment. This suggests that *lar* is a core element of lactic acid homeostasis in *L. plantarum*.

5. Conclusions

Many LAB in kimchi are D-L-lactic acid producers [33]. D-lactic acid is essential for the cell wall synthesis of *L. plantarum* [37,45]. In this study, we performed transcriptional analysis to determine the effect of lactic acid on global gene regulation in *L. plantarum*. We found that isomeric lactic acid acts differently as a signaling molecule in lactate homeostasis. Excess amounts of L-lactic acid increased the expression of the *lar* gene and consequently converted L-lactic acid to D-lactic acid. Moreover, malic acid, which is converted into lactic acid via the malolactic acid fermentation pathway, induces the expression of the *lar* gene and increases the production of D-lactic acid, when compared with acetic acid. Taken together, these results suggest that organic acids can modulate lactic acid production differently by regulating the expression of these genes.

Author contribution statement

Ha-Young Jang, Min Ji Kim, JongHee Lee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Minseo Bae: Performed the experiments; Contributed reagents, materials, analysis tools or data.

In Min Hwang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16520.

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