



Comparative transcriptomic analysis of *Lactiplantibacillus plantarum* RS66CD biofilm in high-salt conditions and planktonic cells

Xiaolin Ao^{1,*}, Jiawei Zhao^{1,*}, Junling Yan¹, Shuliang Liu¹ and Ke Zhao²

¹ College of Food Science, Sichuan Agricultural University, Ya'an, Sichuan, China

² College of Resources, Sichuan Agricultural University, Cheng'du, China

*These authors contributed equally to this work.

ABSTRACT

Background. *Lactiplantibacillus plantarum* (*L. plantarum*), a dominant strain in traditional fermented foods, is widely used in fermentation industry because of its fast acid production. However, *L. plantarum* is easily inactivated due to acidity, high temperature and other factors. The formation of biofilm by bacteria can effectively increase environmental tolerance. Therefore, it is important to improve the environmental tolerance of *L. plantarum* by studying its biofilm formation conditions and regulatory mechanisms.

Methods. After determining a suitable NaCl concentration for promoting biofilm formation, *L. plantarum* was grown with 48 g L⁻¹ NaCl. Differential gene expressions in *L. plantarum* biofilm vs. planktonic cells were analyzed using RNA sequencing and validated using qPCR.

Result. *L. plantarum* RS66CD biofilm formation formed highest amount of when grown at 48 g L⁻¹ NaCl. Altogether 447 genes were up-regulated and 426 genes were down-regulated in the biofilm. KEGG pathway analysis showed that genes coding for D-Alanine metabolism, peptidoglycan biosynthesis, two-component system, carbon metabolism, bacterial secretion system, lysine biosynthesis and fatty acid metabolism were crucial for biofilm formation. In addition, eight other genes related to biofilm formation were differentially expressed. Our results provide insights into the differential gene expression involved in biofilm formation, which can help to reveal gene regulation during *L. plantarum* biofilm formation.

Subjects Food Science and Technology, Genomics, Microbiology, Molecular Biology

Keywords Biofilm, Transcriptomics, Gene expression, *Lactiplantibacillus plantarum*

INTRODUCTION

Lactiplantibacillus plantarum, a facultative heterofermentative lactobacilli, is commonly used in fermenting vegetables (*Alan, Topalcengiz & Dğrak, 2018*), cereals (*Oguntoyinbo & Narbad, 2015*), fermented dairy products (*Zhang et al., 2020*). In addition, *L. plantarum* could be linked with some health effects. For example, clinical studies have found that *L. plantarum* CECT7315 and CECT7316 can enhance the immunity of the elderly (*Mane et al., 2011*). The survivability of *L. plantarum* has been found to be influenced by many

Submitted 23 March 2020

Accepted 9 July 2020

Published 3 August 2020

Corresponding author
Xiaolin Ao, huavslin@163.com,
245317046@qq.com

Academic editor
Joseph Gillespie

Additional Information and
Declarations can be found on
page 14

DOI 10.7717/peerj.9639

© Copyright
2020 Ao et al.

Distributed under
Creative Commons CC-BY 4.0

OPEN ACCESS

factors, e.g., heat treatment, storage temperature and time, acid, microbiota competition and possible presence of bacteriocins or other antimicrobials (Mona *et al.*, 2020; Tripathi & Giri, 2014). Many microorganisms resist the growth-unfriendly environment by forming biofilms (Wu *et al.*, 2019). Therefore, we aimed at improving the environmental tolerance of *L. plantarum* during fermentation and storage by inducing biofilm formation.

Biofilms are composed of microorganisms and an extracellular polymeric matrix that contain polysaccharides, proteins, nucleic acids, and lipids (Flemming & Wingender, 2010). Biofilm can effectively increase the stress tolerance of cells. *L. plantarum* JCM 1149 had greater resistance to organic acids, ethanol and sodium hypochlorite inside biofilm than inside planktonic cells (Kubota *et al.*, 2009). The biofilm-forming *Lactocaseibacillus rhamnosus* not only showed greater heat resistance, but also maintained higher activity in simulated gastrointestinal tract environment (Kiew, Cheow & Hadinoto, 2014). Because the formation of biofilms effectively improves the resistance of microorganisms to extreme environments, studying the mechanism of biofilm formation has become a hot topic.

The formation of biofilms is affected by many factors, such as the roughness of the surface of the carrier, pH, adhesion and gene regulation (Cappitelli, Polo & Villa, 2014). Also, inorganic ions affect the formation of biofilms. Mn^{2+} promoted biofilm formation in *L. plantarum* by eliminating oxidative stress and stimulating the secretion of more extracellular polymer substances (EPS) (Ibusquiza *et al.*, 2015). The formation of the initial biofilm of *Pseudomonas fluorescens* can be promoted by Mg^{2+} , yet higher concentrations lead to blockage of mass transfer channels, affecting the intake of nutrients and inhibiting the further development of biofilm (Song & Leff, 2003; Hoyle, Wong & Costerton, 1992). Fe^{3+} promoted the secretion of EPS and increased the expression level of genes to improve the stability of biofilms in *Staphylococcus epidermidis* (Oliveira, France & Ccerca, 2017). Salt stress by NaCl or KCl promoted biofilm formation in *L. plantarum* and compensated for biofilm phenotypic instability, possibly by regulating the expression of biofilm formation related genes (Valle *et al.*, 2007; Rachid *et al.*, 2000). In fermenting vegetables, 4%–8% of sodium chloride is commonly applied to improve the flavor and inhibit the growth of spoilage microorganisms (Xiong *et al.*, 2012; Zhang *et al.*, 2016; Chun *et al.*, 2020). Therefore, it is relevant to study the biofilm formation of *L. plantarum* in this concentration range.

In recent years, molecular and post-genomic analyses have reported in detail genes specific to the formation of biofilm. For instance, RNA-Seq analysis of *Enterococcus faecalis* isolates identified 163 differentially regulated genes in biofilm compared with planktonic cells, and that of *Salmonella Typhimurium* showed that 618 genes were over-expressed and 897 genes down-expressed in biofilm compared with planktonic cells (Seneviratne *et al.*, 2017). In the biofilm formation of *Bacillus amylobacillus*, the antimicrobial peptide gene *lci* and the drug resistance gene *yvqHI* were among the most up-regulated genes in induction of systemic resistance (Kröber *et al.*, 2016). RNA-seq results showed that added tea tree oil caused differential expression of 304 genes in *Staphylococcus aureus* biofilms compared to planktonic cells (Zhao *et al.*, 2018). Although biofilms made by pathogens have been studied extensively, analyses of *L. plantarum* biofilm formation are still scarce. Therefore,

we studied the differential genes during the formation of *L. plantarum* RS66CD biofilm at high salt concentration by transcriptomics.

MATERIAL AND METHODS

The culture conditions and determination of biofilm formation

L. plantarum RS66CD (GenBank [MN160322](#)) isolated from traditional Sichuan paocai has good fermentation ability. Previous experimental results showed that *L. plantarum* RS66CD was able to form biofilm ([Zhao et al., 2019](#)). Therefore, *L. plantarum* RS66CD was selected as the experimental strain. We hope to promote the biofilm formation of *L. plantarum* under the condition of NaCl concentration of 4%–6% (traditional Sichuan paocai fermentation), so as to improve the bacterial salt tolerance. In this experiment, the biofilm formation of *L. plantarum* RS66CD was tested by growing it in modified MgSO₄ and MnSO₄-free MRS medium with 40, 44, 46, 48, 52, 56 or 60 g L⁻¹ NaCl in 96-well cell culture plates at 37 °C. After 24 h incubation, the culture solution was discarded, and biofilms were stained with 50 µL of 0.5% crystal violet for 5 to 10 min, washed with sterile distilled water for 2 to 3 times, after which the plates were dried at 37 °C, and absorbencies were measured at 490 nm.

The concentration of sodium chloride, at which the maximum amount of biofilm was formed, was used in subsequent experiments. Planktonic cells, which were cultured in MRS liquid medium at 37 °C for 24 h (as control treatment) and biofilm formation cells were fixed in 2.5% glutaraldehyde phosphate buffer solution at 4 °C for 8 h, after which water was removed with ethanol. After gold plating, samples were examined using SEM.

RNA extraction, library preparation, and sequencing

For RNA extraction, cells were collected using centrifugation after culturing at 37 °C for 24 h. Total RNA was extracted using TRIZOL Reagent according the manufacturer's instruction (Invitrogen, California, USA) and genomic DNA was removed using DNase I (TaKara, Shiga, Japan). The quality of RNA determined using 2100 Bioanalyser (Agilent, California, USA). RNA was quantified using the ND-2000 (NanoDrop Technologies).

RNA-seq strand-specific libraries were prepared from 5 µg of total RNA using TruSeq RNA sample preparation Kit from Illumina (San Diego, CA). Shortly, rRNA was removed using RiboZero rRNA removal kit (Epicenter, Stockholm, Swedish) and fragmented using fragmentation buffer. cDNA synthesis, end repair, A-base addition and ligation of the Illumina-indexed adaptors were performed according to the Illumina protocol. Libraries were size selected for cDNA target fragments of 200–300 bp on 2% Low Range Ultra Agarose followed by PCR amplification using DNA polymerase (NEB) for 15 PCR cycles. After libraries were quantified by TBS380, 150 bp*2 paired-end sequencing was done on Illumina Novaseq 6000. The transcriptome sequencing data was submitted to NCBI with the accession number [PRJNA626519](#).

Quality control and mapping

The raw paired end reads were trimmed and quality controlled using Trimmomatic v.0.36 (<http://www.usadellab.org>) with sliding window set to length 4 and Phred quality score 15,

and minimum length to 75. The clean reads were separately aligned to the reference genome (*Lactobacillus plantarum* WCFS1, GenBank: [AL935263](#)) with orientation mode using Rockhopper software (https://cs.wellesley.edu/~btjaden/Rockhopper/user_guide.html). The expression levels of genes were calculated using Rockhopper with default parameters.

Differential gene expression analysis

To identify differentially expressed genes (DEGs) in the biofilm vs. planktonic cells, the expression levels of the transcripts were calculated using the fragments per kilobase of read per million mapped reads (RPKM) method. EdgeR (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>) was used for differential expression analysis. The DEGs were selected using the following criteria: the logarithmic of fold change was greater than 2 and the false discovery rate (FDR) less than 0.05. To understand the functions of the DEGs, GO functional enrichment and KEGG pathway analysis were carried out using Goatools (<https://github.com/tanghaibao/GOatools>) and KOBAS (<http://kobas.cbi.pku.edu.cn/>), respectively. DEGs were significantly enriched in GO terms and metabolic pathways when their Bonferroni-corrected *P*-value was less than 0.05. DEGs in biofilm formation of *L. plantarum* RS66CD were further analyzed using hierarchical clustering and a heat map.

qRT-PCR validation of the transcriptome data

To validate the transcriptome data, six genes were selected for RT-PCR experiments with 16S rRNA gene as the internal reference (Table 1). Total RNA was extracted from *L. plantarum* RS66CD samples using RNA isolation reagent (TIANGEN, Beijing, China) and the first strand cDNA synthesis was carried out using FastKing RT kit (TIANGEN, Beijing, China) according to manufacturer's instructions. RT-PCR amplification using three biological replicates per gene was done in a reaction volume of 20 μ L containing 10 μ L of YBR Green Supermix, 0.4 μ L of forward primer, 0.4 μ L of reverse primer, 0.8 μ L of cDNA and 8.4 μ L of ddH₂O. The qPCR conditions were as follows: predenaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s; fluorescence signals were collected during annealing and extension and the whole process was repeated for 40 cycles. Melting curve analysis is 95 °C for 10 s, 65 °C for 5 s, and 95 °C for 50 s.

The statistical analyses

All the experiments were conducted in triplicate. Data related to the amount of biofilm formation was tested using Duncan's multiple range test in SPSS 22.0 for Windows. All data were reported as mean \pm standard deviation. Differences were taken as statistically significant at *P* < 0.05.

RESULT

Formation of *L. plantarum* RS66CD biofilm

L. plantarum RS66CD formed more biofilm with 48 g L⁻¹ NaCl than with other NaCl concentrations (Fig. 1). Scanning electron microscopy (SEM) revealed that the cells grown in the modified MRS with NaCl were clearly embedded in an extracellular matrix and

Table 1 Genes and primers in the RT-PCR.

Name	Primer sequence (5'-3')	Function
16S rRNA	F-TGAGTGAGTGGCGAACTGGTG R-GCCGTGTCTCAGTCCCAATG	KT025937
<i>groEL</i>	F-GCAAATCGCTTCTGTATCTTC R-CGCTTCCATCCTTATCATTGTC	Chaperonin GroEL (KJ807061)
<i>ftsH</i>	F- ACGACTAATGTGAGTGTTGCTGAA R- ATCCTGCGATGATGGTGTGG	ATP-dependent metalloproteinase HflB (MTU86407)
<i>gyrB</i>	F-AGAAGAGGAAGTTAGAGAAGA R-GCATATCCACTGTTATATTGAAG	DNA topoisomerase (ATP-hydrolyzing) subunit B (MF988195)
<i>alr</i>	F-TAGGATCCATCGAACTCAAACACACCTGCGTC R-CGAAGCTTTGGCAATTCAGTCGACGAGTATC	Alanine racemase (Cao et al., 2006)
<i>LIFabZ1</i>	F- TAACATATGACTAAAAAATACGC R- CAGAAGCTTCAATGCCACATTGC	Regulation of ACP dehydratase (Ma et al., 2014)
<i>LIFabZ2</i>	F-GAACATATGACTGAAGTAAACATTAATG R- GAAAAGCTTACGCCCTAAGGCAAAAAG	ACP dehydrated isomerase (Ma et al., 2014)

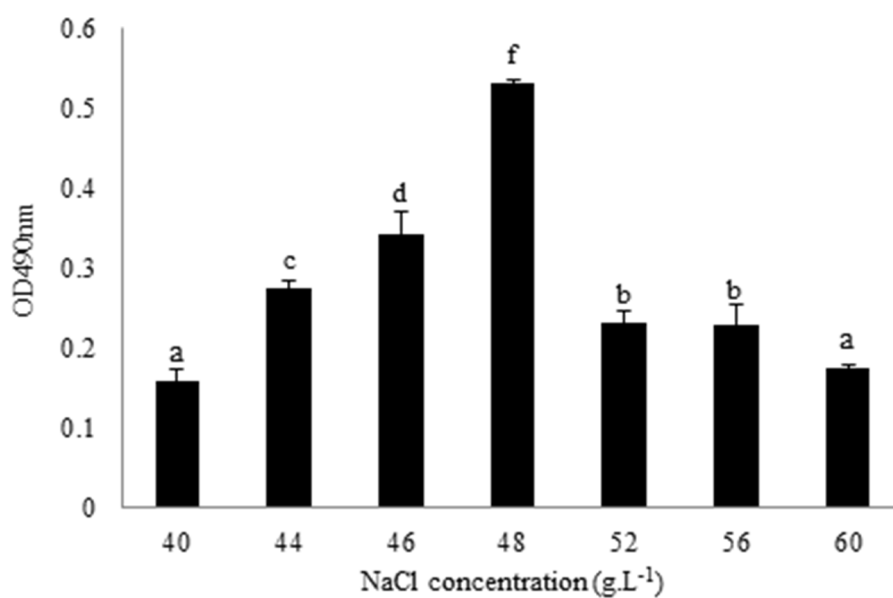


Figure 1 Quantification of *L. plantarum* RS66CD biofilm at different NaCl concentrations. The data columns represent means of the three repetitions. The error bars show standard deviation. Different lowercase letters indicate significant difference ($p < 0.05$).

Full-size  DOI: 10.7717/peerj.9639/fig-1

EPS caused the bacteria to aggregate to form microcolonies (Fig. 2), demonstrating that *L. plantarum* could form a distinct biofilm when grown with NaCl.

Transcriptomic data for biofilm formation of *L. plantarum* RS66CD

On the average, sequencing of the libraries from *L. plantarum* RS66CD biofilm and planktonic cells resulted in $17 \pm 13 \times 10^6$ and $9.3 \pm 0.5 \times 10^6$ clean reads, respectively, out of

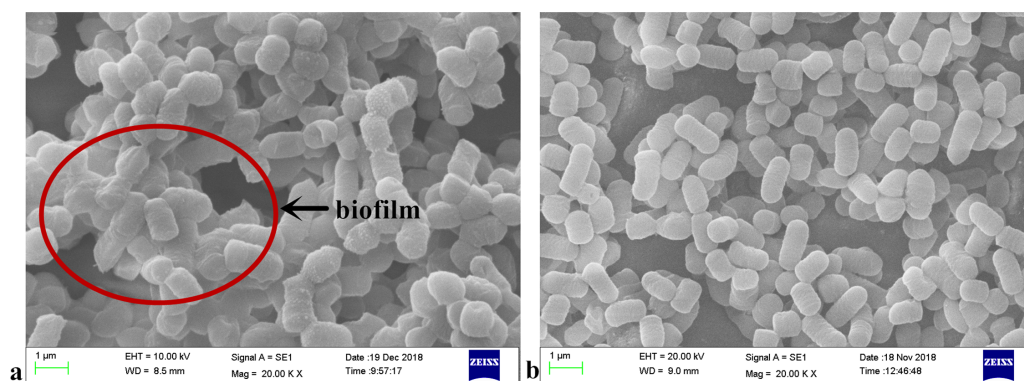


Figure 2 SEM micrographs of *L. plantarum* RS66CD grown in (A) modified MRS medium with NaCl and (B) MRS medium.

Full-size DOI: 10.7717/peerj.9639/fig-2

Table 2 RNA sequencing results for *L. plantarum* RS66CD biofilm and planktonic cells.

	<i>L. plantarum</i> RS66CD biofilm			Planktonic cells		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Clean reads	8581850	10660184	32053428	9674730	9630650	8713450
Clean base	1281768285	1591930909	4765116422	1444240232	1436102731	1301244000
rRNA (%)	0.92	0.89	0.78	0.65	0.63	0.78
Mapped reads	7038442	8769430	26643418	7805908	7837808	6956864
Mapped rate(%)	82.02	82.26	83.12	80.68	81.38	79.84

which $0.86 \pm 0.073\%$ and $0.69 \pm 0.081\%$ were assigned to rRNA (Table 2). The mapping ratio between sequencing samples and reference genome was above 80%.

Differential gene expression and KEGG pathway analysis

The transcribed genes were classified into gene ontology (GO) categories: 1047 genes were classified into biological processes, 660 to cellular components and 208 to molecular functions. Compared to the planktonic cells, 447 genes were up-regulated and 426 genes were down-regulated in the biofilm (Fig. 3).

The DEGs were compared with Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg>) and GO databases (<http://www.geneontology.org>). In the Fig. 4 the abscissa represents the enrichment factor; the vertical coordinate represents the enriched function of the GO term. The larger the circle is, the more differential genes are enriched to this function. Thirty metabolic pathways were enriched in the *L. plantarum* RS66CD biofilm (Fig. 4). In D-alanine metabolism glutathione metabolism, bacterial secretion system, peptidoglycan biosynthesis, two-component system, and carbon metabolism pathways 27 genes were up-regulated and 12 down-regulated in the biofilm compared to the planktonic cells (Table 3, Fig. 5). In addition, five biofilm related genes were up-regulated and four down-regulated in the biofilm (Table 4, Fig. 5).

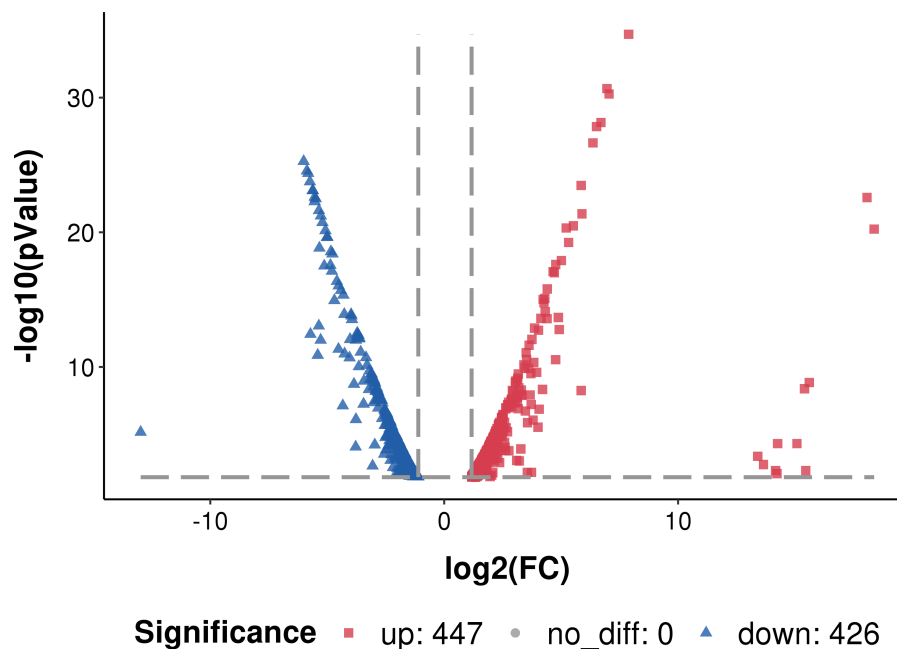


Figure 3 Differentially expressed genes in *L. plantarum* RS66CD biofilm compared to planktonic cells.
*Log₂ FC: fold change in *L. plantarum* RS66CD biofilm compared to planktonic cells.

Full-size DOI: 10.7717/peerj.9639/fig-3

qRT-PCR validation of differential expression

Compared to planktonic cells the expression of *groEL* was 2.4 fold and 2.03 times higher in the biofilm as determined by qPCR and RNA-Seq, respectively, the expression of *ftsH* was 1.07 fold and 1.43 fold higher in biofilm, the expression of *gyrB* was 0.96 and 0.83 fold higher, and the expression of *alr* 1.39 and 1.69 fold higher. For *LlFabZ1* and *LlFabZ2* (encode *fabZ* and regulation of ACP dehydratase and ACP dehydrated isomerase) the expression was 1.4 and 4.3 fold higher in biofilm, as determined by qPCR and RNA-Seq, respectively. As the qRT-PCR validation of differential expression was consistent with the RNA-Seq, the transcriptomics results were considered representative.

DISCUSSIONS

To increase tolerance against adverse environmental conditions, bacteria form biofilms to protect the cells from external forces (Yan & Bonine, 2019). The formation of biofilm has several probable mechanisms including hormone induced, environment stimulation, signaling and gene transfer (Ghafoor, Hay & Rehm, 2011; Ramesh et al., 2019). The main mechanism of biofilm formation can be ascribed to the regulation of transcriptional bacterial physiological metabolism. Biofilms in industrial and clinical context have received considerable attention (Song, Wu & Xi, 2012). In this study we characterized the biofilm formation related gene expression of *L. plantarum* RS66CD.

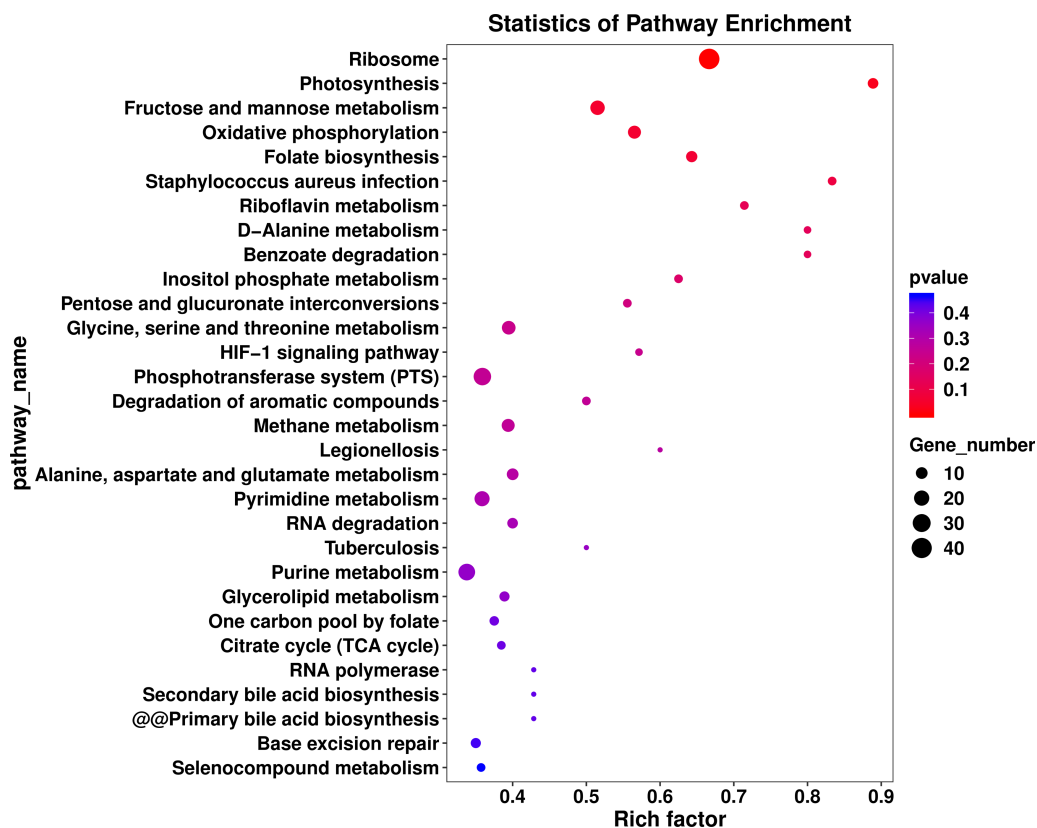


Figure 4 KEGG pathway analysis of differentially expressed genes in *L. plantarum* RS66CD biofilm compared to planktonic cells. The enriched KEGG categories are on the vertical axis. The enrichment factor, i.e., the ratio of the enriched DEGs in the KEGG category to the total genes in that category, is shown on the horizontal axis.

Full-size DOI: 10.7717/peerj.9639/fig-4

D-alanine and peptidoglycan biosynthesis

In our study, D-alanine metabolism and peptidoglycan biosynthesis related genes were up-regulated in the RS66CD biofilm (*dltA*, *dltC*, *ddl*, *alr*). D-alanine, a D-amino acid is abundantly present in the bacterial cell wall (Laura *et al.*, 2014). D-alanine is a structural component in bacterial cell wall peptidoglycan. Bacteria modulate surface charge by attaching D-alanine esters to teichoic acids (Heptinstall, Archibald & Baddiley, 1970), thus D-alanine affects the formation of biofilm.

Alanylation that increases the positive charge of teichoic acid is mediated by products of the *dlt* genes (Nilsson *et al.*, 2016). In the *dlt* operon, *dltA* encodes d-alanine carrier protein ligase, and *dltC* a d-alanyl carrier protein that ensure the ligation of d-alanine to the d-alanyl carrier protein (Neuhaus & Baddiley, 2004). Strains lacking the *dlt* operon have increased antibiotics and acid sensitivity (Boyd *et al.*, 2000). Studies have shown that resistance to gentamicin was significantly reduced in the biofilm formed by *Streptococcus mutans* after the *dltA* was knockout (Nilsson *et al.*, 2016). Interestingly, the reduced antibiotic tolerance of *dltA* mutants was not due to defects in biofilm formation, but rather to more negative charges on the mutant surface. A future knockout experiment will reveal whether, the

Table 3 Differentially expressed genes in biofilm formation related KEGG pathways in *L. plantarum* RS66CD biofilm compared to planktonic cells.

Term	Gene ID	Name	Log ₂ FC	Type	Function
D-Alanine metabolism	gene1728	<i>dltA</i>	1.32	up	D-alanine ligase
	gene2020	<i>ddl</i>	1.39	up	D-ala ligase
	gene433	<i>alr</i>	1.69	up	Alanine racemase
	gene1726	<i>dltC</i>	1.37	up	D-alanine ligase
Peptidoglycan biosynthesis	gene422	<i>murA</i>	1.44	up	Carboxyvinyl transferase
	gene1094	<i>dacA</i>	2.49	up	D-alanyl-D-alanine Carboxypeptidase
	gene1877	<i>mraY</i>	1.65	up	Pentapeptide transferase
	gene2020	<i>ddl</i>	1.39	up	D-ala ligase N-terminal domain protein
	gene707	<i>murB</i>	1.20	up	UDP-pyruvate reductase
	gene1075	<i>lp-1256</i>	1.59	up	Hypothetical protein
Two-component system	gene508	<i>lytR</i>	1.76	up	LytR family transcriptional regulator
	gene1358	<i>glnA</i>	-1.70	down	Type I glutamate-ammonia ligase
	gene939	<i>citG</i>	1.39	up	Triphosphoribosyl-dephospho-CoA synthase
	gene455	<i>ftsH</i>	1.43	up	ATP-dependent metalloproteinase
	gene3028	<i>lamA</i>	1.51	up	DNA-binding response regulator
	gene2569	<i>lp-3019</i>	2.15	up	Membrane protein
	gene2757	<i>lrgB</i>	1.63	up	Murein hydrolase
	gene2756	<i>lrgA</i>	2.65	up	Murein hydrolase
	gene3031	<i>lamB</i>	1.89	up	Accessory gene regulator AgrB
	gene2603	<i>hpk</i>	2.02	up	Histidine protein kinase
	gene635	<i>pstF</i>	-1.28	down	Phosphate ABC transporter substrate-binding protein
	gene963	<i>cydA</i>	-1.93	down	Cytochrome D ubiquinol oxidase
	gene957	<i>mleS</i>	4.65	up	NAD-dependent malic enzyme
	gene2658	<i>lp-3125</i>	3.52	up	Oxidoreductase
	gene964	<i>cydB</i>	-1.79	down	Cytochrome d ubiquinol oxidase
	gene682	<i>rpoN</i>	-1.90	down	RNA polymerase factor
gene4	<i>gyrB</i>	0.83	up	Ica regulatory factor	
Carbon metabolism	gene2989	<i>tkt4</i>	-1.99	down	Transketolase
	gene418	<i>sdhB</i>	1.44	up	L-serine dehydratase
	gene419	<i>sdhA</i>	1.31	up	L-serine dehydratase
	gene163	<i>serC</i>	1.93	up	Phosphoserine aminotransferase
	gene162	<i>serA</i>	1.80	up	D-3-phosphoglycerate dehydrogenase
	gene1635	<i>pps</i>	-1.58	down	Phosphoenolpyruvate synthase
	gene1840	<i>pdhC</i>	-1.59	down	Branched-chain α -keto acid dehydrogenase
	gene1841	<i>pdhB</i>	-1.50	down	α -keto acid dehydrogenase
	gene1842	<i>pdhA</i>	-1.19	down	Pyruvate dehydrogenase
	gene1839	<i>pdhD</i>	-1.51	down	Dihydrolipoyl dehydrogenase
	gene1534	<i>pgm5</i>	-1.17	down	Histidine phosphatase family protein

(continued on next page)

Table 3 (continued)

Term	Gene ID	Name	Log ₂ FC	Type	Function
Bacterial secretion system	gene3155	<i>traK</i>	2.47	up	Conjugal transfer protein
	gene517	<i>radA</i>	1.64	up	DNA repair protein RadA
	gene906	<i>secY</i>	1.35	up	Preprotein translocase subunit

Notes.

^aLog₂FC: fold change in *L. plantarum* RS66CD biofilm compared to planktonic cells.

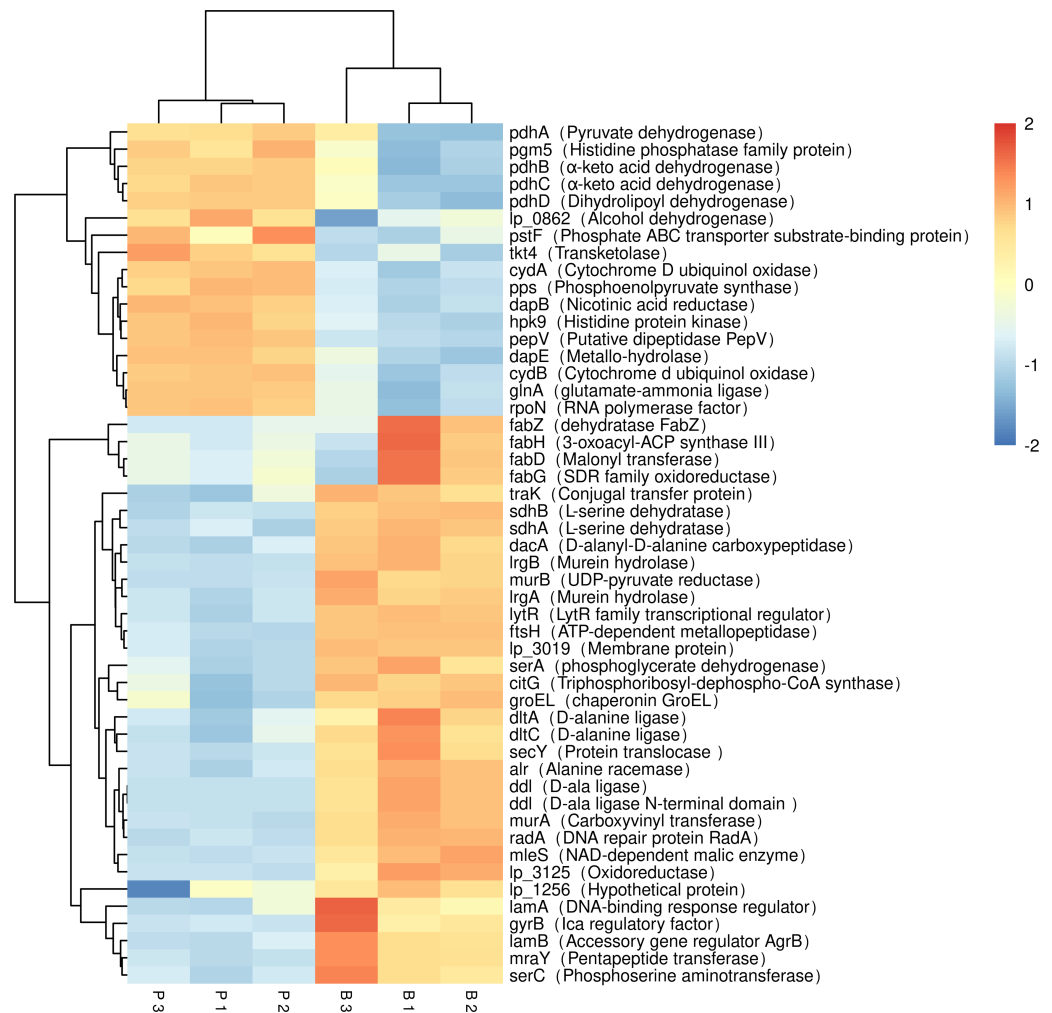


Figure 5 Expression of differentially expressed biofilm related genes in *L. plantarum* RS66CD. Samples in columns and genes on rows. P, planktonic cells; B, biofilm. Differential expression is indicated with the color key.

Full-size DOI: [10.7717/peerj.9639/fig-5](https://doi.org/10.7717/peerj.9639/fig-5)

role of *dltA* of *L. plantarum* RS66CD is similar. Alanine racemase (Alr) converts L-alanine to D-alanine by changing the stereochemical structure of the chiral α -carbon atom of L-alanine, and participates in the synthesis of bacterial cell wall peptidoglycan (Tanner, 2002). *Ddl* is present in both Gram-negative and -positive bacteria and catalyzes the

Table 4 Differentially expressed biofilm formation related genes in *L. plantarum* RS66CD biofilm compared to planktonic cells.

Gene ID	Name	Log ₂ FC	Type	Function
gene747	<i>lp-0862</i>	-1.32	down	Alcohol dehydrogenase
gene2442	<i>dapE</i>	-1.71	down	Metallo-hydrolase
gene1604	<i>dapB</i>	-1.18	down	4-hydroxy-tetrahydrodipicolinate reductase
gene1139	<i>pepV</i>	-1.43	down	Putative dipeptidase PepV
gene632	<i>groEL</i>	2.03	up	chaperonin GroEL
gene1439	<i>fabG</i>	1.40	up	SDR family oxidoreductase
gene1438	<i>fabD</i>	1.33	up	Malonyltransferase
gene1435	<i>fabZ</i>	1.25	up	dehydratase FabZ
gene1436	<i>fabH</i>	1.33	up	3-oxoacyl-ACP synthase III

Notes.

^aLog₂ FC: fold change in *L. plantarum* RS66CD biofilm compared to planktonic cells.

formation of D-Ala-D-Ala dipeptide in an early step in peptidoglycan synthesis, which can promote the synthesis of bacterial cell walls (*Ameryckx et al., 2018*).

The *dacA* encoded D-alanine carboxypeptidase is a low molecular mass penicillin-binding protein (LMM PBP) (*Despreaux & Manning, 1993*). LMM PBPs may regulate cell morphology and mediate the formation of biofilm (*Young, 2006*). *murA* and *murB* in the *mur* enzyme family are involved in the synthesis of propeptides of peptidoglycans (UDP-MurNAc) and the regulation of bacterial osmotic pressure (*Hrast et al., 2014; Hattersley et al., 2011*). Our results imply that during the formation of *L. plantarum* RS66CD biofilm, the up-regulation of the peptidoglycan synthesis related genes promote the synthesis of peptidoglycan and improve the stability of the bacteria; plausibly, changes in the cell surface due to the up-regulation of the D-alanine biosynthesis related genes increase cell aggregation and promote the biofilm formation.

Two-component system

Two-component system is a signaling pathway that regulates the gene expression of bacteria and coordinates responses to environmental stimulus, mainly through adaptation to changes in the environment (osmolarity, light, temperature and oxygen) (*Sieuwerts et al., 2010*), and regulation of developmental pathways and behaviors (sporulation, biofilm formation, competence and chemotaxis) (*Biondi, 2013*).

In our study, the differentially expressed genes related to the two-component system included both up-regulated (*hytR*, *citG*, *lrg* operon, *lam* operon, *ftsH*, *lp-3125*, *mleS*) and down-regulated genes (*pstF*, *cyd* operon, *glnA*, *rpoN*). The gene *citG* is essential for holo-acyl carrier protein (ACP) synthesis in *Escherichia coli* and catalyzes the formation of a prosthetic group precursor from ATP and dephospho-CoA (*Schneider, Dimroth & Bott, 2000*). Bacterial ACPs are the donors of the acyl moiety involved in the biosynthesis of fatty acids, playing an important role in increasing the ability of form biofilm (*Silvia et al., 2008*). *lrgA* and *lrgB* are presumably involved in the formation of the murein hydrolase transport channel to regulate signal transmission and then to control programmed cell death (*Zhang et al., 2018*). *lamA*, *lamB* and *ftsH* participates in the accessory gene regulator

(AGR) quorum sensing system. The AGR system influences the formation of staphylococcal biofilms during the structuring and dispersion phases (Cheraghi et al., 2017). *FtsH* gene may regulate adhesion proteins. Some studies have shown that the *ftsH* gene increases the amount of biofilm formation and is related to multiple environmental stress responses (Bove et al., 2012). Further experiments with *ftsH* mutant strain should reveal it affects the formation of biofilm by *L. plantarum* RS66CD and reduces the salt tolerance of the strain. The *agr* operon includes *agrB*, *agrD*, *agrC*, and *agrA*. *agrB* is regarded as a factor to regulate biofilm dispersal, because biofilm biomass is inversely correlated with *agrB* expression (Li et al., 2018). *CydA* and *cydB* regulate cytochrome bd ubiquinol oxidase formation. Cytochrome bd panthenol oxidase is a terminal oxidase involved in aerobic respiration of prokaryotes (Hazan et al., 2016). It funnels electrons coming from NADH and ubiquinol to cytochrome *c*, and it is also capable of producing significant amounts of the free radical superoxide. *L. plantarum* lacks superoxide dismutase, thus it must reduce the formation of free radical superoxides to promote biofilm formation (Bazil, 2017). Due to changes in osmotic pressure, it can also lead to programmed cell death and decreased respiratory intensity. This may explain the down-regulation of *cydA* and *cydB* and the up regulation of *lp_3125* and *mleS* that participate in aerobic respiratory metabolism and simultaneously reduced NAD(P)⁺ to NAD(P)H. These genes were enriched in this pathway mainly through sense external signal stimulation and influence the expression of quorum sensing system that leads to increases in the adhesion proteins thereby promoting the biofilm formation. This can resist the osmotic pressure changes caused by the addition of NaCl. *L. plantarum* also reduced the production of free radical superoxide by regulating oxidase activity to promote the formation of biofilm and increase the salt tolerance.

Carbon metabolism

One the roles of carbon metabolism is to help bacteria to adapt to the environment when it changes. In our study, the differentially expressed genes related to the carbon metabolism included both up-regulated and down-regulated genes. The *lytR* family genes that encode putative proteins *FlmA*, *FlmB*, and *FlmC* were up-regulated in the biofilm. The proteins might be involved in cell wall integrity, cell growth, and cell adhesion, and in the developmental process leading to biofilm formation (Muscariello et al., 2013). Given the important role of *lytR* family protein genes in the biofilm formation process, the role of *flm* gene must be investigated separately in future experiments. The genes *serA* and *serC* participate in the formation of phosphoserine aminotransferase (P-Ser-HPr) that belongs to the carbohydrate phosphotransferase system. The inactivation of *Neisseria meningitidis* *hprK* strongly diminished cell adhesion (Deutscher et al., 2005). *serA* and *serC* increase the adhesion of strains by increasing the content of P-Ser-HPr, which promotes the formation of biofilm. Pyruvate dehydrogenase(Pdh) participates in many physiological processes, such as carbon metabolism, aerobic respiration and fatty acid metabolism. *PdhA*, *PdhB* and *PdhC* are involved in the formation of a pyruvate dehydrogenase complex which belongs to a family of alpha-keto-acid dehydrogenase complexes (Neveling et al., 1998). In our study, the *pdh* genes were down-regulated, which may be related to the change in osmotic pressure inside biofilm, leading to a decrease in respiratory intensity and redox reactions, and to less

intense energy metabolism. Out of the other down-regulated genes *pps* gene catalyzes the phosphorylation of pyruvate and participates in the TCA cycle (Smyer & Jeter, 1989). *pgm5* gene regulates histidine phosphatase that is mainly involved in the synthesis of ATP, and *tkt4* encodes a key enzyme of the non-oxidative branch of the pentose phosphate pathway. NADPH participates in reductive biosyntheses and defense against oxidative stress (Wood, 1968). These down-regulated genes reduce bacterial damage by improving the oxidative stress response. The biofilm formation can be promoted to resist the change of osmotic pressure by enhancing the bacterial adhesion, participating in the anabolism of serine which constitutes biofilm. Our study found that the genes related to energy metabolism and respiratory intensity were inhibited due to the changes of osmotic pressure.

Bacterial secretion system

Bacteria secrete many proteins to adapt to their living environment. Bacterial secretion system affects the formation of biofilm by regulating the entry and exit of substances which further affects energy metabolism and signal transmission. Protein export across the cell envelope of Gram-positive (G+) bacteria is relatively simple as only one membrane needs to be passed (Glöckner et al., 2008). In our study, three bacterial secretion system genes (*traK*, *radA* and *secY*) were up-regulated in the biofilm. Out of the up-regulated genes, *traK* encodes a transfer protein that regulates substance entry and exit and signaling. The C terminus of *radA* is related to *LonB* protease, an serine protease that regulates capsular polysaccharide synthesis (Ishibashi et al., 2006). In many eubacterial *radA* orthologs, active-site serine residue is replaced by alanine, which may be related to the involvement of L-alanine in biofilm formation. *SecY* has been reported to be a transmembrane protein and the most important component of the Sec protein secretion system (Flower, 2007). The *secY* encodes a transmembrane protein in the general secretory (Sec) pathway that is responsible for translocation of extracytoplasmic proteins across the plasma membrane in G+ bacteria. The above genes regulate protein secretion to synthesize biofilm, and reduce the damage of osmotic pressure to strain through regulates substance entry and exit. Studies have shown that, the biofilms of *L. plantarum* were reduce the density by destroy extracellular matrix (Fernández Ramírez et al., 2015), which further indicates that the formation of related proteins can promote biofilm formation.

Other key genes

Lysine can inhibit biofilm formation via inducing the production of reactive oxygen species and malondialdehyde (Ge et al., 2018). The genes in the Lysine biosynthesis pathway mainly inhibit the synthesis of redox-related enzymes and hydrolases (Table 4). Alcohol dehydrogenase are a group of oxidoreductases which encoded by *lp_0862* gene during the stage of formation (Hu, Pu & Bai, 2019).

Fatty acids are the main components of cell membrane and participate in the physiological metabolism of bacteria. By regulating the type and composition of fatty acids, bacteria can transmit signals, regulate the fluidity of cell membranes, maintain membrane stability, and increase environmental tolerance. In our study, four of the up-regulated genes (*fabG*, *fabD*, *fabH*, *fabZ*) were related to fatty acid metabolism, mainly

involved in the synthesis of type II fatty acids. The genes *fabD* and *fabH* encode ACP transacylase that catalyzes the formation of malonyl ACP, and 3-ketoacyl-ACP synthase III that catalyzes the condensation of malonyl ACP and acetyl-CoA to form 3-keto butyryl ACP, respectively. The enzyme encoded by *fabZ* gene is mainly involved in the cyclic reaction of fatty acid synthesis and promotes a new round of synthetic reactions. The products of fatty acid synthesis include long-chain fatty acids, branched-chain fatty acids and unsaturated fatty acids (UFA). Among them, long-chain fatty acids can be used as signal transduction substances (Zhou et al., 2015). UFA has a low melting point which affects the physical properties of cell membranes, making them important molecules in regulating bacterial cell membrane fluidity. Synthesizing branched-chain fatty acids is a way to cope with unfavorable environments (Santiago et al., 2013). Comparison of biofilm components of *Staphylococcus aureus* and *Pseudomonas aeruginosa* with planktonic cells revealed that biofilm growth mode leads to a decrease in the uneven-numbered chain phospholipids, and accumulation of long chain lipids (Benamara et al., 2011; Maria et al., 2019). This indicates that the accumulation of fatty acids could improve the stability of biofilm. Combined with the results of RNA-Seq, it is shown that *L. plantarum* can affect the fluidity of cell membrane and maintain the stability of biofilm by regulating the type and composition of fatty acids, which can increase the environmental tolerance. Some studies have shown that *fadD* and *fabH* were involved in fatty acid and lipid biosynthesis pathway during the formation of biofilm by *L. plantarum* DB200 (Angelis et al., 2015). In addition, the heat stress protein (*GroEL*) also exhibited chloroprene and immune modulatory-properties to improve the aggregation of bacteria and environmental resistance (Pessione, 2012). In future experiments, the biofilm formation mechanism can be further analyzed by comparing the fatty acid contents on the surface of planktonic cells and biofilm forming strains.

In summary, this study provided important insights into gene expression during the formation of biofilm by *L. plantarum*. With the formation of biofilm, transcriptome profile changed significantly. Specifically, genes coding for D-alanine metabolism, peptidoglycan biosynthesis, two-component system, carbon metabolism, bacterial secretion system and fatty acid metabolism were found to be crucial for biofilm formation. The results will pave way for a more comprehensive understanding of the mechanisms involved in biofilm formation.

ACKNOWLEDGEMENTS

The authors acknowledge Associate Professor Petri Penttinen, Sichuan Agricultural University, for his help in revising the manuscript.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The authors received no funding for this work.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Xiaolin Ao conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Jiawei Zhao conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Junling Yan and Ke Zhao analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Shuliang Liu conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in [Data S1–S3](#). The transcriptome sequencing data is available at NCBI: [PRJNA626519](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.9639#supplemental-information>.

REFERENCES

- Alan Y, Topalcengiz Z, Dğrak M. 2018. Biogenic amine and fermentation metabolite production assessments of *Lactobacillus plantarum* isolates for naturally fermented pickles. *LWT* **98**:322–328 DOI [10.1016/j.lwt.2018.08.067](#).
- Ameryckx A, Thabault L, Pochet L, Leimanis S. 2018. 1-(2-Hydroxybenzoyl)-thiosemicarbazides are promising antimicrobial agents targeting D-alanine-D-alanine ligase in *bacterio*. *European Journal of Medicinal Chemistry* **159**:324–338 DOI [10.1016/j.ejmech.2018.09.067](#).
- Angelis MD, Siragusa S, Campanella D, Cagno RD. 2015. Comparative proteomic analysis of biofilm and planktonic cells of *Lactobacillus plantarum* DB200. *Proteomics* **15**(13):2244–2257 DOI [10.1002/pmic.201400363](#).
- Bazil JN. 2017. Analysis of a functional dimer model of ubiquinol cytochrome c oxidoreductase. *Biophysical Journal* **113**:1599–1612 DOI [10.1016/j.bpj.2017.08.018](#).
- Benamara H, Rihouey C, Jouenne T, Alexandre S. 2011. Impact of the biofilm mode of growth on the inner membrane phospholipid composition and lipid domains in *Pseudomonas aeruginosa*. *BBA- Biomembranes* **1808**(1):98–105 DOI [10.1016/j.bbamem.2010.09.004](#).
- Biondi EG. 2013. *Two-Component Systems[M]*. New York: Springer, 2116–2305.
- Bove P, Capozzi P, Garofalo C, Rieu A, Spano G, Fiocco D. 2012. Inactivation of the *ftsH* gene of *Lactobacillus plantarum* WCFS1: effects on growth, stress tolerance, cell surface properties and biofilm formation. *Microbiological Research* **167**(4):187–193 DOI [10.1016/j.micres.2011.07.001](#).

- Boyd DA, Cvitkovitch DG, Bleiweis AS, Kiriukhin MY, Debabov DV, Neuhaus FC, Hamilton IR. 2000. Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *Journal of Bacteriology* **182**(21):6055–6065 DOI [10.1128/JB.182.21.6055-6065.2000](https://doi.org/10.1128/JB.182.21.6055-6065.2000).
- Cao Q, Zhao Z, Zhang YZ, Wang Y, Ding JY. 2006. Cloning, sequence analysis and expression of alanine racemase gene in *Pseudomonas putida*. *Acta Microbiologica Sinica* **46**(1):80–84.
- Cappitelli F, Polo A, Villa F. 2014. Biofilm formation in food processing environments is still poorly understood and controlled. *Food Engineering Reviews* **6**(2014):29–42 DOI [10.1007/s12393-014-9077-8](https://doi.org/10.1007/s12393-014-9077-8).
- Cheraghi S, Pourgholi L, Shafaati M, Fesharaki SH, Jalali A. 2017. Molecular analysis of virulence genes and the accessory gene regulator (*agr*) types among methicillin resistant *Staphylococcus aureus* (MRSA) strains. *Journal of Global Antimicrobial Resistance* **10**:315–320 DOI [10.1016/j.jgar.2017.06.009](https://doi.org/10.1016/j.jgar.2017.06.009).
- Chun BH, Kim KH, Jeong SE, Jeon CO. 2020. The effect of salt concentrations on the fermentation of doenjang, a traditional Korean fermented soybean paste. *Food Microbiology* **86**:Article 103329 DOI [10.1016/j.fm.2019.103329](https://doi.org/10.1016/j.fm.2019.103329).
- Despreaux CW, Manning RF. 1993. The *dacA* gene of *Bacillus stearothermophilus* coding for d-alanine carboxypeptidase: cloning, structure and expression in *Escherichia coli* and *Pichia pastoris*. *Gene* **131**:35–41 DOI [10.1016/0378-1119\(93\)90666-Q](https://doi.org/10.1016/0378-1119(93)90666-Q).
- Deutscher J, Herro R, Bourand A, Mijakovic I, Poncet S. 2005. P-Ser-HPr—a link between carbon metabolism and the virulence of some pathogenic bacteria. *BBA-Proteins and Proteomics* **1754**(1–2):118–125 DOI [10.1016/j.bbapap.2005.07.029](https://doi.org/10.1016/j.bbapap.2005.07.029).
- Fernández Ramírez MD, Smid EJ, Abee T, Groot MNN. 2015. Characterisation of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates. *International Journal of Food Microbiology* **207**(17):23–29 DOI [10.1016/j.ijfoodmicro.2015.04.030](https://doi.org/10.1016/j.ijfoodmicro.2015.04.030).
- Flemming HC, Wingender J. 2010. The biofilm matrix. *Nature Reviews Microbiology* **8**(9):623–633 DOI [10.1038/nrmicro2415](https://doi.org/10.1038/nrmicro2415).
- Flower AM. 2007. The SecY translocation complex: convergence of genetics and structure. *Trends in Microbiology* **15**(5):203–210 DOI [10.1016/j.tim.2007.03.001](https://doi.org/10.1016/j.tim.2007.03.001).
- Ge Y, Wei Y, Li C, Chen YR. 2018. Reactive oxygen species metabolism and phenylpropanoid pathway involved in disease resistance against *Penicillium expansum* in apple fruit induced by ϵ -poly-L-lysine. *Journal of the Science of Food and Agriculture* **98**(13):5082–5088 DOI [10.1002/jsfa.9046](https://doi.org/10.1002/jsfa.9046).
- Ghafoor A, Hay LD, Rehm BHA. 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Applied and Environmental Microbiology* **77**(15):5238–5246 DOI [10.1128/AEM.00637-11](https://doi.org/10.1128/AEM.00637-11).
- Glöckner G, Weissenberger CA, Weinmann E, Jacobi S, Schunder E, Steinert M, Hacker J, Heuner K. 2008. Identification and characterization of a new conjugation/type IVA secretion system (*trb/tra*) of *Legionella pneumophila* Corby localized on two mobile genomic islands. *International Journal of Medical Microbiology* **298**(5–6):411–428 DOI [10.1016/j.ijmm.2007.07.012](https://doi.org/10.1016/j.ijmm.2007.07.012).

- Hattersley GJ, Pérez-Velázquez J, Chappell MJ, Bearup D, Roper D, Dowson C, Bugg T, Evans ND. 2011. Indistinguishability and identifiability of kinetic models for the MurC reaction in peptidoglycan biosynthesis. *Computer Methods & Programs in Biomedicine* 104(2):70–80 DOI 10.1016/j.cmpb.2010.07.009.
- Hazan R, Que YA, Maura D, Strobel B, Majcherczyk PA, Hopper LR. 2016. Auto poisoning of the respiratory chain by a quorum-sensing-regulated molecule favors biofilm formation and antibiotic tolerance. *Current Biology* 26(2):195–206 DOI 10.1016/j.cub.2015.11.056.
- Heptinstall S, Archibald AR, Baddiley J. 1970. Teichoic acids and membrane function in bacteria. *Nature* 225:519–521 DOI 10.1038/225519a0.
- Hoyle BD, Wong CKW, Costerton JW. 1992. Disparate efficacy of tobramycin on Ca²⁺, Mg²⁺, and HEPES treated *Pseudomonas aeruginosa* biofilms. *Canadian Journal of Microbiology* 38(11):1214–1218 DOI 10.1139/m92-201.
- Hrast M, Sosič I, Šink R, Gobec S. 2014. Inhibitors of the peptidoglycan biosynthesis enzymes MurA-F. *Bioorganic Chemistry* 55:2–15 DOI 10.1016/j.bioorg.2014.03.008.
- Hu ZH, Pu J, Bai YJ. 2019. Characterisation of five alcohol dehydrogenases from *Lactobacillus reuteri* DSM20016. *Process Biochemistry* 86:73–79 DOI 10.1016/j.procbio.2019.08.010.
- Ibusquiza PS, Groot MN, DEBÁN-Valles A, Abee T, Besten HMW. 2015. Impact of growth conditions and role of sigB on *Listeria monocytogenes* fitness in single and mixed biofilms cultured with *Lactobacillus plantarum*. *Food Research International* 71:140–145 DOI 10.1016/j.foodres.2015.03.001.
- Ishibashi T, Isogai M, Kiyohara H, Hosaka M, Chiku H, Koga A, Yamamoto T, Uchiyama Y, Mori Y, Hashimoto J, Ausió J, Kimura S, Sakaguchi K. 2006. Higher plant RecA-like protein is homologous to RadA. *DNA Repair* 5(1):80–88 DOI 10.1016/j.dnarep.2005.07.010.
- Kiew TY, Cheow WS, Hadinoto K. 2014. Importance of biofilm age and growth medium on the viability of probiotic capsules containing *Lactobacillus rhamnosus* GG biofilm. *LWT-Food Science and Technology* 59(2):956–963 DOI 10.1016/j.lwt.2014.07.053.
- Kröber M, Verwaaijen B, Wibberg D, Winkler A, Pühler A, Schlüter A. 2016. Comparative transcriptome analysis of the biocontrol strain *Bacillus amyloliquefaciens* FZB42 as response to biofilm formation analyzed by RNA sequencing. *Journal of Biotechnology* 231(10):212–223 DOI 10.1016/j.jbiotec.2016.06.013.
- Kubota H, Senda S, Tokuda H, Tokuda H, Uchiyama H, Nomura N. 2009. Stress resistance of biofilm and planktonic *Lactobacillus plantarum* subsp *plantarum* JCM 1149. *Food Microbiology* 26(6):592–597 DOI 10.1016/j.fm.2009.04.001.
- Laura A, Akbar E, Hermoso JA, Pedro MA, Cava F. 2014. Peptidoglycan remodeling by the coordinated action of multispecific enzymes. *Microbial Drug Resist* 20(3):190–198 DOI 10.1089/mdr.2014.0047.
- Li T, Li SR, Jiang B, Hu XM. 2018. Therapeutic Targeting of the *Staphylococcus aureus* Accessory Gene Regulator (*agr*) System. *Frontiers in Microbiology* 9:Article 55 DOI 10.3389/fmicb.2018.00055.

- Ma JC, Luo B, Hu Z, Wang HH. 2014.** Identification and function research of *fabZ1* and *fabZ2* of *Lactococcus lactis*. *Progress in Biochemistry and Biophysics* **41(8)**:777–786.
- Mane J, Pedrosa E, Loren V, Gassull MA, Espadaler J, Cuñé J, Audivert S, Bonachera MA, Cabré E. 2011.** A mixture of *Lactobacillus plantarum* CECT7315 and CECT7316 enhances systemic immunity in elderly subjects. A dose–response, double-blind, placebo-controlled, randomized pilot trial. *Nutricion Hospitalaria* **26**:1611–1613.
- Maria IPL, Rudy MR, Trier S, Herrfurth C, Feussner I, Bernal A, Manu FS, Leidy C. 2019.** Variations in carotenoid content and acyl chain composition in exponential, stationary and biofilm states of *Staphylococcus aureus*, and their influence on membrane biophysical properties. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1861(5)**:978–987 DOI [10.1016/j.bbamem.2019.02.001](https://doi.org/10.1016/j.bbamem.2019.02.001).
- Mona M, Abdallah NA, El-Shafei K, Tawfik NF, El-Sayed HS. 2020.** Survivability of alginate-microencapsulated *Lactobacillus plantarum* during storage, simulated food processing and gastrointestinal conditions. *Heliyon* **6(3)**:e03541 DOI [10.1016/j.heliyon.2020.e03541](https://doi.org/10.1016/j.heliyon.2020.e03541).
- Muscariello L, Marino C, Capri U, Vastano V, Marasco R, Sacco M. 2013.** CcpA and three newly identified proteins are involved in biofilm development in *Lactobacillus plantarum*. *Journal of Basic Microbiology* **51(1)**:62–71.
- Neuhaus FC, Baddiley J. 2004.** A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiology and Molecular Biology Reviews* **67**:686–723.
- Neveling U, Klasen R, Bringer MS, Sahn H. 1998.** Purification of the pyruvate dehydrogenase multienzyme complex of *Zymomonas mobilis* and identification and sequence analysis of the corresponding genes. *Journal of Bacteriology* **180(6)**:1540–1548 DOI [10.1128/JB.180.6.1540-1548.1998](https://doi.org/10.1128/JB.180.6.1540-1548.1998).
- Nilsson M, Rybtke M, Givskov M, Høiby N, Twetman S, Nielsen TT. 2016.** The *dlt* genes play a role in antimicrobial tolerance of *Streptococcus mutans* biofilms. *International Journal of Antimicrobial Agents* **48(3)**:298–304 DOI [10.1016/j.ijantimicag.2016.06.019](https://doi.org/10.1016/j.ijantimicag.2016.06.019).
- Oguntoyinbo FA, Narbad A. 2015.** Multifunctional properties of *Lactobacillus plantarum* strains isolated from fermented cereal foods. *Journal of Functional Foods* **17**:621–631.
- Oliveira F, France Â, Ccerca N. 2017.** *Staphylococcus epidermidis* is largely dependent on iron availability to form biofilms. *International Journal of Medical Microbiology* **307(8)**:552–563 DOI [10.1016/j.ijmm.2017.08.009](https://doi.org/10.1016/j.ijmm.2017.08.009).
- Pessione E. 2012.** Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Frontiers in Cellular and Infection Microbiology* **2(86)**:1–12.
- Rachid SK, Ohlsen U, Wallner J, Hacker J, Hecker M, Ziebuhr W. 2000.** Alternative transcription factor sigma B is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. *Journal of Bacteriology* **182(32)**:6824–6826 DOI [10.1128/JB.182.23.6824-6826.2000](https://doi.org/10.1128/JB.182.23.6824-6826.2000).
- Ramesh S, Sivasubramanian S, Veera RA, Karutha PS. 2019.** Synergistic antibiofilm efficacy of undecanoic acid and auxins against quorum sensing mediated

- biofilm formation of luminescent *Vibrio harveyi*. *Aquaculture* **498**:162–170
DOI [10.1016/j.aquaculture.2018.08.038](https://doi.org/10.1016/j.aquaculture.2018.08.038).
- Santiago B, Marek M, Faustoferri RC, Quivey RG. 2013.** The *Streptococcus mutans* aminotransferase encoded by *ilvE* is regulated by *CodY* and *CcpA*. *Journal of Bacteriology* **195**(16):3552–3562 DOI [10.1128/JB.00394-13](https://doi.org/10.1128/JB.00394-13).
- Schneider K, Dimroth P, Bott M. 2000.** Identification of triphosphoribosyl-dephospho-CoA as precursor of the citrate lyase prosthetic group. *FEBS Letters* **483**:165–168
DOI [10.1016/S0014-5793\(00\)02105-0](https://doi.org/10.1016/S0014-5793(00)02105-0).
- Seneviratne CJ, Suriyanarayanan T, Swarup S, Chia KHB, Nagarajan N, Zhang C. 2017.** Transcriptomics analysis reveals putative genes involved in biofilm formation and biofilm-associated drug resistance of *Enterococcus faecalis*. *Journal of Endodontics* **43**(6):949–955 DOI [10.1016/j.joen.2017.01.020](https://doi.org/10.1016/j.joen.2017.01.020).
- Sieuwert S, Molenaar D, Sacha AFT, Beerthuyzen M, Stevens MJ, Janssen PWA, Ingham CJ, De Bok FA, De Vos WM, Van Hylckama Vlieg JE. 2010.** Mixed-culture transcriptome analysis reveals the molecular basis of mixed-culture growth in *Streptococcus thermophiles* and *Lactobacillus bulgaricus*. *Applied and Environmental Microbiology* **76**:7775–7784 DOI [10.1128/AEM.01122-10](https://doi.org/10.1128/AEM.01122-10).
- Silvia AS, Ramos CG, Almeida F, Luís MS, Wopperer J. 2008.** *Burkholderia cenocepacia* J2315 acyl carrier protein: a potential target for antimicrobials' development. *Microbial Pathogenesis* **45**(5–6):331–336 DOI [10.1016/j.micpath.2008.08.002](https://doi.org/10.1016/j.micpath.2008.08.002).
- Smyer JR, Jeter RM. 1989.** Characterization of phosphoenolpyruvate synthase mutants in *Salmonella typhimurium*. *Archives of Microbiology* **153**:26–32
DOI [10.1007/BF00277536](https://doi.org/10.1007/BF00277536).
- Song B, Leff LG. 2003.** Influence of magnesium ions on biofilm formation by *Pseudomonas fluorescens*. *Microbiological Research* **161**:355–361.
- Song LY, Wu JF, Xi CW. 2012.** Biofilms on environmental surfaces: evaluation of the disinfection efficacy of a novel steam vapor system. *American Journal of Infection Control* **40**(10):926–930 DOI [10.1016/j.ajic.2011.11.013](https://doi.org/10.1016/j.ajic.2011.11.013).
- Tanner ME. 2002.** Understanding nature's strategies for enzyme-catalyzed racemization and epimerization. *Accounts of Chemical Research* **35**(3):237–246
DOI [10.1021/ar000056y](https://doi.org/10.1021/ar000056y).
- Tripathi MK, Giri SK. 2014.** Probiotic functional foods: survival of probiotics during processing and storage. *Journal of Functional Foods* **9**(1):225–241
DOI [10.1016/j.jff.2014.04.030](https://doi.org/10.1016/j.jff.2014.04.030).
- Valle J, Vergarairigaray M, Merino N, Penadés JR, Lasa I. 2007.** σ B regulates IS256-mediated *Staphylococcus aureus* biofilm phenotypic variation. *Journal of Bacteriology* **189**(7):2886–2896 DOI [10.1128/JB.01767-06](https://doi.org/10.1128/JB.01767-06).
- Wood T. 1968.** Physiological functions of the pentose phosphate pathway. *Cell Biochemistry & Function* **4**(4):241–247.
- Wu YC, Cai P, Jing XX, Jing XX, Niu XK. 2019.** Soil biofilm formation enhances microbial community diversity and metabolic activity. *Environment International* **132**:105–116.

- Xiong T, Guan QQ, Song SH, Hao MY, Xie MY. 2012.** Dynamic changes of lactic acid bacteria flora during Chinese sauerkraut fermentation. *Food Control* **26**(1):178–181 DOI [10.1016/j.foodcont.2012.01.027](https://doi.org/10.1016/j.foodcont.2012.01.027).
- Yan J, Bonine LB. 2019.** Surviving as a community: antibiotic tolerance and persistence in bacterial biofilms. *Cell Host & Microbe* **26**:15–21 DOI [10.1016/j.chom.2019.06.002](https://doi.org/10.1016/j.chom.2019.06.002).
- Young KD. 2006.** The selective value of bacterial shape. *Microbiology and Molecular Biology Review* **70**:660–703 DOI [10.1128/MMBR.00001-06](https://doi.org/10.1128/MMBR.00001-06).
- Zhang F, Gao J, Wang B, Huo DX, Wang ZX, Zhang JC, Shao YY. 2018.** Whole-genome sequencing reveals the mechanisms for evolution of streptomycin resistance in *Lactobacillus plantarum*. *Journal of Dairy Science* **101**(4):2867–2874 DOI [10.3168/jds.2017-13323](https://doi.org/10.3168/jds.2017-13323).
- Zhang QS, Chen G, Shen WX, Wang Y, Zhang WX, Chi YL. 2016.** Microbial safety and sensory quality of instant low-salt Chinese paocai. *Food Control* **59**:575–580 DOI [10.1016/j.foodcont.2015.06.041](https://doi.org/10.1016/j.foodcont.2015.06.041).
- Zhang SS, Xu ZS, Qin LH, Kong J. 2020.** Low-sugar yogurt making by the co-cultivation of *Lactobacillus plantarum* WCFS1 with yogurt starter cultures. *Journal of Dairy Science* **103**:3045–3054 DOI [10.3168/jds.2019-17347](https://doi.org/10.3168/jds.2019-17347).
- Zhao JW, Ao XL, Cai YM, Liu SL, Chen AJ, Wan H, Xu F, Wang F, He JY. 2019.** Effect of metal ions on the biofilm formation and environmental tolerance of *Lactobacillus Plantarum* RS66CD. *Food and Fermentation Industries* **45**(21):46–52.
- Zhao XC, Liu ZH, Liu ZJ, Meng RZ. 2018.** Phenotype and RNA-seq based transcriptome profiling of *Staphylococcus aureus* biofilms in response to tea tree oil. *Microbial Pathogenesis* **123**:304–313 DOI [10.1016/j.micpath.2018.07.027](https://doi.org/10.1016/j.micpath.2018.07.027).
- Zhou L, Yu YH, Chen XP, Diab A, Wang HH, He YW. 2015.** The multiple DSF-family QS signals are synthesized from carbohydrate and branched-chain amino acids via the FAS elongation cycle. *Scientific Reports* **5**:13294 DOI [10.1038/srep13294](https://doi.org/10.1038/srep13294).