J. Dairy Sci. 102:10838–10844
https://doi.org/10.3168/jds.2019-16593

Short communication: Complete genome sequence of *Lactobacillus plantarum* J26, a probiotic strain with immunomodulatory activity

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ABSTRACT

Lactobacillus plantarum J26, a significant probiotic isolated from Chinese traditional fermented dairy products, exerts a positive immunomodulatory effect by regulating the expression of immune-related genes. We investigated expression of the cytokines IL-1α, IL-1β, IL-6, and tumor necrosis factor-α in the intestinal tract of mice stimulated by *L. plantarum* J26. In vivo, these cytokines were upregulated, peaked on d 5, and then decreased to the control level, indicating that *L. plantarum* J26 could induce expression of the genes encoding these proinflammatory cytokines. Teichoic acids produced by *L. plantarum* are recognized as key immunomodulatory molecules involved in the regulation of the host immune response. To better understand the genetic basis of this immunomodulatory mechanism, we sequenced and analyzed the whole genome of *L. plantarum* J26. The genome of *L. plantarum* J26 contains a circular chromosome and 4 circular plasmids. *Lactobacillus plantarum* J26 was predicted to synthesize ribitol-type backbones of wall teichoic acid. Furthermore, orthologous average nucleotide identity (OrthoANI) values showed that the genome was highly similar (>98.00%) to other *L. plantarum* strains, especially to *L. plantarum* ST-III and JDM1. The genomic data of *L. plantarum* J26 provide a genetic basis to further elucidate its mechanism of immunoregulation and will facilitate its application in the functional dairy food industry.
Key words: *Lactobacillus plantarum* J26, complete genome sequence, immunomodulatory activity, cytokine

Short Communication

Lactobacillus constitutes a large and diverse genus within the lactic acid bacteria and its genetic diversity is larger than that of a normal family (Sun et al., 2015). *Lactobacillus plantarum* is a gram-positive bacterium (Hugenholtz, 1998) that inhabits relatively abundant ecological niches and plays a significant role in food microbiology and human nutrition because of its fermentative and probiotic functions (Salvetti et al., 2012). Recently, numerous studies have investigated the health benefits of *L. plantarum*, such as effects on oxidative stress regulation (Li et al., 2014), reduction of cholesterol level (Barreto et al., 2014), and management of bacterial composition in feces (Goossens et al., 2003). Among these properties, the potential of *L. plantarum* to inhibit inflammation has received extensive attention. *Lactobacillus plantarum* GB-LP2 has also shown antiviral effects against the influenza virus in mice (Choi et al., 2015). Similarly, *L. plantarum* PS128 reduced LPS-induced proinflammatory cytokine production in a mouse macrophage cell model (Liu et al., 2015).

Teichoic acid (**TA**), the major component of cell walls of gram-positive bacteria, is recognized as key immunomodulatory molecule involved in the regulation of host immune response. For example, heat-killed *L. plantarum* MYL26 and bacterial cell wall extracts are able to reduce LPS-induced inflammation by impairing toll-like receptor 4 (TLR4)-nuclear factor κB (NF-κB) signal transduction in vitro, suggesting that constituents of bacterial cell wall help attenuate inflammation (Chiu et al., 2013). In addition, an investigation found that D-alanylation of TA abolished the pro- and anti-inflammatory response in vivo (Smelt et al., 2013). However, immunomodulatory capability varies in different strains, probably because the types and structures of TA are significantly different (Han et al., 2003).

Lactobacillus plantarum J26 is a common probiotic strain isolated from traditional fermented dairy products. Our recent investigation showed that strain J26 could alleviate oxidative stress by modulating the production of antioxidant enzymes significantly in vitro (Hou et al., 2019). In the current study, we confirmed that the strain exhibits potent immunomodulatory activity. To better understand the immunomodulatory mechanism of *L. plantarum* J26 and obtain detailed

Received March 10, 2019.
Accepted July 30, 2019.
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insight into the genetic basis, we analyzed the whole genome sequence of *L. plantarum* J26 combined with biochemical assays.

Lactobacillus plantarum J26 was isolated from Chinese traditional fermented dairy products in Inner Mongolia (China). It was grown in de Man, Rogosa, and Sharpe (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, China) broth at 37°C for 16 h through 2 propagation steps. Genomic DNA was extracted using the Qiagen DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Quantity and quality determinations of genomic DNA in *L. plantarum* J26 were conducted by Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The genomic DNA was sequenced by Illumina Miseq (insert size of 400 bp; Illumina Inc., San Diego, CA) with paired-end sequencing mode and Pacbio sequencing (20,000 bp template library; Pacific Biosciences, Menlo Park, CA) with standard sequencing mode using next-generation sequencing technology and single molecule real-time (**SMRT**) technology. A total of 3,221,902 reads were obtained. Genome assembly was performed using A5-miseq v20150522 (Coil et al., 2015) and CANU (Koren et al., 2017) software for the data obtained by second- and third-generation sequencing platforms. Then, the assembled results were confirmed by MUMmer software (http://mummer.sourceforge.net/) to determine the positional relationship between contigs and to fill gaps (Delcher et al., 2003). Finally, a complete genome without gaps was constructed after the correction using Pilon software (Walker et al., 2014). Gene prediction was carried out by GeneMarkS (http://topaz.gatech.edu/). The predictions of transfer (t)RNA, rRNA, and small (s)RNA were identified using tRNAscan-SE (Lowe and Eddy, 1997), rRNAmmer (Lagesen et al., 2007), and Rfam (Gardner et al., 2009) softwares, respectively. Genome annotation was accomplished by National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (Pruitt et al., 2012) and confirmed by BLAST analysis (www.ncbi.nlm.nih.gov/protein). Using the multiple sequence alignment software MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) to align the core gene sequences of the *L. plantarum* J26 and other species, we constructed a phylogenetic tree based on the core genes (Kumar et al., 2016). Furthermore, the average nucleotide identity of the genome sequence between the *L. plantarum* J26 and other reference strains was evaluated by orthologous average nucleotide identity (**OrthoANI**; http://www.ezbiocloud.net/tools/orthoani) tool.

The expression of immune-related genes was evaluated in vivo. All of the procedures used here were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University in China, and the animal experiments were performed following the Northeast Agricultural University guidelines for Laboratory Animals Care and Use.

Forty BALB/cl mice, 8 wk old and 18 to 22 g of BW, were provided by Heilongjiang Province Medical University Laboratory Animal Center (Harbin, China). Mice were randomly divided into 2 groups and acclimated in pathogen-free cages under controlled conditions (22°C ± 2°C, relative humidity 55% ± 5%, 12-h light/dark cycles) with free access to water and a standard diet for 7 d. After this period, the experimental group was given 0.3 mL of skim milk containing *L. plantarum* J26 (10^8 cfu/mL) each day. The mice in the control group were given skim milk without *L. plantarum* J26. The mice were killed at 1, 3, 5, or 7 d after supplementation of bacteria. The colon and ileum tissue were removed, washed with cold saline, and placed in liquid nitrogen (Jiang et al., 2016). To evaluate the mRNA expression levels of *IL1A*, *IL1B*, *IL6*, and *TNFA*, RNA of colon and ileum was extracted using a commercial kit (RNAprep Tissue Kit, Tiangen Biotech, Shanghai, China) and converted to cDNA using a PrimeScript RT

Table 1. The base sequence, annealing temperatures, and sizes of PCR products for target gene-specific primers

Target gene	Primer sequence[1] (5′→3′)	Annealing temperature (°C)	Product size (bp)
GAPDH	F: GCCTGGAGAAACCTGCC	55.4	200
	R: ATACCAGGAAATGAGCTTGACA	56.7	
IL1A	F: TCTGCCATTGACCATCTC	50.7	183
	R: AATCTTCCCGTTGCTTG	50.8	
IL1B	F: AAGTTGACGGACCCCA	50.6	126
	R: GTGATACTGCCTGCCTGA	51.3	
IL6	F: GAAGTGATTCTTACGCA	42.5	247
	R: GTTTAGGTGGAGGTGTC	43.0	
TNFA	F: CGTAGCAAACCACCAAG	49.3	149
	R: CCGTGAAGAGAACCTGG	50.4	

[1]F = forward; R = reverse.

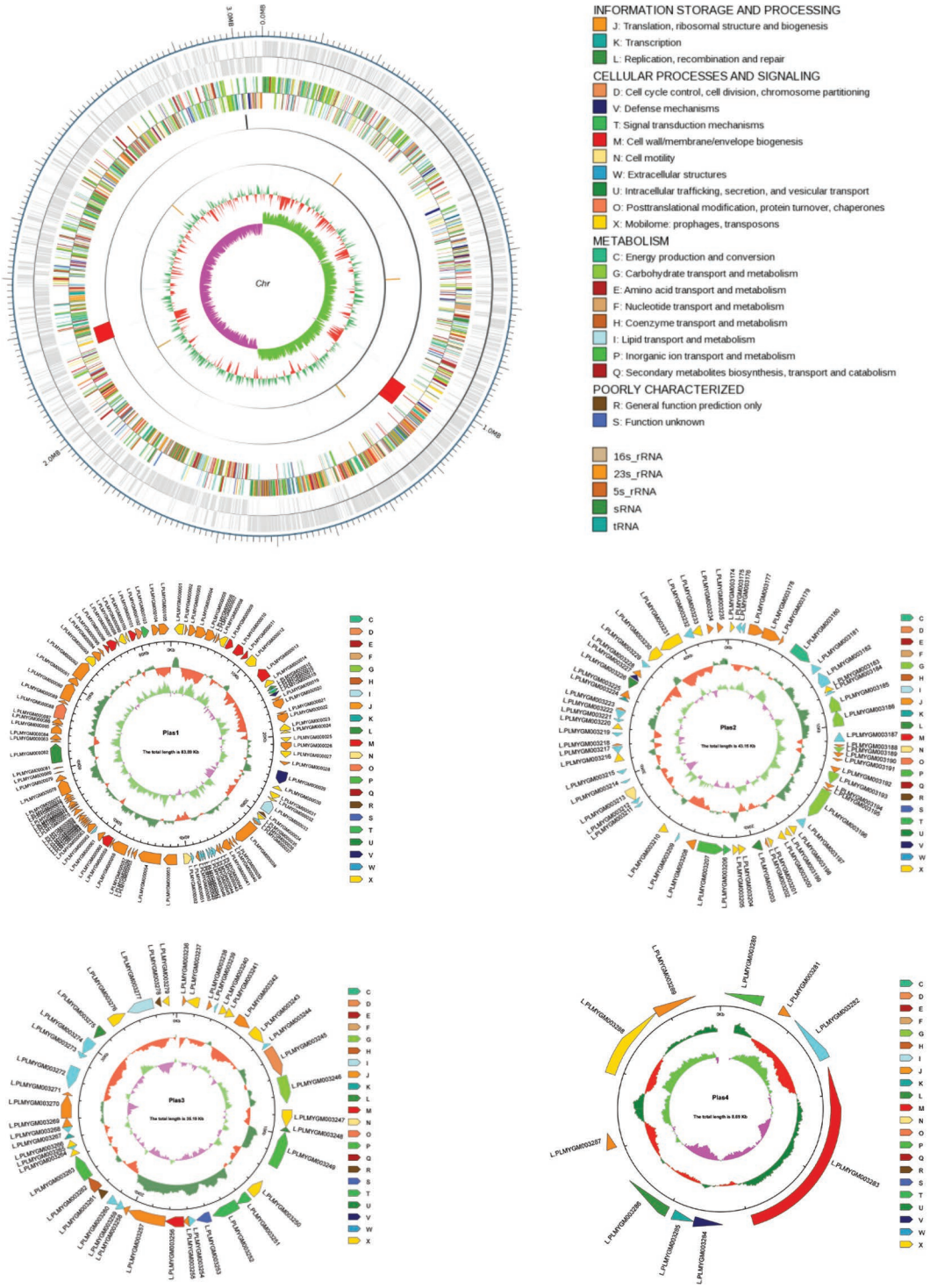

Figure 1. Circular genome map of *Lactobacillus plantarum* J26. From periphery to center: genome sequence (ring 1 and 2), Cluster of Orthologous Groups of proteins (COG) annotated coding sequences (ring 3 and 4), intact prophages in red and clustered regularly interspaced short palindromic repeats (CRISPR) in black (ring 5), noncoding (nc)RNA genes (ring 6), GC content (ring 7), and GC skew [(G+C)/(G+C)] (ring 8), where values >0 are in green, and values <0 are in purple. The smaller maps show the genome map of plasmids in *L. plantarum* J26. The image was created using the software Circos (http://circos.ca/software/download/circos/).

Table 2. Features of *Lactobacillus plantarum* J26 genome

Feature[1]	Chromosome	Plasmid 1	Plasmid 2	Plasmid 3	Plasmid 4
Size (bp)	3,096,468	83,889	43,153	35,191	8,686
GC content (%)	44.79	41.14	40.30	45.48	35.92
Predicted genes	3,068	105	62	44	10
Protein-coding genes (CDS)	3,068	105	62	44	10
rRNA operons	16	0	0	0	0
tRNA	71	0	0	0	0
sRNA	1	2	1	1	1

[1]CDS = coding sequence; tRNA = transfer RNA; sRNA = small RNA sequences.

reagent kit (Takara, Dalian, China). The expression of immune-related genes was analyzed by using real-time quantitative PCR (ABI Prism 7500 system; Applied Biosystems, Foster City, CA). Approximately 2 μL of the RNA sample was added to the 20-μL total reaction volume according to the requirements of the SYBR PremicExTaqTMII (PerfectReal-time) kit (Takara). The *GAPDH* gene was used as the reference gene. To amplify the selected immune-related genes *IL1A*, *IL1B*, *IL6*, and *TNFA* and *GAPDH*, specific primers were designed using Primer 5.0 software (Premier Biosoft, USA; http://www.premierbiosoft.com/primerdesign/index.html) as shown in Table 1. The protocol for the amplification reactions was as follows: 95°C for 30 s (denaturation) and 40 cycles of 95°C for 5 s and 60°C for 34 s (amplification and quantification). The results were expressed as relative values after normalization to *GAPDH* mRNA (Jiang et al., 2016).

The complete genome of *L. plantarum* J26 comprises a circular chromosome (3,096,468 bp) and 4 circular plasmids: pJ26p1 (83,889 bp), pJ26p2 (43,153 bp), pJ26p3 (35,191 bp), and pJ26p4 (8,686 bp), with GC contents of 44.79, 41.14, 40.30, 45.48, and 35.92%, respectively (Figure 1). Among the 3,289 identified protein-coding genes, 3,068 were in the chromosome and 105, 62, 44, and 10 in plasmids pJ26p1 to pJ26p4, respectively. Furthermore, the chromosome included 16 rRNA operons, 71 tRNA, and 1 sRNA (Table 2). Each gene was assigned to a functional category according to the Clusters of Orthologous Groups database (www.ncbi.nlm.nih.gov/COG; Table 3). Among 23 functional categories, the most abundant were assigned to transcription (248 genes); 236 genes were assigned to carbohydrate transport and metabolism; and 137 genes were responsible for cell wall, membrane, and envelope biogenesis.

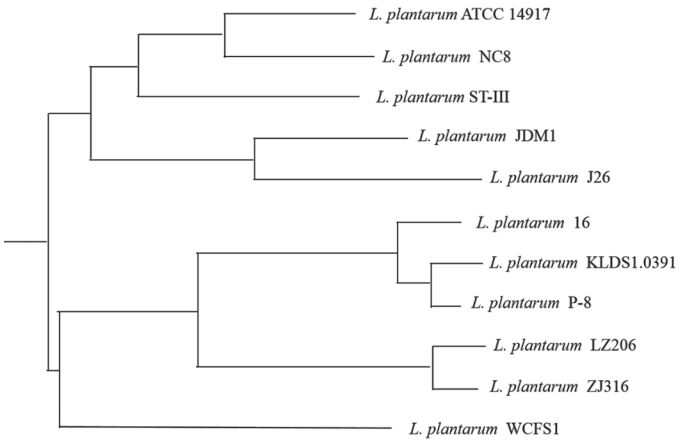

Figure 2. Phylogenetic tree based on core gene sequence showing the phylogenetic relationships of 11 *Lactobacillus plantarum* strains. Phylogenetic analysis was evaluated by MUSCLE software (https://www.ebi.ac.uk/Tools/msa/muscle/).

A phylogenetic tree was constructed based on homology of core genes of *L. plantarum* J26 with related strains. Phylogenetic tree analysis (Figure 2) showed that *L. plantarum* J26 was more closely related to *L. plantarum* JDM1 than to other *L. plantarum* strains and evolved from the common ancestor compared with ST-III (which contains TA synthesis protein B instead of TA synthesis proteins TagF1 and TagF2 (Liu et al., 2015). The genome of J26 was compared with 10 other *L. plantarum* strains. The OrthoANI values (Table 4) indicated that the genome of *L. plantarum* J26 was closest to *L. plantarum* JDM1 (99.30% OrthoANI), followed by *L. plantarum* 5-2 (98.73%) and *L. plantarum* WCFS1 (98.71%). Generally, two genomes are considered the same species when the ANI value is higher than 95 to 96% (Lee et al., 2016). Consequently, *L. plantarum* J26 was confirmed to belong to the species of *L. plantarum.*

Intestinal epithelial cells stimulated by microbes can secrete cytokines and pattern recognition receptors to participate in appropriate immune regulation (Round and Mazmanian, 2009). As shown in Figure 3, we investigated mRNA expression of proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α in the intestinal tract of mice stimulated with *L. plantarum* J26. These cytokines were all upregulated, with expression peaking at d 5, and then decreasing to control levels. Moreover, the expression of *IL1A*, *IL1B*, *IL6*, and *TNFA* was sig-

Table 3. Cluster of Orthologous Groups of proteins (COG) categories of coding proteins in *Lactobacillus plantarum* J26

COG class	Name	Count	Proportion (%)
C	Energy production and conversion	111	4.21
D	Cell cycle control, cell division, chromosome partitioning	36	1.37
E	Amino acid transport and metabolism	219	8.31
F	Nucleotide transport and metabolism	102	3.87
G	Carbohydrate transport and metabolism	236	8.96
H	Coenzyme transport and metabolism	126	4.78
I	Lipid transport and metabolism	95	3.61
J	Translation, ribosomal structure and biogenesis	203	7.71
K	Transcription	248	9.42
L	Replication, recombination and repair	114	4.33
M	Cell wall/membrane/envelope biogenesis	137	5.20
N	Cell motility	17	0.65
O	Posttranslational modification, protein, turnover, chaperones	86	3.26
P	Inorganic ion transport and metabolism	129	4.90
Q	Secondary metabolites biosynthesis, transport and catabolism	28	1.06
R	General function prediction only	237	9.00
S	Function unknown	161	6.11
T	Signal transduction mechanisms	118	4.48
U	Intracellular trafficking, secretion, and vesicular transport	18	0.68
V	Defense mechanisms	76	2.89
W	Extracellular structures	3	0.11
X	Mobilome: prophages, transposons	134	5.09

nificantly different from that in the control group ($P < 0.01$) on d 5, indicating that *L. plantarum* J26 was able to trigger the mRNA expression of the genes encoding these proinflammatory cytokines in vivo, results consistent with our previous study with another *L. plantarum* strain (Jiang et al., 2016). Although IL-1α, IL-1β, IL-6, and TNF-α are considered proinflammatory cytokines, the specific mechanism by which *L. plantarum* J26 regulates their expression is unknown. We found no evidence that the immunomodulatory activity of *L. plantarum* J26 was directly related to certain host cells; our results indicate that the immune responses induced by J26 were likely linked to the type of immune-related cytokines, which is the focus of our further research.

The immunomodulatory mechanism of J26 was further investigated by analysis of the whole genome of *L. plantarum* J26. Teichoic acids consist of 2 types: wall teichoic acid (**WTA**) and lipoteichoic acid (**LTA**). For *L. plantarum* WCFS1, D-alanylated TA in the cell walls account for several host immunomodulatory effects (Smelt et al., 2013). Because TA are recognized as immunoregulatory molecules, the TA-related genes of *L. plantarum* J26 have been searched by in silico analysis. The synthesis genes encoding the WTA backbones are *tag* and *tar* homologs, whereas those of LTA are *ltaS*. The genes encoding LTA synthase (*ltaS*), TA glycosylation proteins (*gtcA1*, *gtcA2*, *gtcA3*), and D-alanylation protein (*dltX*) were found in the *L. plantarum* J26 genome. Two gene regions, *tagD1-tagF2* (*tagD1*, *tagF1*, and *tagF2*) and *tarI-tarL* (*tarI*, *tarJ*, *tarK* and *tarL*), were regarded as the synthesis genes of glycerol-type and ribitol-type backbones of WTA. In *L. plantarum* J26, only a glycerol-3-phosphate cytidylyltransferase gene *tagD1* was detected. The genes encoding TA synthesis protein F (*tagF1* and *tagF2*) were absent. However, there are 2 TA synthesis protein B genes (*tagB1*

Table 4. Average nucleotide identity (ANI; %) values between the strains and different *Lactobacillus* plantarum species

Strain code	16	5-2	JDM1	LPL-1	LZ206	LZ227	LZ95	WCFS1	J26	ST-III	ZJ316
16	100.00	98.71	98.90	99.07	99.12	98.88	98.80	98.76	98.53	98.73	99.11
5-2	98.71	100.00	98.94	98.73	98.76	98.71	99.07	99.00	98.73	99.00	98.73
JDM1	98.90	98.94	100.00	98.87	98.72	98.83	98.96	98.95	99.30	98.87	98.75
LPL-1	99.07	98.73	98.87	100.00	98.70	98.80	98.74	98.79	98.70	98.67	98.70
LZ206	99.12	98.76	98.72	98.70	100.00	99.27	98.73	98.69	98.51	98.66	99.71
LZ227	98.88	98.71	98.83	98.80	99.27	100.00	98.67	98.66	98.40	98.66	99.31
LZ95	98.80	99.07	98.96	98.74	98.73	98.66	100.00	98.93	98.71	99.89	98.83
WCFS1	98.76	99.00	98.95	98.79	98.69	98.66	98.93	100.00	98.71	98.99	98.77
J26	98.53	98.73	99.30	98.70	98.51	98.40	98.71	98.71	100.00	98.62	98.56
ST-III	98.73	99.00	98.87	98.67	98.66	98.66	99.89	98.99	98.62	100.00	98.73
ZJ316	99.11	98.75	98.75	98.70	99.71	99.31	98.83	98.77	98.56	98.73	100.00

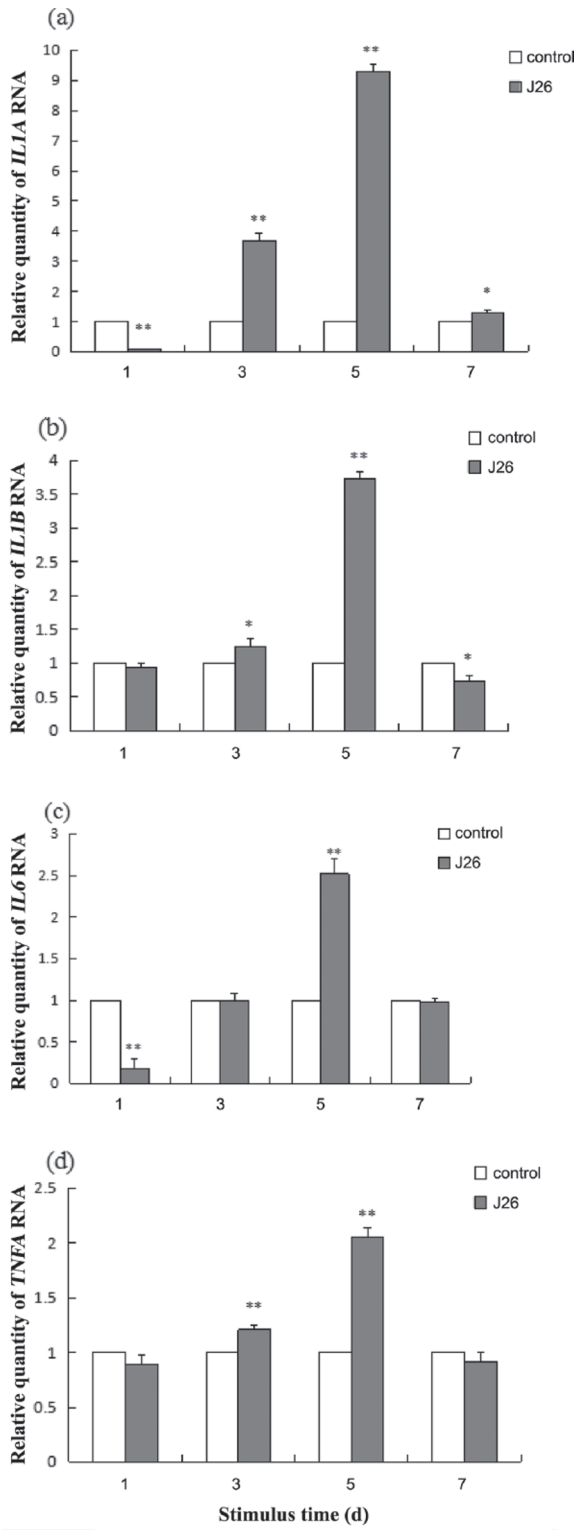

Figure 3. Expression of *IL1A*, *IL1B*, *IL6*, and *TNFA* genes induced by *Lactobacillus plantarum* J26 in mice. Mice were intragastrically administered 0.3 mL of *L. plantarum* J26 (gray bars) or an equal amount of sterilized skim milk (white bars) for 7 d, and the expression of *IL1A* (a), *IL1B* (b), *IL6* (c), and *TNFA* (d) was determined. Results are expressed as mean ± SD of 3 independent determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

and *tagB2*) instead, and *tagB1* and *tagB2* found in *L. plantarum* were supposed as *tarK* and *tarL*. The *tarI* and *tarJ* genes were annotated as D-ribitol-5-phosphate cytidylyltransferase and ribitol-5-phosphate 2-dehydrogenase. Hence, we propose that *L. plantarum* J26 contains *tarI-tarL* region and synthesizes ribitol-type backbones of WTA. Besides, 2 TA transporters (permease protein, *tag*; and ATP-binding protein, *tagH*), subunits of the ABC transporter complex, were identified and may be associated with the immunomodulatory effects of *L. plantarum* J26.

In the current study, we demonstrated that the *L. plantarum* J26 is a probiotic strain with immunomodulatory activity (regulating the expression of immune-related genes). The genome data of *L. plantarum* J26 provide substantial information for understanding the genetic basis of its probiotic properties. In addition, we discovered genes involved in key immunomodulatory molecules (teichoic acids) in the genome of J26. These results contribute to the development of immune-related functional dairy foods containing lactic acid bacteria. Further investigation into the molecular mechanism by which the TA-related gene products affect the host immune response are ongoing. The complete genomic sequence of *L. plantarum* J26 has been deposited in National Center for Biotechnology Information under GenBank accession nos. CP033616, CP033617, CP033618, CP033619, and CP033620.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Beijing; project number 31871828) and the National High-Level Talents Special Support Program of China (810098).

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