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Research Article

# Identification and expression of key genes related to 1-deoxynojirimycin biosynthesis in *Streptomyces lavendulae*

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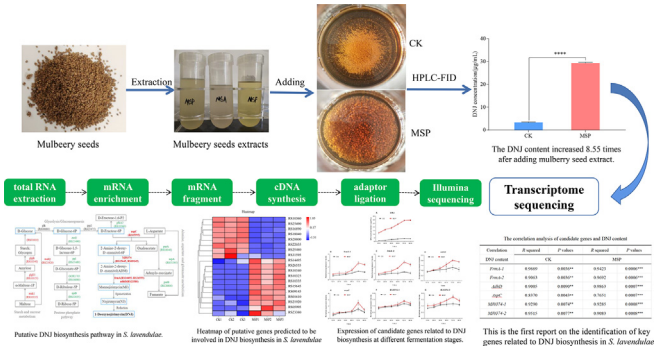
## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* *Streptomyces lavendulae* is a bacterium that produces 1-deoxynojirimycin (DNJ), an alkaloid with various beneficial pharmacological properties. While a putative DNJ biosynthesis metabolite pathway has been proposed for this bacterium, the related key genes have not yet been identified.
*Results:* In this study, DNJ production during *S. lavendulae* fermentation was induced by mulberry seed extracts and the key genes related to DNJ biosynthesis under those experimental conditions were identified using transcriptome sequencing. Gene expression was then analyzed using real-time qPCR, and related to metabolite levels in the DNJ pathway. The mulberry seed polysaccharide extract (MSP) induced DNJ yield in *S. lavendulae* broth more effectively than the mulberry seed alkaloid and flavonoid extracts. Supplementation with 3.00 mg/mL MSP resulted in 27.19 μg/mL DNJ, which was 8.55 times than that in the control group (3.18 μg/mL). Transcriptome sequencing revealed 2328 differentially expressed genes (DEGs) in MSP vs. unsupplemented-control comparisons, of which 1151 DEGs were up-regulated and 1177 DEGs were down-regulated in MSP. Functional enrichment analysis revealed four pathways of DNJ biosynthesis. Six key genes related to DNJ biosynthesis were then validated using real-time qPCR and the analysis of intermediate metabolites. These were, *aspC*, encoding a putative transaminase that produces 2-amino-2-deoxy-D-mannitol-6-phosphate; *MJ0374-1* and *MJ0374-2*, encoding a phosphatase that produces 2-amino-2-deoxy-D-mannitol; and *frmA-1*, *frmA-2*, and *adhD*, which encode a Zn-dependent dehydrogenase that produces mannojirimycin.
*Conclusions:* This is the first study to identify the genes relevant for DNJ biosynthesis in *S. lavendulae*, and

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it paves the way for engineering the bacterium to achieve for industrial-scale DNJ production.

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## 1. Introduction

Deoxynojirimycin (DNJ) is a natural alkaloid that possesses a variety of beneficial pharmacological properties, such as anti-diabetic, anti-virus, anti-tumor, and anti-inflammatory properties [1,2]. It is produced by plants, such as mulberry (*Morus* L.) [3] and Asiatic dayflower (*Commelina communis*) [4], and various microorganisms, such as *Bacillus* sp. [5] and *Streptomyces* sp. [6].

Considering rapid microbial growth, there is an increased interest in identifying and using DNJ from microbial sources [7,8,9]. Nojirimycin (NJ) was the first natural azasugar related to DNJ synthesis isolated from the fermentation broth of *Streptomyces* [10]. DNJ, its more stable derivative, was synthesized from NJ and later isolated from diverse natural sources. Optimization of fermentation conditions of *Bacillus subtilis* [11] and *Bacillus methylotrophicus* [12] and construction of genetically engineered strains of *Escherichia coli* [13] and *Bacillus amyloliquefaciens* [14] have since been reported for the industrial-scale production of DNJ.

The DNJ biosynthesis pathway has been investigated in some species. In mulberry leaves, lysine was identified as the precursor, which is then converted to cadaverine, 5-aminopentanal, piperideine, finally resulting in DNJ [15]. In addition, Hardick et al. concluded that glucose is the precursor for DNJ biosynthesis in *S. subrutilus* and *B. subtilis* [16,17]. In a synthetic process, DNJ was generated through a C2-N-C6 cyclization reaction. In *Commelina communis*, glucose was also the precursor of DNJ, but it was formed via a C1-N-C5 cyclization reaction [4]. The production of DNJ by *S. lavendulae*, the main DNJ-producing strain, was first reported in 1985 [6]. Wu et al. [18] reported that fructose-6-phosphate (F6P) is the precursor of DNJ in this bacterium. DNJ biosynthesis is followed by amination, dephosphorylation, oxidation, cyclization, dehydration, and reduction reactions. Considering the above findings, it can be concluded that the DNJ biosynthetic routes differ between various species, thus, the enzymes and genes involved in DNJ biosynthesis are also different.

The enzymes and genes involved in DNJ biosynthesis have been reported in some microorganisms. Three genes related to DNJ biosynthesis have been identified in *B. amyloliquefaciens* [19] and *B. subtilis* [20]. These are *gabT1*, *yktc1*, and *gutB1*, and encode putative transaminase, phosphatase, and Zn-dependent dehydrogenase, respectively. Furthermore, Beal and Horenstein [21] showed that the three key genes (*gabT1*, *yktc1*, and *gutB1*) related to DNJ biosynthesis in *S. subrutilis* ATCC 27467 are related to those in *Bacillus velezensis* FZB42. However, the key genes involved in DNJ biosynthesis in *Streptomyces* sp. have not yet been identified. In a previous research, we attempted conducted to clone the genes whose products are involved in the synthesis of DNJ in *Streptomyces lavendulae* based on the sequences of *Bacillus*. sp *gabT1*, *yktC1*, and *gutB*. However, we did not obtain positive results, which was consistent with the findings of Wu et al. [18]. Therefore, further research is required to identify the key genes involved in DNJ biosynthesis in *Streptomyces lavendulae.*

It is reported that mulberry seeds contain the highest amounts of DNJ among all tissues, such as roots, fruits and leaves [22]. We have previously shown that mulberry extract supplementation significantly improves DNJ yield in the fermentation broth of *S. lavendulae*. In the current study, we compared the efficiency with which mulberry seed polysaccharide, alkaloid, and flavonoid extracts induce DNJ production in the fermentation broth of *S. lavendulae*. The best-inducing extract was then used for transcriptome sequencing analysis of *S. lavendulae* to identify differentially expressed genes (DEGs) involved in DNJ biosynthesis in this strain. Gene expression was then validated by real-time qPCR (RT-qPCR). Finally, changes in DNJ content and the levels of its precursor and intermediate metabolites during *S. lavendulae* fermentation were correlated with specific gene expression. Our results provide new technical insights for engineering the bacterium to achieve industrial-scale DNJ production.

## 2. Materials and methods

### 2.1. Strain and growth conditions

The DNJ-producing strain *S. lavendulae* ATCC 8664 was purchased from BeNa Culture Collection (Henan, China). The strain was activated by growing on ISP-2 medium consisting of yeast extract (4.0 g/L), malt extract (10.0 g/L), glucose (4.0 g/L), and agar (20.0 g/L) at 28°C for 3 d.

### 2.2. Preparation of mulberry seed extracts

Mulberry (*Morus alba* L.) seeds were collected from a mulberry plantation of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). Three plants per variety were randomly selected. Matured fruits harvested from each variety were homogenized and were washed with distilled water to separate the seeds.

Dried mulberry seeds were ground in a blender to obtain a fine powder (particle diameter: 0.2–0.5 mm) for extraction of total alkaloids, total flavonoids, and polysaccharides. Total alkaloids extraction was carried out using acidified ethanol (25% ethanol −0.05 mol/L HCl) in the reaction of dried mulberry seeds 2.5 g and acidified ethanol 50 mL, assisted by ultrasonic disruption. Total flavonoids were extracted using 1 g sample in 30 mL of 80% ethanol solvent, assisted by ultrasonic disruption. The total alkaloids and total flavonoids were detected as previously described, with minor modifications [23].

The polysaccharide was extracted by the Sevag method and determined using a previously described method [24].

### 2.3. DNJ extraction and determination

DNJ was extracted as previously described, with minor modifications [18]. Briefly, 1000 µL of fermentation broth of *S. lavendulae* was sterilized at 121°C for 15 min. After centrifugation at 13,400 × *g* for 15 min, 500 µL of the supernatant was transferred to a 1.5 mL tube containing 500 µL of 0.05 mol/L HCl. The mixture was vortex-mixed for 1 min, ultrasonicated for 40 min, and then centrifuged at 13,400 × *g* for 15 min. DNJ levels in the supernatant were determined using the HPLC-FD (Shimadzu, Japan) system as described previously [25]. For the analysis, 15 µL aliquot of the filtrate was injected into the Inertsil ODS-SP C-18 HPLC column (5 µm, 4.6 mm × 250 mm) (Shimadzu) and separated using a

mobile phase of acetonitrile: 0.1% acetic acid (45:55, v/v), at a flow rate of 1 mL/min. All determinations were performed in triplicate.

### 2.4. RNA extraction

Total RNA was extracted from the bacterial fermentation broth using the TRIzol-based method (Life Technologies, CA, USA). RNA quality was measured using 1% agarose gel electrophoresis (Bio-Rad, USA) and Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

### 2.5. cDNA library construction and sequencing

rRNA was depleted from 1 μg total RNA using the Illumina MRZB12424 Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, San Diego, CA, USA). The first strand of cDNA was synthesized using ProtoScript II Reverse Transcriptase (New England BioLabs, Ipswich, MA, USA) at 25°C for 10 min, 42°C for 15 min, and 70°C for 15 min. The second strand of cDNA was synthesized using the NEBNext Second Strand Synthesis Reaction Buffer and dATP, dGTP, dCTP, and dUTP mix (New England BioLabs) at 16°C for 1 h. The resultant double-stranded cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and end-repaired with NEBNext End Repair Reaction Buffer and Enzyme Mix (New England BioLabs) at 20°C for 30 min and 65°C for 30 min. Sequencing adapters were then attached using NEBNext Adaptor for Illumina (New England BioLabs) at 20°C for 15 min. The double-stranded cDNA was then degraded using the USER enzyme mix (New England BioLabs) at 37°C for 15 min, and the reaction products were purified using Agencourt AMPure XP beads (Beckman Coulter). Finally, the index-coded samples were clustered using the cBot Cluster Generation System using NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs). After clustering, sequencing was performed using the Illumina Novaseq 6000 platform with paired-end 150 base reads.

### 2.6. Sequencing data filtering

Raw sequencing data were filtered by removing reads with $\geq$ 10% unidentified nucleotides (N), those with > 50% bases with phred quality scores of $\leq$ 20, and those aligned to the barcode adapter, using FASTP (version 0.18.0) [26]. High-quality trimmed reads were mapped to the reference genome using Bowtie2 (version 2.2.8) allowing no mismatches to identify known genes [27]. Gene expression was calculated using RSEM [28].

### 2.7. DEG analysis

Fragments per kilobase of transcript per million fragments mapped (FPKM) were used to determine gene expression to eliminate the influence of gene length and sequencing depth on the calculated gene expression. The edgeR package (http://www.r-project.org/) was used to identify DEGs across samples with fold changes $\geq$ 2 and a false discovery rate-adjusted *P* (*Q*-value) < 0.05. Data were further subjected to enrichment analysis of Gene Ontology (GO) functions (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg), and *Q*-value < 0.05 was used as a threshold.

### 2.8. RT-qPCR

cDNA was generated from DNA-free total RNA using reverse-transcription with TRUEscript RT MasterMix (Aidlab, China) and a random hexamer primer. Next, 2 × SYBR Green qPCR Mix (Aidlab, China) was used in a final volume of 20 μL. qPCR reaction was performed in 96-well plates using an Applied Biosystems 7300 Fast Real-Time PCR system (Thermo Fisher Scientific, USA) with the following thermal cycling protocol: initial incubation at 95°C for 45 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method as previously reported [29], with *hardB* (F: 5′-CGCGGCATGCTCTT CCT-3′, R: 5′-AGGTGGCGTACGTGGAGAAC-3′) as the reference gene [30]. All assays were performed in triplicate.

### 2.9. Determination of glucose, glucose-6-phosphate (G6P), F6P, and fructose-1,6-diphosphate (FDP) levels

Glucose and F6P levels in the fermentation broth were determined following the method described by other researchers, using a UV-2100 spectrophotometer (UNICO, Shanghai, China), and with sample measurements performed at 625 nm and 300 nm, respectively [31,32]. G6P and FDP levels were determined using the G6P ELISA Kit (PYRAM, China) and FDP ELISA Kit (PYRAM), respectively, following the manufacturer's instructions, and a microplate reader (TECAN, Switzerland) set at 450 nm.

### 2.10. Statistical analysis

Data were statistically analyzed using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance (ANOVA) was used to determine significant differences between samples, and *t*-tests were used for correlation analysis. *P*-value of 0.05 was the statistical significance threshold in all statistical analyses. All calculations were performed three times.

### 2.11. GenBank accession numbers of six key genes related to DNJ biosynthesis in S. lavendulae

The GenBank accession number of *aspC* was WP_030235063.1 that of *MJ0374-1* and *MJ0374-2* was WP_030240563.1 and WP_078950191.1, respectively, and that of *frmA1, frmA1* and *adhD* was WP_030241140.1, WP_030238888.1 and WP_030238535.1, respectively.

## 3. Results

### 3.1. Effect of mulberry seed extracts on DNJ yield in the fermentation broth of S. lavendulae

To determine the ability of mulberry seed extracts to induce DNJ production in *S. lavendulae*, bacterial culture was supplemented with different amounts of three different extracts (i.e., total alkaloid, polysaccharide, and total flavonoid extracts) and DNJ yield in culture broth at the fastest bacterial growth stage was then determined. As shown in Fig. 1, DNJ yield was highest upon supplementation with 0.32 mg/mL alkaloid extract (Fig. 1A), 3.00 mg/mL polysaccharide extract (Fig. 1B), and 0.06 mg/mL flavonoid extract (Fig. 1C), and was equal to 26.10 μg/mL, 27.19 μg/mL, and 21.74 μg/mL, respectively. Compared with the DNJ yield in unsupplemented control cultures (3.18 μg/mL), the supplementation increased the yield 8.20, 8.55, and 6.84 times, respectively. These observations indicated that mulberry seed polysaccharide (MSP) extract is an effective inducer of DNJ yield in *S. lavendulae* culture broth. Therefore, the fermentation broth of MSP cultures (0.32 mg/mL MSP) and control (CK) cultures was selected for subsequent transcriptome sequencing analysis.

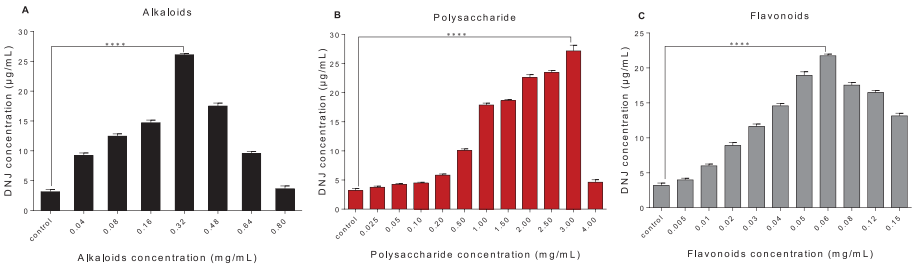


**Fig. 1. DNJ content in the fermentation broth of *Streptomyces lavendulae*.** **(A)** Mulberry seed alkaloid extracts at different concentrations; **(B)** Mulberry seed polysaccharide extracts at different concentrations; **(C)** Mulberry seed flavonoid extracts at different concentrations. **** indicates $P < 0.0001$.

### *3.2. Transcriptome sequencing, clustering, and functional enrichment of DEGs related to DNJ biosynthesis*

To identify potential genes of *S. lavendulae* involved in DNJ biosynthesis, broth from MSP and CK cultures was analyzed using RNA sequencing. Overall, 2328 genes were differentially expressed, among which 1151 were up-regulated and 1177 were down-regulated by MSP.

GO functional annotations of DEGs revealed the enrichment of 36 functional terms. Among these terms, 14 were related to biological processes, 11 to molecular functions, and 11 to cellular components (Fig. 2A). In the biological process category, the DEGs were mainly involved in cellular, metabolic, and single-organism processes. In the molecular function category, the DEGs were mainly involved in catalytic activity and binding. In the cellular component category, the DEGs were mainly involved in cell and cell parts (Fig. 2A). The top 21 pathways with low *Q*-value were selected for significance analysis based on KEGG analysis. As shown in Fig. 2B, the DEGs in the MSP vs. CK group comparisons were mainly enriched in the metabolism category, including metabolic pathway, biosynthesis of secondary metabolites, and microbial metabolism in diverse environments.

### *3.3. DNJ biosynthesis pathway in S. lavendulae*

For an overview of the DNJ biosynthesis pathway in *S. lavendulae*, the transcript levels of genes involved in DNJ synthesis and the main related metabolites in CK and MSP samples were compared. Fig. 3A presents a schematic representation of the deduced DNJ metabolism, including core metabolites and enzymes, in *S. laven-*

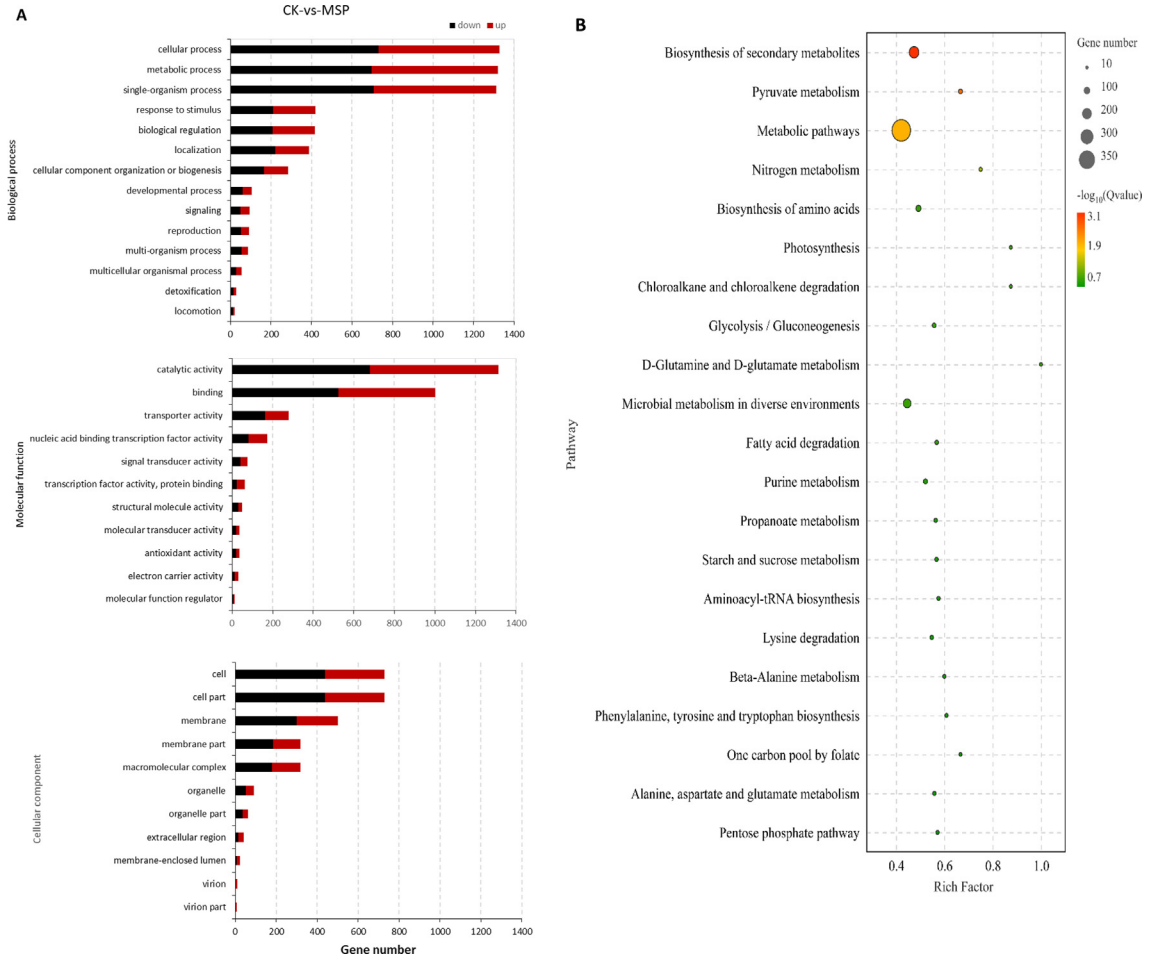


**Fig. 2. Differentially expressed gene (DEG) statistics and functional enrichment in CK vs. MSP samples.** **(A)** The functional categories of DEGs based on GO enrichment; **(B)** KEGG pathway enrichment of DEGs.

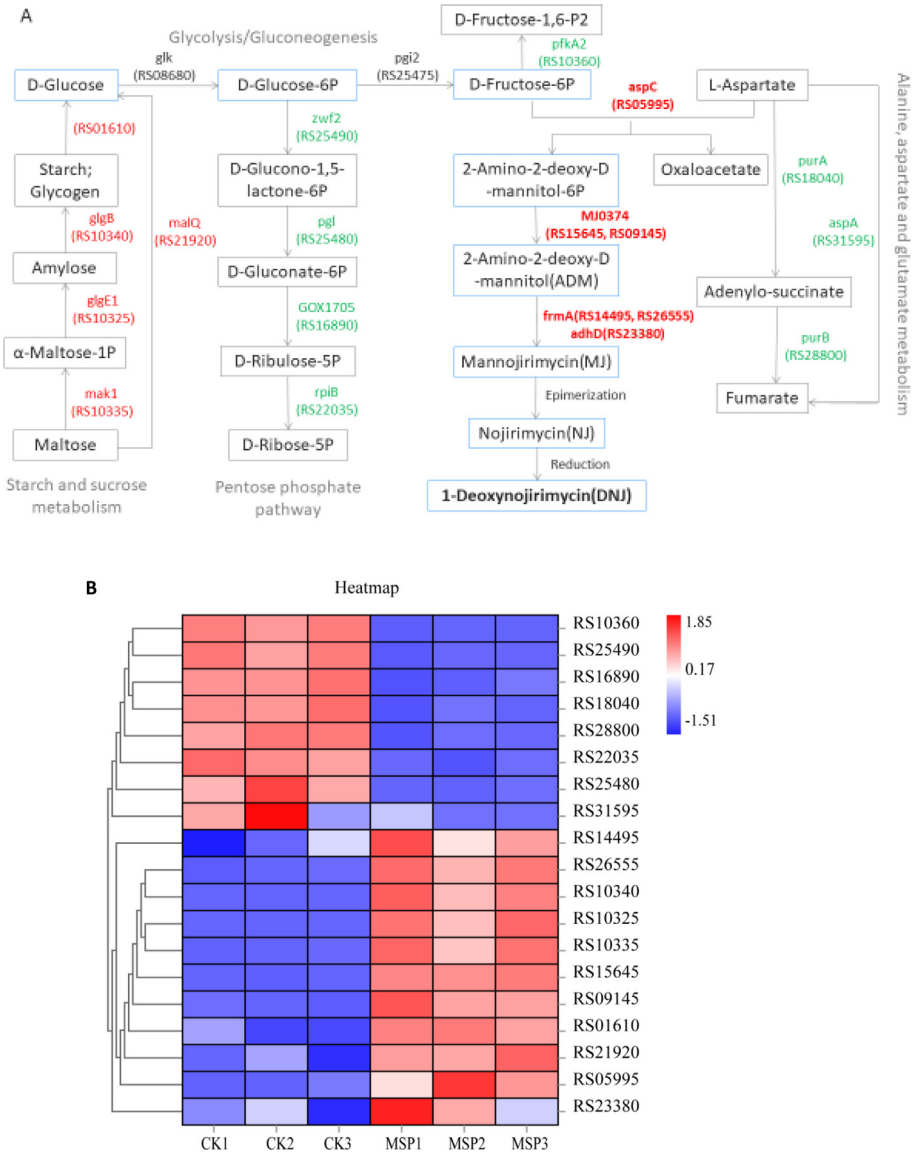


**Fig. 3. DEGs related to DNJ biosynthesis in *Streptomyces lavendulae*. (A)** Putative DNJ biosynthesis pathway in *Streptomyces lavendulae*. The genes in red color indicate up-regulation, and the genes in green color indicate down-regulation. glgB, 1,4-alpha-glucan branching enzyme; glgE1, alpha-1,4-glucan–maltose-1-phosphate maltosyltransferase; mak1, phosphotransferase; malQ, 4-alpha-glucanotransferase; glk, ROK family protein; pgi2, glucose-6-phosphate isomerase; zwf2, glucose-6-phosphate dehydrogenase; pgl, 6-phosphogluconolactonase; GOX1705, decarboxylating 6-phosphogluconate dehydrogenase; rpiB, ribose-5-phosphate isomerase; pfkA2, 6-phosphofructokinase; aspC, aspartate/tyrosine/aromatic aminotransferase; MJ0374, phosphatase PAP2 family protein; frmA, Zn-dependent alcohol dehydrogenase; adhD, Zn-dependent alcohol dehydrogenase; purA, adenylosuccinate synthase; aspA, aspartate ammonia-lyase; purB, adenylosuccinate lyase. **(B)** Heatmap of putative genes predicted to be involved in DNJ biosynthesis in *Streptomyces lavendulae*.

*dulae*. Five genes involved in starch and sucrose metabolism were up-regulated in MSP samples compared with CK samples. Starch and sucrose are sources of glucose, the DNJ precursor. The main three enzymes, aminotransferase (encoded by RS05995), phosphatase (RS15645 and RS09145), and Zn-dependent dehydrogenase (RS14495, RS26555, and RS23380), shown in Fig. 3A, may play an important role in the DNJ biosynthesis in *S. lavendulae*.

Fig. 3B shows the expression patterns of 19 genes predicted to be involved in DNJ biosynthesis in the bacterium. Among these, 8 genes (colored green in Fig. 3A) were down-regulated and 11 genes (colored red in Fig. 3A) were up-regulated in the MSP group compared with the CK group.

### 3.4. Validation of DEG expression using RT-qPCR

Next, 19 DEGs identified using RNA sequencing were validated using RT-qPCR. In MSP vs. CK group comparisons, five genes related to starch and sucrose metabolism (Fig. 4A) and six genes related to DNJ biosynthesis (Fig. 4C) were up-regulated in MSP samples and five genes involved in pentose phosphate (Fig. 4B) and three genes involved in alanine, aspartate, and glutamate metabolism (Fig. 4D) were down-regulated in MSP samples. The RT-qPCR results were consistent with the RNA sequencing data.

### 3.5. Expression of key genes related to DNJ biosynthesis in S. lavendulae

The DNJ yield during *S. lavendulae* growth is shown in Fig. 5A. After supplementation of *S. lavendulae* culture with MSP, DNJ levels in the fermentation broth dynamically increased. The highest DNJ levels were noted 72 h after inoculation in both MSP and CK samples.

To validate the roles of the putative genes related to DNJ biosynthesis, DNJ content and gene expression were analyzed at

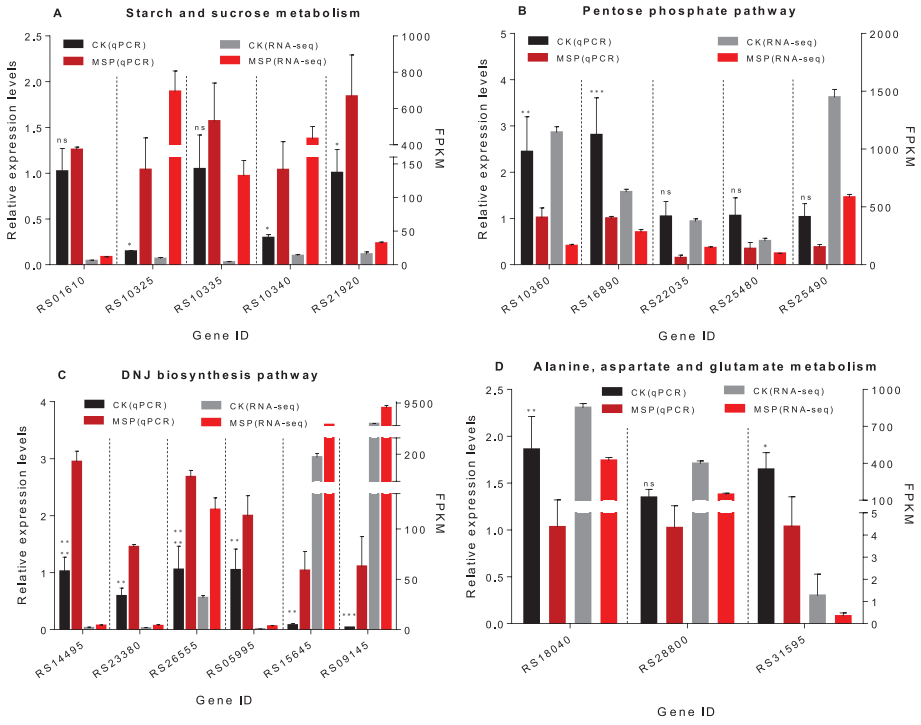


**Fig. 4. Validation of the expression of DEGs using RT-qPCR. (A)** Genes related to starch and sucrose metabolism; **(B)** Genes related to the pentose phosphate pathway; **(C)** Genes related to the DNJ biosynthesis pathway; **(D)** Genes related to alanine, aspartate, and glutamate metabolism. Note: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significance. The significance comparison indicates CK (qPCR) to MSP (qPCR).

different fermentation times (after 12 h, 24 h, 36 h, 48 h, 72 h, and 96 h of fermentation). The expression of six candidate genes (*frmA-1*, *frmA-2*, *adhD*, *aspC*, *MJ0374-1*, and *MJ0374-2*) in MSP and CK samples was compared. For these genes, gene expression increased with an increasing fermentation time and was the highest after 72 h of fermentation. The expression of all genes in MSP culture was higher than that in CK culture (Fig. 5B-G). The gene expression trend was consistent with the changes in DNJ levels.

To understand the relationship between DNJ levels and key gene expression, correlation analysis was performed (Table 1). The analysis revealed that the expression of the six genes was positively correlated with DNJ levels in both MSP samples ($P < 0.001$) and CK samples ($P < 0.01$).

### *3.6. Changes in DNJ precursor and intermediate metabolite levels induced by MSP*

Glucose is the precursor of DNJ, and F6P, G6P, and FDP are the intermediate metabolites of DNJ biosynthesis in *S. lavendulae* [13]. To understand the effect of MSP on their content during DNJ biosynthesis, changes in DNJ, F6P, G6P, and FDP levels were determined after 0 h, 24 h, 48 h, and 96 h of fermentation in CK and MSP samples. As shown in Fig. 6A, DNJ levels increased rapidly in MSP samples, but increased gradually in CK samples, with an increasing fermentation time.

The glucose, G6P, F6P, and FDP levels are shown in Fig. 6B-E. The glucose levels in MSP samples were high even after 72 h of fermentation, while they decreased gradually in CK samples during 0 h-72 h of fermentation (Fig. 6B). G6P and F6P levels in MSP samples were significantly higher than those in CK samples. The changes in G6P and F6P levels in the two groups showed a similar trend from 0 h to 72 h of fermentation (Fig. 6C and Fig. 6D). As shown in Fig. 6E, FDP levels in the two groups increased gradually from 0 h to 72 h of culture, without significant differences between the two groups. These observations indicate that a high level of glucose generates a sufficient amount of precursors for the synthesis of G6P and F6P intermediates, and that G6P and F6P are mainly used for DNJ synthesis.

## 4. Discussion

DNJ is a polyhydroxy piperidine alkaloid with beneficial pharmacological properties. In this study, the key genes involved in DNJ biosynthesis in *S. lavendulae* ATCC 8664 were identified, and their expression correlated with the production of various metabolic precursors and DNJ by this bacterium.

Mulberry seeds contain many nutrient substances [33] and biologically active compounds, such as polysaccharides, alkaloids, and flavonoids [34,35]. Thus, mulberry seed extracts can provide nutrients that improve the growth of microorganisms when added to the culture medium. Here, alkaloids, flavonoids, and polysaccharides extracted from mulberry seeds were used to induce DNJ production during bacterial fermentation. As shown in Fig. 1, the extent of the induction of DNJ production by these extracts was concentration-dependent. Among the extracts and concentrations tested, the MSP extract was the most effective inducer when used at 3.00 mg/mL. These observations suggested that MSP provides the precursor and intermediate metabolites for DNJ biosynthesis in *S. lavendulae* ATCC 8664. The observed changes in glucose, F6P, and G6P levels during fermentation shown in Fig. 6 are consistent with the changes in DNJ levels.

According to previous studies, glucose is the precursor [16] and F6P, G6P, and FDP are the intermediate metabolites of DNJ biosynthesis in *Streptomyces* sp. [18]. In addition, an amino group is required during DNJ biosynthesis, and it was confirmed that alanine, aspartate, and glutamate can be used as amino donors for DNJ synthesis [36]. In the current study, transcriptome sequencing revealed four pathways related to DNJ biosynthesis in *S. lavendulae* ATCC 8664. The proposed scheme of DNJ biosynthesis is presented in Fig. 3A. Among the four pathways, the starch and sucrose

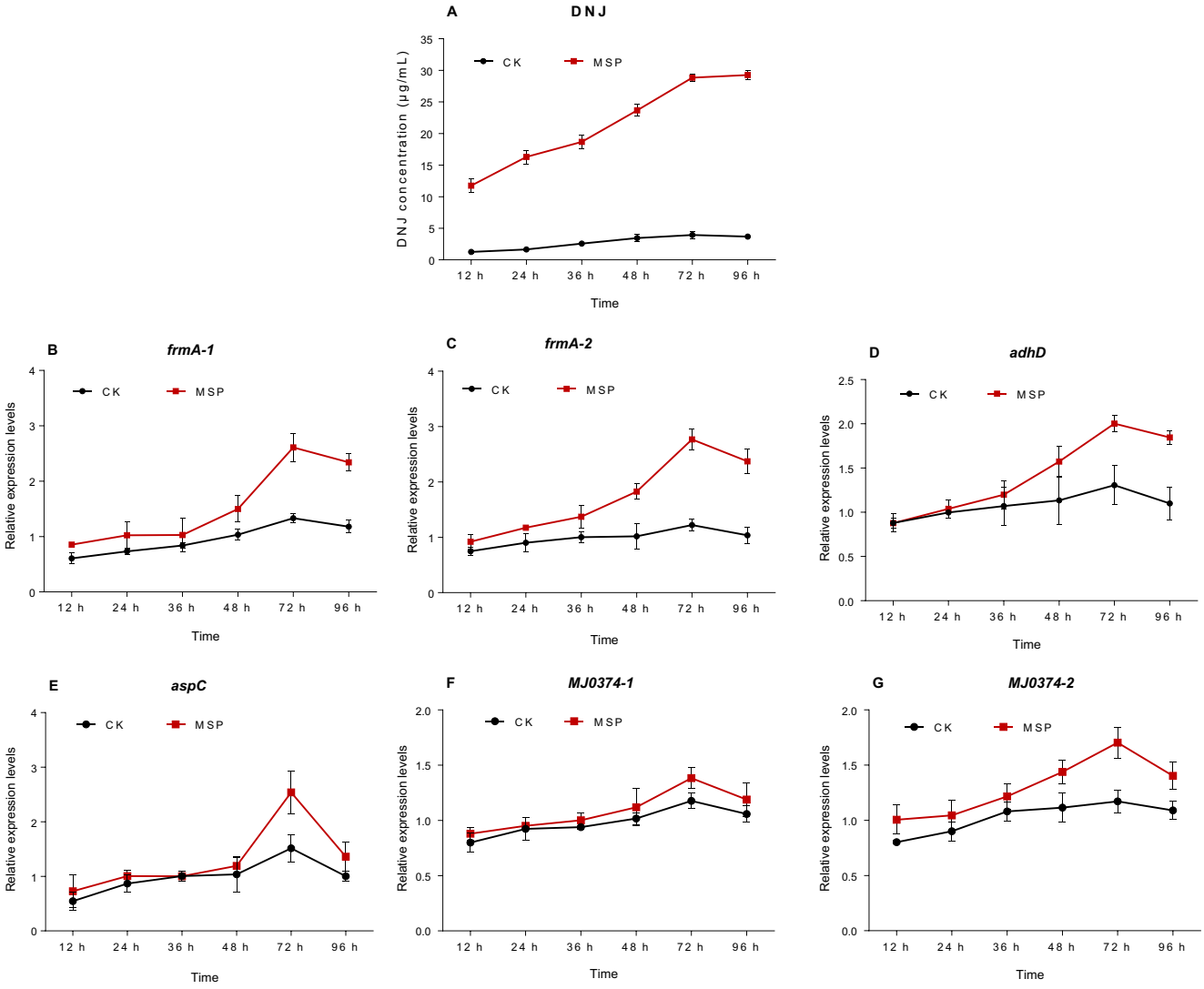


**Fig. 5. Expression of candidate genes related to DNJ biosynthesis at different fermentation stages.** **(A)** The growth curve of DNJ content in *Streptomyces lavendulae*; **(B)** *frmA-1*, RS14495; **(C)** *frmA-2*, RS26555; **(D)** *adhD*, RS23380; **(E)** *aspC*, RS05995; **(F)** *MJ0374-1*, RS15635; **(G)** *MJ0374-2*, RS09145.

**Table 1**
The correlation analysis of candidate gene expression and DNJ levels.

| Correlation | *R* squared | *P*-values | *R* squared | *P*-values |
|---|---|---|---|---|
| DNJ content | CK | | MSP | |
| *FrmA-1* | 0.9669 | 0.0036** | 0.9423 | 0.0006*** |
| *FrmA-2* | 0.9063 | 0.0036** | 0.9692 | 0.0006*** |
| *AdhD* | 0.9005 | 0.0090** | 0.9863 | 0.0007*** |
| *AspC* | 0.8370 | 0.0043** | 0.7651 | 0.0007*** |
| *MJ0374-1* | 0.9290 | 0.0074** | 0.9285 | 0.0008*** |
| *MJ0374-2* | 0.9515 | 0.0077** | 0.9083 | 0.0008*** |

Note: ** Significantly correlated at 0.01 level (two-tailed). *** significantly correlated at 0.001 level (two-tailed).

metabolism pathway is related to glucose biosynthesis; the pentose phosphate pathway is related to G6P metabolism [13]; the glycolysis/gluconeogenesis pathway is related to glucose, G6P, and F6P metabolism; and the alanine, aspartate, and glutamate metabolism pathway provides amino acids and interacts with F6P in DNJ biosynthesis [36].

In *B. subtilis* MORI 3 K-85, *B. amyloliquefaciens* 140 N, and *B. velezensis* K26, DNJ biosynthesis is initiated by three proteins, GabT1, YktC1, and GutB1, which catalyze transamination, dephosphorylation, and oxidoreduction [20,19,37]. In *B. subtilis* MORI 3 K-85, the *gabT1*, *yktC1*, and *gutB1* genes encode a putative transaminase, phosphatase, and Zn-dependent dehydrogenase, respectively [20]. Beal and Horenstein [21] reported that these three proteins are significantly different in *S. subrutilis* ATCC 27467 and *B. velezensis* FZB42. Park et al. [38] investigated a gene cluster containing orthologs of these genes in region 20, and suggested their potential for the production of DNJ in the *Streptomyces* spp. JCM 3382 strain. However, the distribution of the three protein sequences varies

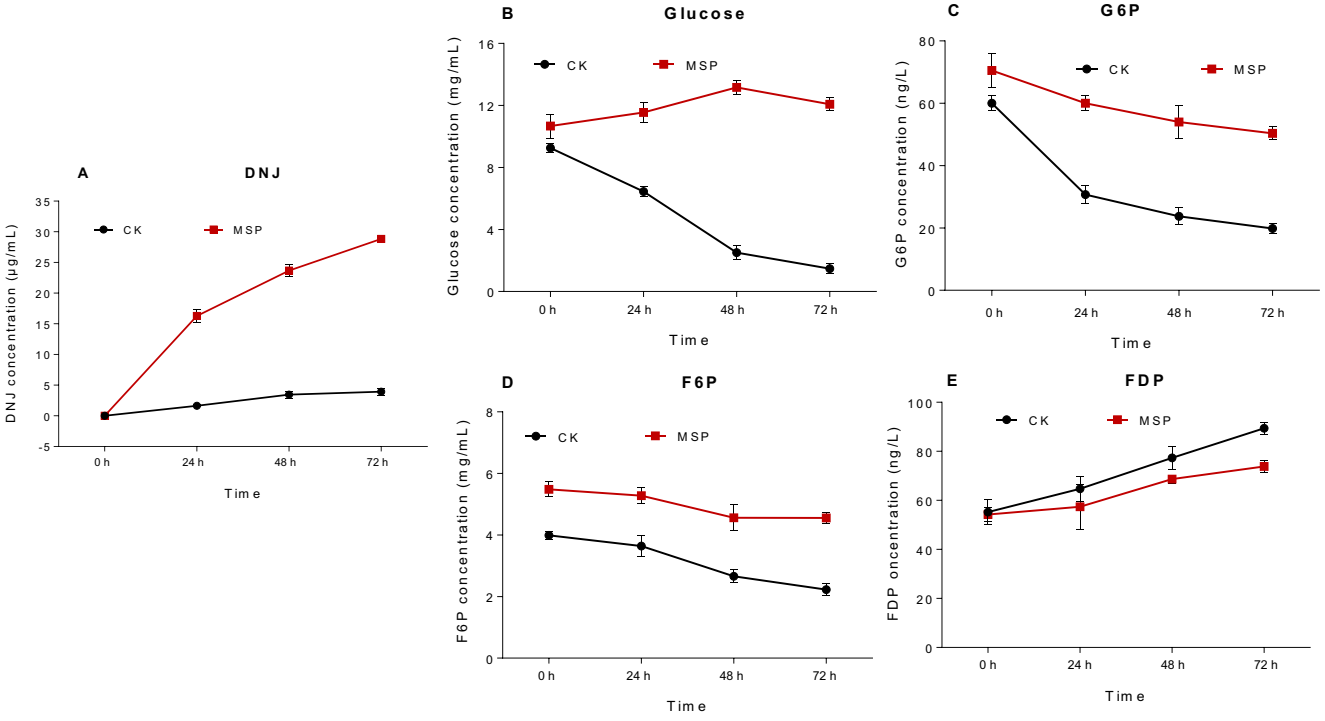


**Fig. 6. Changes in DNJ and its precursor and intermediate contents during fermentation in MSP and CK samples.** **(A)** DNJ; **(B)** Glucose; **(C)** F6P (fructose-6-phosphate); **(D)** G6P (glucose-6-phosphate); **(E)** FDP (fructose-1,6-bisphosphate).

greatly across the 2062 analyzed *Streptomyces* genomes. While homologs of *gabT1* and *gutB1* are commonly identified in the DNJ gene cluster, *yktC1* has been identified in only few *Streptomyces* genomes. Taken together, the existence of several types of clusters suggests that different strains may produce different DNJ derivatives or different types of azasugars.

The first committed step of DNJ biosynthesis critically involves a transamination reaction. In this study, *aspC* (coding for a putative transaminase), *MJ0374-1* and *MJ0374-2* (coding for a phosphatase), and *frmA-1, –2* and *adhD* (coding for a Zn-dependent dehydrogenase) were identified in *S. lavendulae*. Their expression, shown in Fig. 5, suggested that these genes play an important role in DNJ biosynthesis.

AspC is used to catalyze the transfer of the amino group between acidic amino acids and α-ketoacids [36]. The results of this study indicate that AspC may transfer the amino group from aspartate to F6P to produce 2-amino-2-deoxy-D-mannitol-6P. The function of AspC is similar to that of GabT1 reported elsewhere [36]. It has been reported that a *Streptomyces* phosphatase is involved in dephosphorylation during chemical compound synthesis [39]. The data presented in the current study suggest that MJ0374-1 and MJ0374-2 may be responsible for the dephosphorylation of 2-amino-2-deoxy-D-mannitol-6P to produce 2-amino-2-deoxy-D-mannitol (ADM). FrmA-1, FrmA-2, and AdhD catalyze hydrogen atom from the hydroxyl group in ethanol to form acetaldehyde [40], which is the same process involved in the transformation of ADM to mannojirimycin (Fig. 3A).

## 5. Conclusions

In this study, supplementation of *S. lavendulae* culture with different mulberry seed extracts induced DNJ yield in the fermentation broth. MSP was a more effective inducer than mulberry alkaloid and flavonoid extracts. At 3.00 mg/mL MSP, DNJ production reached the highest level, 27.19 μg/mL, which was 8.55 times than that in the unsupplemented control group (3.18 μg/mL). Comparative transcriptome analysis of MSP and CK samples revealed 2328 DEGs, of which 1151 DEGs were up-regulated and 1177 DEGs were down-regulated in MSP samples. KEGG enrichment analysis of the DEGs revealed four pathways related to DNJ biosynthesis, i.e., the starch and sucrose metabolism pathway, pentose phosphate pathway, glycolysis/gluconeogenesis pathway, and alanine, aspartate, and glutamate metabolism pathway. Six key genes (*aspC*, *MJ0374-1*, *MJ0374-2*, *frmA-1*, *frmA-2*, and *adhD*) related to DNJ biosynthesis in *S. lavendulae* were validated using RT-qPCR and the analysis of intermediate metabolite levels. Of note, *aspC* encodes a putative transaminase that produces 2-amino-2-deoxy-D-mannitol-6P. *MJ0374-1* and *MJ0374-2* encode a phosphatase that produces ADM. Finally, *frmA-1, frmA-2,* and *adhD* encode a Zn-dependent dehydrogenase that produces mannojirimycin. To the best of our knowledge, this is the first report on the identification of key genes related to DNJ biosynthesis in *S. lavendulae*. This study provides new insights into the regulation of DNJ biosynthesis in *S. lavendulae* and the large-scale application of DNJ from this bacterium in future.

## Author contributions

- Study conception and design: XD Xin; ZZ Gui.
- Data collection: XD Xin; XP Jiang; BX Niu; MQ Zhang; XM Xu.
- Analysis and interpretation of results: R Zhang; H Li.
- Draft manuscript preparation: XD Xin.
- Revision of the results and approval of the final version of the manuscript: ZZ Gui.

## Conflict of interest

Zhongzheng Gui reports was provided by Jiangsu University of Science and Technology. Zhongzheng Gui reports a relationship with Jiangsu University of Science and Technology that includes: employment.

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