

# Cellobiose inhibits the release of deoxynivalenol from transformed deoxynivalenol-3-glucoside from *Lactiplantibacillus plantarum*

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## ABSTRACT

The masked mycotoxin deoxynivalenol-3-glucoside (D3G) has been reported to be a detoxification product in plants, but can be hydrolyzed into its toxic precursor, deoxynivalenol (DON). Herein, we reported that *Lactiplantibacillus plantarum* (*L. plantarum*) NMM.1, isolated from Inner Mongolia raw milk, can efficiently transform D3G to DON in a short time. The global transcriptome microarray profiling of *L. plantarum* NMM.1 revealed differential expression of genes related to the phosphotransferase system (PTS) when D3G was used as the sole carbohydrate source. By adding an exogenous carbon source, we found that cellobiose efficiently inhibited the conversion of D3G into its precursor toxin by *L. plantarum* NMM.1. Overall, substrate depletion studies, transcriptome analysis, and carbohydrate intervention studies of *L. plantarum* NMM.1 suggested that cellobiose could be used to prevent the transformation of D3G into its free native DON by *L. plantarum*, thereby preventing harm to the human body.

## 1. Introduction

Deoxynivalenol-3-glucoside (D3G) belongs to a group of biologically modified mycotoxins that are biosynthesized under the influence of plant, animal, or bacterial enzymes (Bryla et al., 2018). The precursor toxin of D3G is deoxynivalenol (DON), which is a toxic mycotoxin primarily produced by *Fusarium* fungi, and occurs predominantly in cereal grains (Chain et al., 2017). D3G usually co-occurs with its prototype toxin DON; in some cereal and cereal-based products, it has an even higher incidence than DON (Malachova et al., 2011; Sasanya et al., 2008). According to a 2016 report, the most frequently found mycotoxins in wheat flour samples (n = 359) from Shandong Province, China were DON (97.2%), nivalenol (40.4%), and D3G (33.4%), with the mean contamination levels in positive samples being 86.7 µg/kg, 3.55 µg/kg, and 3.34 µg/kg, respectively (Li et al., 2016). A 2021 report showed that D3G was present in almost all samples of naturally contaminated forage maize in Northern Germany with amounts of up to 3,038 µg/kg and a wide range of concentrations (Birr et al., 2021). Human exposure to D3G is mainly through the consumption of grains and grain-based products, while farm and companion animals are exposed to D3G mainly through the consumption of cereal grains, cereal by-products, and forage maize. DON has serious toxic effects on humans and animals and is also

phytotoxic (Kahlert et al., 2019; Payros et al., 2016; Pinton & Oswald, 2014; Wipfler et al., 2019; Yang et al., 2020; Yu et al., 2018). D3G, mainly produced by the glucosylation reaction, is considered a detoxification of DON in plants, with no or low toxicity to microbial cells, plants, and mammals (Berthiller et al., 2011; Broekaert et al., 2016; Pierron et al., 2016; Suzuki & Iwahashi, 2015; Yang et al., 2017). However, D3G can release DON in some cases. It has been reported that D3G can be converted into DON in some food processing processes, such as dough extrusion, fermentation, and steaming (Wu et al., 2017; Zhang & Wang, 2015). Studies have also shown that some microbiota in the small and large intestines of animals can rapidly hydrolyze D3G and release DON both *in vitro* and *in vivo* (Broekaert et al., 2017; Gratz et al., 2018; Nagl et al., 2014). More importantly, the release of DON owing to D3G ingestion can also be detected in human feces, which increases the toxic burden in exposed individuals (Gratz et al., 2013; Mengelers et al., 2019).

*Lactobacillus* is a complex genus that mainly obtains metabolic energy from carbohydrate fermentation; its fermentation modes mainly include homofermentation and heterofermentation (Zhao & Ganzle, 2018). *Lactiplantibacillus plantarum*, a homofermentative model organism, is found in diverse ecological niches and is used in many food fermentation processes and commercially available probiotic products,

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thereby occurring in human diet (Seddik et al., 2017). *Lactiplantibacillus plantarum* is a facultative anaerobe that exists in the upper gastrointestinal tract of humans and animals and absorbs diverse carbohydrate-rich substrates through various inherent metabolic pathways (Panwar & Kapoor, 2020). In *L. plantarum*, carbohydrate transport is mediated by members of the ATP-binding cassette (ABC) superfamily of ABC transporters, secondary transporters of the major facilitator superfamily (MFS), or phosphotransferase systems (PTS) (Zhao & Ganzle, 2018). Research showed that *L. plantarum* DSM 20174<sup>T</sup> (ATCC 14917<sup>T</sup>) can convert D3G into its prototype toxin, DON (Berthiller et al., 2011; Daud et al., 2020). As *L. plantarum* is a main component of the human gastrointestinal flora, it is necessary to circumvent the release of DON to reduce its harm to the human body.

In our study, a strain of *L. plantarum* that efficiently transformed D3G into DON was isolated from raw milk collected from Inner Mongolia. In order to identify the transformation efficiency of the NMM.1 strain, we supervised the consumption of D3G and the production of DON at different time points and reaction conditions. To increase our insights into the microbial mechanisms involved in the transformation of D3G, global transcriptome microarray profiling of *L. plantarum* NMM.1 using D3G as the sole carbohydrate source was carried out. According to the results of transcriptome, the gene expression of some specific carbohydrate metabolism pathways was significantly changed which may indicated that the transformation of D3G by *L. plantarum* NMM.1 was related to its carbohydrate metabolism. Accordingly, we investigated the effects of several saccharides including glucose, fructose, sucrose, maltose, trehalose, and cellobiose on the production of DON. Based on the substrate depletion studies, transcriptome analysis, and carbohydrate intervention studies of *L. plantarum* NMM.1, we aimed to find a way to efficiently inhibit D3G transformation, thereby applied that to control mycotoxin pollution in food and to prevent the harm to the human body caused by release of DON from *L. plantarum*.

## 2. Materials and methods

### 2.1. Isolation and taxonomic characterization of bacteria with D3G-transformation activity

Strain NMM.1 was isolated from raw milk samples collected from Xilin Gol League in Inner Mongolia by repeated MRS plate streaking and was characterized as *L. plantarum* by searching database 16S ribosomal RNA sequences (Bacteria and Archaea) and blasted the 16S rRNA sequence with sequences from type material using Megablast (Optimize for highly similar sequences) combined with Mole-BLAST (MUSCLE multiple alignment) with the neighbor joining tree method and maximum sequence difference at 0.75 based on BLAST (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). The type strain *L. plantarum* ATCC 14917<sup>T</sup> and *L. plantarum* NMM.1 were used in the present study. The *L. plantarum* strains were propagated microaerobically at 37 °C in de Man, Rogosa, and Sharpe broth (MRS) (Meilunbio, Dalian, China). A single colony of *L. plantarum* from an MRS agar plate was inoculated in a falcon tube containing MRS broth and grown for 24 h at 37 °C. Then, the *L. plantarum* cells were recovered by centrifugation (5,000 g, 10 min, 4 °C), washed twice with saline magnesium buffer (100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl, pH 8.0), and adjusted to an OD = 2.0 with a buffer containing 5 µg/mL (10.9 µM) D3G purchased from Sigma-Aldrich (St. Louis, MO, USA). After being treated with D3G at different time points, the supernatants were collected for LC-MS/MS quantification. The transformation rate was calculated following the formula:

$$\text{Transformation rate (\%)} = n_{\text{DON}}/n_{\text{D3G}} \times 100$$

### 2.2. Analysis of toxins released by LC-MS/MS

Deoxynivalenol and deoxynivalenol-3-glucoside standard solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard

assay conditions with substrate were 5 µg/mL substrate concentration, saline magnesium buffer (100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl, pH 8.0), and 37 °C; the assay was stopped by heat inactivation (100 °C, 10 min). The samples were vortexed with a quarter of the volume of acetonitrile and further filtered through a 0.22-µm nylon filter. The filtered solution was then taken and stored in chromatographic sample bottles at -20 °C until the LC-MS/MS analysis. The transformation activity of *L. plantarum* strains toward D3G was assessed using an Accela 1250 UPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a TSQ Vantage<sup>TM</sup> (Thermo Fisher Scientific, San Jose, CA, USA) triple stage quadrupole mass spectrometer. The AgilentExtend-C18 (100 mm × 4.6 mm, 3.5 µm) chromatographic column with the mobile phase consisted of water containing 5 mM ammonium acetate (A) and methanol (B). The injection volume was 10 µL with a flow rate of 0.35 mL/min at 30 °C. The gradient was as follows: 0 min 15% B, 1 min 15% B, 6.5 min 90% B, 8.5 min 90% B, 9 min 15% B, and 12 min 15% B. Mass spectrometry analysis was carried out in both positive (ESI<sup>+</sup> 3.5 kV) and negative (ESI<sup>-</sup> 2.5 kV) ionization modes using selected reaction monitoring (SRM). For the MS/MS analysis, the running parameters were as follows: the vaporizer temperature and capillary temperature were both 300 °C; 50 psi sheath gas pressure was combined with 5 psi aux gas pressure. Raw data were analyzed using Xcalibur<sup>TM</sup> software (Thermo Fisher Scientific, San Jose, CA, USA; 2011).

### 2.3. Cytotoxic effect of D3G transformation products on human cells

In order to simulate the reaction environment of *L. plantarum* and D3G in the intestine so as to assess the cytotoxic effect of the D3G hydrolysis products on human cells, HIEC cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA) and penicillin/streptomycin (BioInd, Kibbutz Beit, Israel). After plating, when the cells reached 80% confluence in a 6-cm dish, they were harvested to conduct the cytotoxicity assay. Next, we used 96-well plates and plated  $1 \times 10^4$  cells per well in 100 µL of culture medium RPMI 1640 supplemented with 10% FBS. The plates were incubated in a 5% CO<sub>2</sub> oven at 37 °C for 24 h to allow cell adhesion. After 24 h of incubation, the cells were treated with 0.5 µg/mL D3G, 1 h reaction supernatant of *L. plantarum* in saline magnesium buffer (10:90, v/v) and 1 h reaction supernatant of *L. plantarum* in saline magnesium buffer (10:90, v/v) containing 5 µg/mL D3G for 24 h. All additional reagents were sterilized and each test was performed in triplicate. Cell survival assays were performed using the Cell Counting Kit-8 (CCK-8) (Dojingdu, Kumamoto, Japan) according to the manufacturer's instructions. Cell viability was calculated as the main experimental parameter using the following formula:

$$\text{Cell viability (\%)} = 100 * (\text{mean OD450 in sample group} - \text{OD450 in blank}) / (\text{mean OD450 in control group} - \text{OD450 in blank})$$

### 2.4. The effect of D3G on the growth of *L. plantarum* and assay of D3G-transformation activity under different conditions

A single colony of *L. plantarum* NMM.1 from the MRS agar plate was inoculated in a falcon tube containing MRS broth and grown at 37 °C by shaking it at 220 rpm for 24 h. Then, the bacterial solution was transferred to an MRS liquid medium containing 5 µg/mL at a ratio of 1:50 and the OD value was calculated at different time points. When the grown culture had an OD of 1.4, the cells were recovered by centrifugation (5,000 g, 10 min, 4 °C), washed twice with saline magnesium buffer (100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl, pH 8.0), resuspended in OD = 2 using saline magnesium buffer, and lysed by extensive sonication. The lysate was filtered through a 0.45-µm syringe filter and Halt Protease Inhibitor Cocktail (Thermo Fisher, Mississauga, Canada)

without EDTA was added. The lysate was then inoculated with a final concentration of 5 µg/mL of D3G for 24 h. The same OD of cells was collected or heat-inactivated at 121 °C for 15 min and then treated with 5 µg/mL D3G. For buffers of different pH levels, we used L-(+)-lactic acid (Sangon, Shanghai, China) to adjust the pH of the buffer to 4, 5, 6, and 7 and then inoculated *L. plantarum* NMM.1 cells with D3G according to the aforementioned methods.

## 2.5. RNA extraction and sequencing

*L. plantarum* NMM.1 cells were collected by centrifugation (5,000 g, 10 min, 4 °C) when the grown culture reached the OD value of 1.4. Then pellet obtained was washed twice by using saline magnesium buffer (100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl, pH 8.0), and adjusted to an OD = 2.0 with saline magnesium buffer or a buffer containing 5 µg/mL D3G (St. Louis, MO, USA). After being treated with buffer for 1 h, cells were collected for RNA extraction. Each group had three replicates. Total RNA was extracted using an RNAiso Plus Kit (Takara, Beijing, China) according to the manufacturer's recommendations. To eliminate genomic DNA contamination, DNase I (Takara) was used. The total RNA was further subjected to quality control using an Agilent 2100 Bio-analyzer according to the instructions of the Agilent RNA 6000 Nano Assay Protocol (Agilent Technologies, Waldronn, Germany). Libraries were prepared using the Illumina TruSeq stranded RNA sample preparation Kit; then, the Ribo-Zero Magnetic kit (Illumina, San Diego, CA, USA) was used to remove the rRNA, randomly fragments the mRNA into small fragments of approximately 150 bp, and then use the mRNA as a template. Random primers (Illumina) and a SuperScript double-stranded cDNA synthesis kit (Invitrogen) were used to reverse the synthesis of double-stranded cDNA. We used dUTP instead of dTTP to synthesize the second strand of cDNA, so that it was quenched in the subsequent PCR amplification process. The synthesized double-stranded cDNA was added to End Repair Mix to make it into a blunt end, the 5'-end was phosphorylated, and an A base was added to the 3'-end, so that indexing adapters were ligated. The products were then purified and amplified using Phusion DNA polymerase (NEB, Ipswich, MA, USA) (15 PCR cycles) to create the final cDNA library. After library validation and quantification using TBS380 (Picogreen), an Illumina HiSeq X Ten (2 × 150 bp) sequencer with two paired-ends was used for sequencing.

## 2.6. Reads and statistical analysis of differential gene expression

All reads were quality-filtered to keep only sequences with Phred-Scores higher than 20 and a percentage of N < 10%. The reads were trimmed for the presence of TruSeq adapters and, according to a FastQC quality control, bases other than A, G, C, and T nucleotides at the 5'-end were statically removed. Reads smaller than 25 nucleotides were discarded. The reads were mapped to the reference genome *L. plantarum* ATCC 14917<sup>T</sup> ENA| Accession: ACGZ02000000 (<https://www.ebi.ac.uk/ena/>) using by Bowtie (<http://bowtie-bio.sourceforge.net/index.shtml>). All reads were filtered against annotated *L. plantarum* genes (90% identity over 80% of the sequence). For expression analysis, only uniquely mapped exon read counts were considered. The read counts were then analyzed using the DESeq2 package (v1.10). Alternatively, we used the trimmed mean of the M-values method implemented in the ENMM.1eR package (v3.12). Pairwise comparisons were made with ENMM.1eR and DESeq2 using the conditional maximum likelihood (qCML) method and the Cox-Reid profile-adjusted likelihood method, respectively (Lanver et al., 2018). The RSEM software was used to quantitatively analyze gene expression levels respectively and perform PCA analysis. In both analyses, genes with a |log<sub>2</sub> fold change| ≥ 1 and P value < 0.05, were considered differentially expressed.

## 2.7. Annotation of clusters of orthologous groups of proteins (COG) and enrichment analysis of DEGs

The function annotation of all genes was performed using Diamond (<https://github.com/bbuchfink/diamond>) and the eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups, <http://eggnoG.embl.de/>) database was used as a reference. GO term enrichment analysis was performed with Goatools (<https://github.com/tanghaibao/GOatools>) using Fisher's exact test. The Bonferroni method was used to correct the P value (FDR). When the FDR was < 0.05, the GO function was considered to be significantly enriched. KEGG pathway enrichment analysis was performed using KOBAS 2.0 (<http://kobas.cbi.pku.edu.cn/home.do>). The test and P value correction methods were same to GO term enrichment analysis. When the corrected P value of enrichment was more than 0.05, an uncorrected P value < 0.05, was also considered for analysis.

## 2.8. Real time quantitative PCR (q-RT-PCR)

*L. plantarum* NMM.1 was cultured (in duplicates) in saline magnesium buffer and buffer containing 5 µg/mL D3G for 1 h (as described in RNA extraction section). The pellet obtained was freeze in liquid nitrogen and stored in - 80 °C till further usage. The stored cells were pelleted and subjected to lysozyme treatment (200 mg/mL) for 1 h at 37 °C. Thereafter, total RNA was extracted by using Trizol reagent. 1 µg of isolated RNA from each sample was subjected to remove genomic DNA contamination and cDNA synthesis (PrimeScript™ RT reagent Kit with gDNA Eraser, Takara, Beijing, China) as described by suppliers. Each sample was quantified using TB Green Premix Ex Taq II (Takara, Beijing, China) following the instructions of the manufacturer. Expression of selected differentially expressed genes in D3G fed *L. plantarum* NMM.1 was compared with control (buffer without D3G) in QuantStudio™ Real-Time PCR System (Applied Biosystems™) and the 16 S rRNA coding gene was used as the housekeeping control (Panwar & Kapoor, 2020; Reverón et al., 2018). The primers employed in this experiment were listed in Table S4. Three replicates were employed for every gene. The 2<sup>-ΔΔCt</sup> method was used to calculate the expression level of every gene.

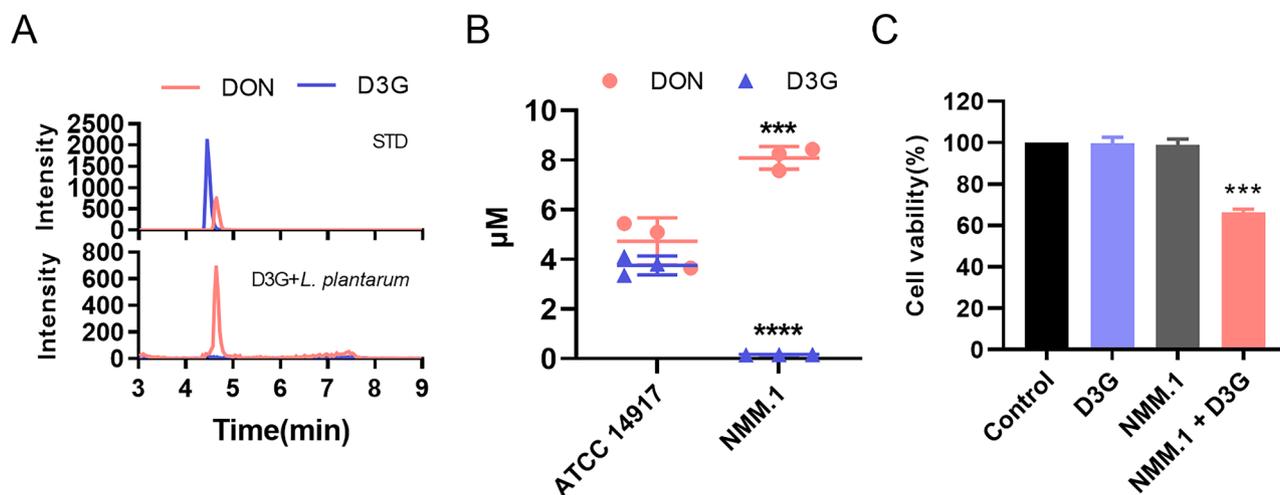
## 2.9. Statistical analysis

Data were analyzed using two-tailed unpaired one-way or two-way ANOVA with a post hoc Bonferroni test for comparisons to assess the differences between groups using Prism software (GraphPad Prism 6, Dr. Harvey Motulsky, San Diego, CA, USA; <http://www.graphpad.com/scientific-software/prism/>). All probabilities are two-sided, and a value of P < 0.001 was considered statistically significant. The data represented the mean ± SD of three independent experiments.

## 3. Results

### 3.1. Identification of a highly active strain of *L. Plantarum* efficiently transforming D3G into DON

A single strain with high efficient D3G transformation ability, phylogenetically identified as an *L. plantarum* strain, was purified using the plate streak method and had high homology with the model strain *L. plantarum* ATCC 14917<sup>T</sup>, designated *L. plantarum* NMM.1. The 16S rDNA sequence data has been submitted to the GenBank databases under accession number OK500273. In LC-MS profiles, by using a toxin standard solution as calibration, we found that after *L. plantarum* NMM.1 incubated with D3G for 24 h, D3G disappeared; however, at the 4.46 min mark, a substance consistent with DON standard solution was detected, which indicated that DON was produced (Fig. 1A). Additionally, this strain transformed almost 5 µg/mL (10.9 µM) D3G into 8.1 µM DON (74.3%) in 4 h with a cell optical density (OD) value of 2, while



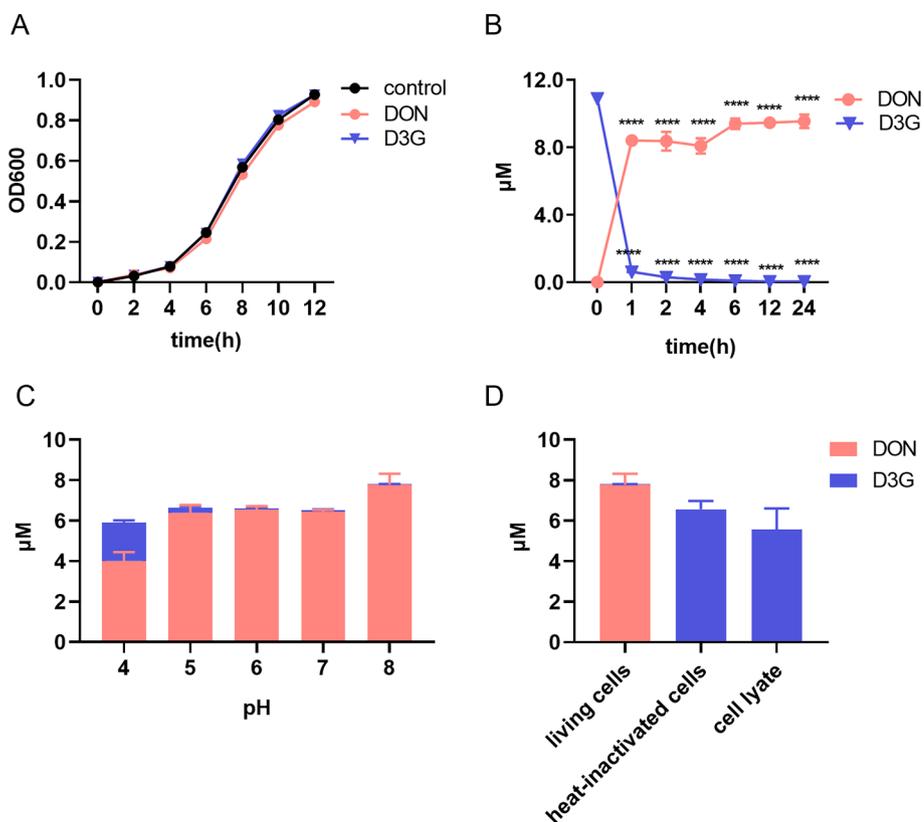
**Fig. 1.** *Lactobacillus plantarum* NMM.1 isolated from Inner Mongolia raw milk highly transformed D3G into the toxic free native DON. (A) Determination of D3G and its metabolites in *L. plantarum* NMM.1. STD, standard DON and D3G solution; peaks of D3G and DON were monitored by LC-MS/MS. (B) Comparison of D3G transformation efficiency between model strain *L. plantarum* ATCC 14917<sup>T</sup> and the isolated strain *L. plantarum* NMM.1. (C) Viability of human intestinal epithelial cells (HIECs) treated with 0.5 µg/mL D3G, 10% of cell medium volume of 1 h reaction supernatant of *L. plantarum* NMM.1 in saline magnesium buffer and 10% of cell medium volume of 1 h reaction supernatant of *L. plantarum* NMM.1 in saline magnesium buffer containing 5 µg/mL D3G for 24 h. The data represented the mean ± SD of three independent experiments, \*\*\* means  $p < 0.001$ .

ATCC 14917<sup>T</sup> only transformed D3G into 4.7 µM DON (43%) (Fig. 1B). This is the first time that the D3G transformation capacity of a strain was more efficient than that of the model strain.

### 3.2. Cytotoxicity assessment of D3G transforming products produced by *L. Plantarum* NMM.1

As *L. plantarum* is a strain of intestinal microorganisms and the intestine is one of the main organs exposed to mycotoxins, human

intestinal epithelial cells (HIECs) were used for toxicity assessment in the present study. Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8) (Dojingdo) to detect the cytotoxicity of products released by *L. plantarum* NMM.1 when in contact with D3G. As is shown in Fig. 1C, cell viability was not affected by D3G and *L. plantarum* NMM.1 metabolites, but it decreased by approximately 35% under a treatment of 10% transformation products of D3G produced by *L. plantarum* NMM.1 (Fig. 1C and Fig. S1). Therefore, we conclude that the transformation products released by *L. plantarum* NMM.1 containing



**Fig. 2.** Production of deoxynivalenol (DON) by *L. plantarum* NMM.1. (A) Growth curves of *L. plantarum* NMM.1 in MRS medium containing 5 µg/mL D3G or DON. (B) Concentration variation of DON and D3G at different time point during *L. plantarum* NMM.1 contacting with D3G. (C) D3G transformation ability of *L. plantarum* NMM.1 under different pH environment. (D) Efficiency of D3G transformation of *L. plantarum* NMM.1 at different locations of action. Levels of D3G and DON were measured by LC-MS/MS. \*\*\*\* means  $p < 0.0001$ .

DON and were toxic to human cells, while D3G or the metabolites of the *L. plantarum* strain were not cytotoxic.

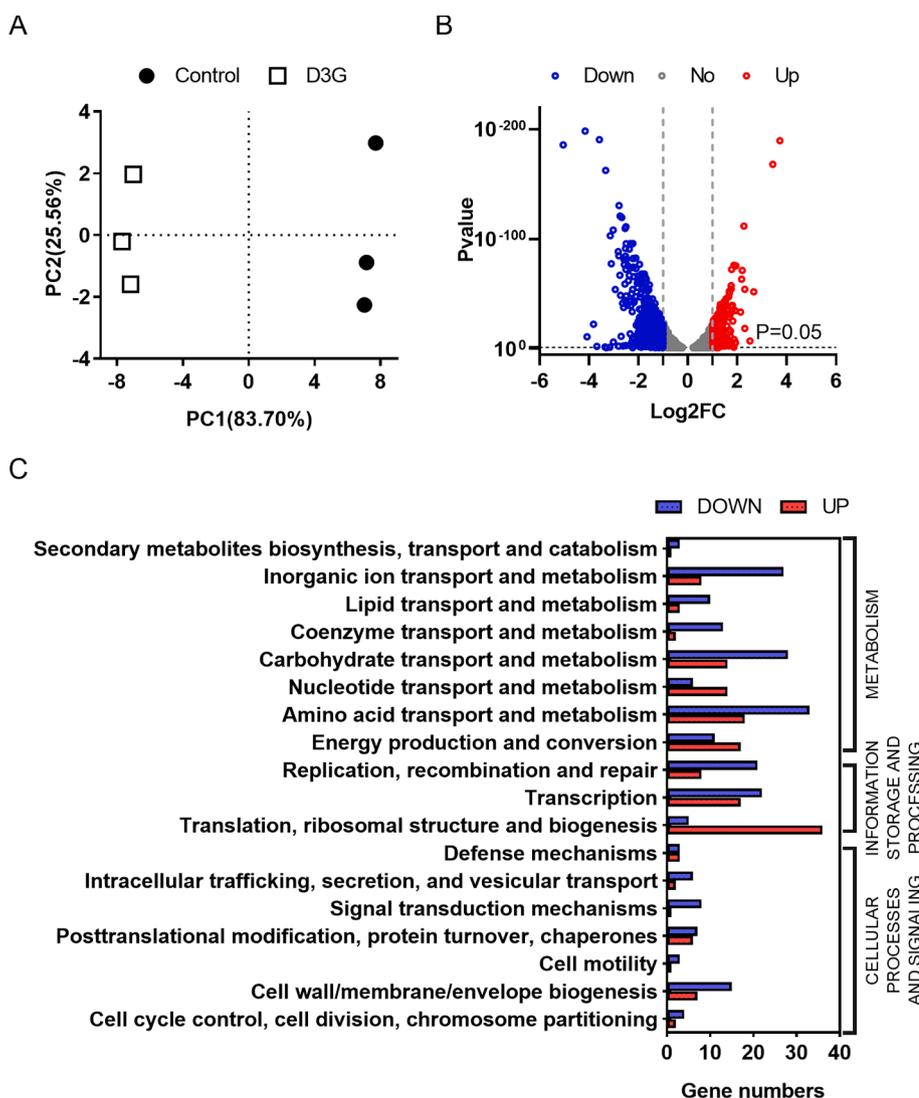
### 3.3. Determination of the transformation capacity of *L. Plantarum* NMM.1

To investigate the critical factors of *L. plantarum* NMM.1 transforming D3G, we initially evaluated the effect of D3G on *L. plantarum* NMM.1 growth. As shown in Fig. 2A, the growth curve of the strain propagated in MRS medium containing either 5 µg/mL D3G or DON was not significantly different from that of the control group in terms of bacterial growth and proliferation. After excluding the toxic effect of D3G on bacterial cells, we explored the conversion rate of *L. plantarum* NMM.1 to D3G. We sampled and tested at different time points, and found that this strain was not only highly efficient, but also quick in transforming D3G. In just 1 h, the concentration of D3G was reduced by about 95%. Moreover, as the reaction time increased, D3G was gradually exhausted and the DON content tended to stabilize (Fig. 2B). Thus, strain NMM.1 displayed extremely high D3G transformation activity. As *L. plantarum* produces a large amount of lactic acid during growth, it is more acid-tolerant than other species. In order to explore whether the pH in the environment could promote or inhibit the D3G transformation ability of *L. plantarum* NMM.1, we adjusted the pH of the reaction buffer using L-(+)- lactic acid and found that the transformation efficiency of

this strain was higher under alkaline conditions (pH = 8), while it was significantly reduced under acidic conditions (pH = 4) (Fig. 2C). This may indicate a preference for D3G transformation by *L. plantarum* in weak alkaline intestinal environments. In order to identify the pattern of *L. plantarum* NMM.1 transforming D3G, we set up three groups, including high-temperature inactivated cells, living cells, and lysate of cells and treated them with 5 µg/mL of D3G. As is shown in Fig. 2D, heat-inactivated cells and cell lysate showed no transforming capacity of D3G, while DON was only produced in the living cells, thereby indicating that the production of DON was the response of living *L. plantarum* NMM.1 cells to the sole carbon source D3G rather than cell wall absorption or intracellular material.

### 3.4. Overview of the transcriptomic response during contact with D3G

To expand our insights into the biological processes and gene circuits involved in the responses to D3G of *L. plantarum* NMM.1, a transcriptomic profile analysis was carried out after 1 h of exposure to 5 µg/mL of D3G. The RNA-seq data has been submitted to GEO database under accession number GSE185970 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185970>). The PCA analysis showed a significant distinction between the control group and D3G treatment group samples (Fig. 3A). The impact of D3G transformation on the transcriptomic profile of *L. plantarum* was evaluated by sorting all genes



**Fig. 3.** Differential expression genes between control group and D3G treatment group samples and functional categories that were upregulated and downregulated in the presence of D3G. (A) PCA analysis of triplet of control and 5 µg/mL D3G treatment group samples. (B) Volcano map of differential gene expression. (C) Number of *L. plantarum* differential expression genes in functional categories by Clusters of Orthologous Groups of proteins (COG) annotation that was upregulated and downregulated.

whose transcript levels showed a fold change (FC)  $\geq 2$  (P value  $< 0.05$ ). The analysis revealed that 647 transcripts were affected, among which 230 were upregulated and 417 were downregulated (Fig. 3B). These differentially expressed genes were functionally classified according to their Clusters of Orthologous Groups (COGs) categories (Fig. 3C). We observed that among the affected genes, excluding those whose function was unknown, the largest group corresponded to metabolism, information storage, and processing (Fig. 3C).

### 3.5. Gene ontology (GO) pathway terms analysis of differentially expressed genes (DEGs)

Genes showing a 2.0-fold or greater FC (P value  $< 0.05$ ) were used to conduct a functional enrichment analysis of *L. plantarum* NMM.1 in contact with D3G according to their GO pathway terms using Goatools. Terms with P values  $< 0.05$ , were considered significant. The analysis showed that DEGs were significantly enriched in many biological processes (Fig. S3). The GO category mainly includes biological processes (BP), cellular components (CC), and molecular functions (MF). Among these enriched pathways, the largest number of upregulated genes was enriched in the BP category, followed by CC (Fig. S3). In the BP category, the analysis showed that the most significantly upregulated biological processes were the metabolic process (GO: 0008152) and cellular process (GO: 0009987). In the CC category, cellular component (GO: 0005575) was the largest part. In MF, RNA binding (GO: 0003723) was the most significant molecular function (Fig. S3). According to the GO enrichment analysis, we observed that among the three categories, the largest number of down-regulated genes were enriched in the MF category with an uncorrected P value  $< 0.05$  (Table S1 and Fig. S3). The most significantly downregulated molecular functions were phosphotransferase activity, alcohol group as acceptor (GO: 0016773) and transcription regulator activity (GO: 0140110, GO: 0003700) (Fig. 4B).

### 3.6. KEGG enrichment analysis of DEGs

Genes involved in ribosome (KEGG map03010) was significantly enriched by Goatools. This response included a 2 to 6.4-fold change in the induction of 22 genes encompassing ribosomal proteins that made up ribosome subunits. Upregulated and downregulated genes were also enriched owing to ABC transporters (KEGG map02010) (Table S2). However, downregulated genes were mainly enriched in saccharides,

phosphate, and vitamin transporters, such as organic ion, galactose oligomer/ maltooligosaccharide, *sn*-Glycerol 3-phosphate, phosphate, phosphonate, glutamine, and biotin transporters, while the enrichment of upregulated genes in the ABC transporter pathway was various (Table S2). Moreover, genes was upregulated in the aminoacyl-tRNA biosynthesis pathway (KEGG map00970) including L-Aspartyl-tRNA, L-Asparaginyl-tRNA, Glycyl-tRNA, and L-Isoleucyl-tRNA, while genes encoding for L-Arginyl-tRNA were downregulated. In addition, a relatively large number of genes were enriched in the phosphotransferase system (PTS) (KEGG map02060) (Table S2). The gene expression profile within this category indicates an increase in mannose and fructose metabolism and downregulated genes coded for enzymes involved in cellobiose/diacetylchitobiose, galatosamine, and galactitol metabolism.

### 3.7. Changes in carbohydrate metabolism triggered by the transformation of D3G in *L. plantarum* NMM.1

As the sole carbon source, D3G has an essential effect on carbohydrate metabolism as it is not a high-quality carbon source for bacterial growth compared to other saccharides. The GO analysis revealed that the PTS system accounted for a large proportion of the downregulated pathways. Consistent with this expression profile we noticed that the KEGG pathway analysis also revealed that the transcript changes in the PTS system and many transporters were related to carbohydrate metabolism (Fig. 4). By combining the GO analysis with the KEGG pathway enrichment analysis, we summarized the expression patterns of enriched genes related to saccharide metabolism pathways in the phosphotransferase system (PTS) term (Fig. 5). In cellobiose operon, gene encoding for cellobiose phosphotransferase (*celB*), which is specific for a particular substrate is involved in a successive transfer of the phosphate group to amino acids and gene encoding for 6-phospho- $\beta$ -glucosidase (*bgIA*), which recognizes phosphorylated  $\beta$ -glucosides at C1 position and is generally involved in hydrolysis of cellobiose were both downregulated. In trehalose metabolism pathway, the expression of gene encoding for trehalose-6-phosphate hydrolase (*treC*) was also significantly lower than the control group. The downregulation of gene encoding for trehalose phosphotransferase and maltose phosphotransferase (*crr*) was not significant. However, the expression of genes related to sucrose and maltose metabolism which were indicated by grey arrows in Fig. 5 was unchanged. The KEGG orthologies depicted in Fig. 5 are explained in Table S3.

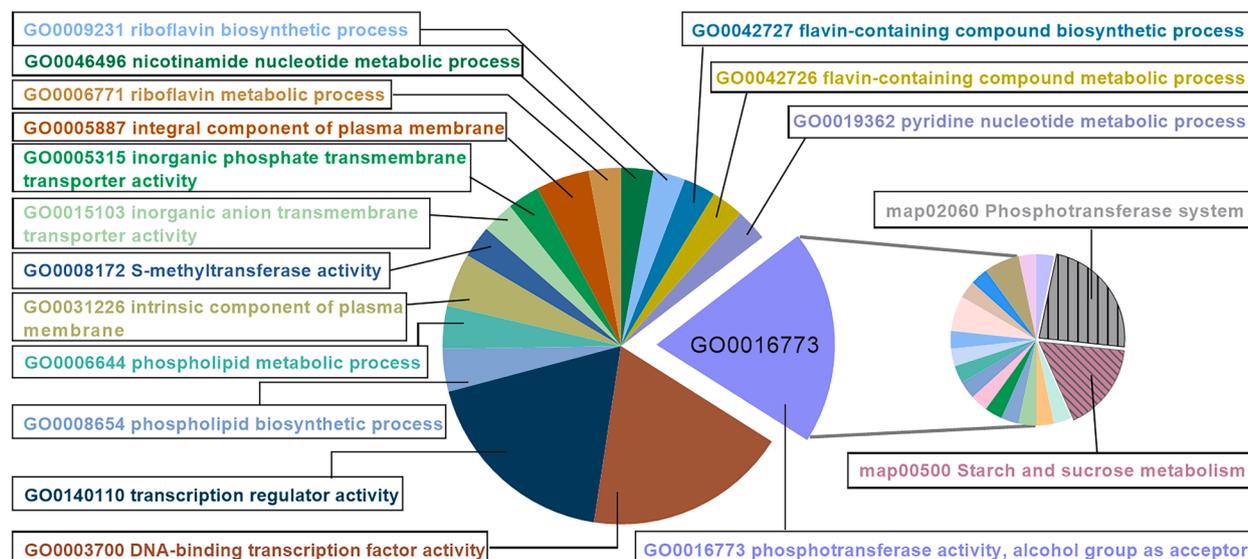
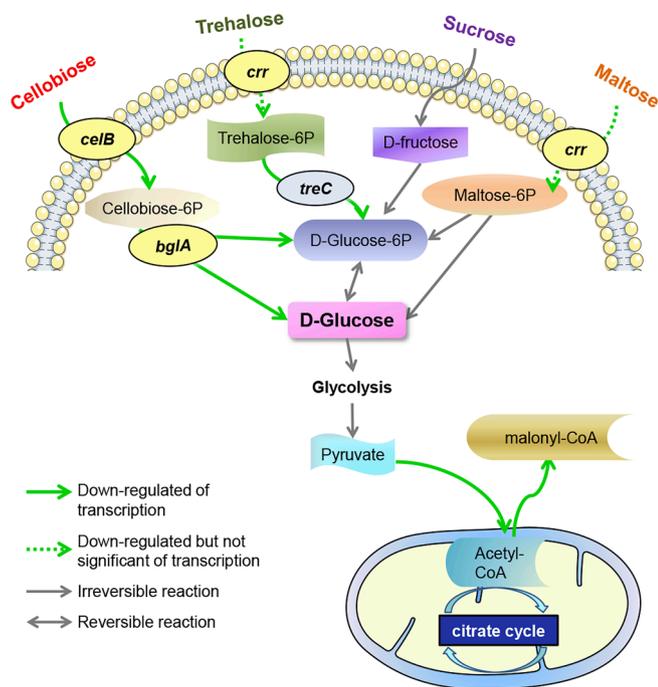


Fig. 4. GO enrichment analysis of downregulated genes in *L. plantarum* NMM.1 when in contact with 5  $\mu\text{g}/\text{mL}$  D3G. Big pie chart shows GO terms proportion distribution of downregulated genes and small pie chart shows KEGG enrichment analysis of GO0016773 term.



**Fig. 5.** Graphical representation of carbohydrate metabolic pathways transcriptionally affected by D3G in *L. plantarum* NMM.1. Green arrows represent downregulated or, if dotted arrows, downregulated but not significant ( $\log_2 FC < 1$ ). KEGG Orthology with no change was represented by grey arrows. Pathways were based on GO database and KEGG database.

### 3.8. Inhibitory effect of saccharides on the conversion of D3G by *L. plantarum* NMM.1

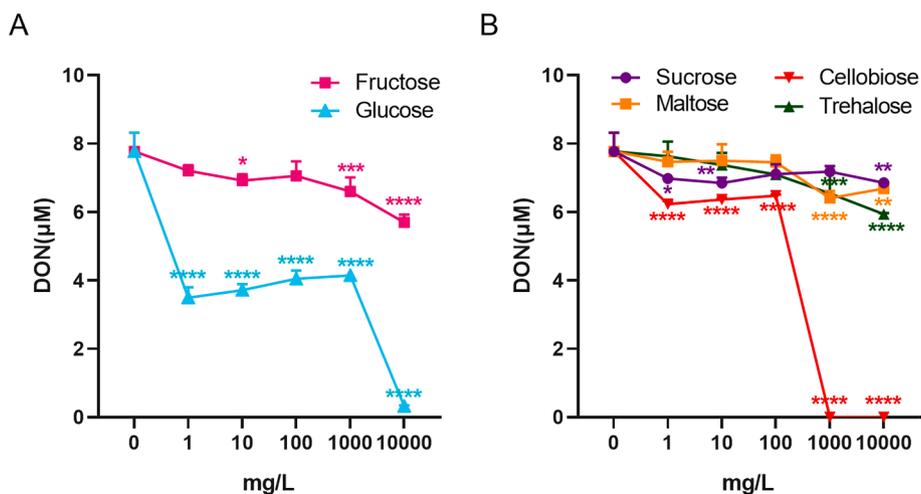
As shown in Fig. 5, the expression of genes related to the saccharide metabolism pathway was affected by the D3G transformation. In order to explore whether exogenously added saccharides would competitively inhibit the transformation of D3G and whether glucose, the product of D3G transformation, would inhibit the transformation of D3G, we added 1–10000 mg/L gradient concentrations of glucose, fructose, maltose, sucrose, trehalose, and cellobiose in the reaction matrix. The addition of the monosaccharide glucose had an obvious impact on the transformation efficiency of D3G. When the glucose concentration was only 1 mg/L, the production of DON decreased by approximately 55%, while as the glucose concentration increased, the production of DON appeared

slightly increased until it was completely suppressed (Fig. 6A). The addition of 1–10,000 mg/L of fructose, the precursor substance of glucose *in vivo*, led to the decrease of the production of DON in a dose-dependent manner while this was reduced by 26.8% at most (Fig. 6A). As is shown in Fig. 5, among the metabolic pathways of several extracellular carbohydrates, only the expression of KEGG orthology genes related to the metabolism of trehalose, maltose and cellobiose was inhibited. In addition to *crr*, several other orthologous genes belonging to PTS were significantly downregulated; among these, *treC* was not a part of PTS (Table S3). Among the aforementioned kinds of carbohydrates that were added exogenously, cellobiose was the most efficient in inhibiting D3G conversion, as it achieved 100% inhibition at a concentration of 1 g/L (Fig. 6B). However, although the gene expression in the trehalose and maltose metabolic pathways was affected, the highest inhibitory effect was only 23.6% in a dose-dependent manner, similar to the inhibitory effect of fructose. Extracellular sucrose and maltose did not incur significant changes in genes related to metabolic pathways. Additionally, they only inhibited DON production by only approximately 10% at most and did not show a significantly dose-dependent manner (Fig. 6B). This suggested that the cellobiose metabolism of *L. plantarum* NMM.1 had a significant inhibitory effect on the transformation of D3G, thereby exhibiting potential to be used as a safe inhibitor of the process of D3G transformation into its precursor toxin DON by *L. plantarum*.

## 4. Discussion

In the natural environment, *Fusarium* sp. infects plants and synthesizes DON, and the plant modifies DON into the non-toxic D3G under the action of uridine diphosphate glycosyltransferases (UGTs) through the defense response. D3G can be transformed by *L. plantarum* which is part of the gastrointestinal microbiota and then releases the free native toxin DON, which increases toxicity to the human body (Fig. S4).

The strain NMM.1, possessing high D3G conversion activity, was isolated from Inner Mongolia raw milk which is an indispensable food source for humans. Based on the results of our study, we concluded that D3G can be efficiently transformed by *L. plantarum* NMM.1 to release the precursor toxin, DON, when used as the sole carbon source (Fig. 1A). This phenomenon is consistent with the result of the previous study (Berthiller et al., 2011). D3G is generally considered to have no toxicity or low toxicity. The results of our study showed that D3G did not have a significant effect on the growth of *L. plantarum* in the MRS culture medium (Fig. 2A). It has been reported that D3G is not toxic to human gastric epithelial cells (GES-1) (Yang et al., 2017). We evaluated the



**Fig. 6.** Effect of different concentrations of monosaccharide (A) and disaccharides, and polysaccharides (B) on the DON production ability of *L. plantarum* NMM.1. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$ : Saccharides treated group compared with saccharides-free group.

cytotoxicity of D3G using HIECs and found that it had no effect on cell viability while DON reduced cell viability in a dose-dependent manner (Fig. S1). Substrate depletion studies have shown that the D3G transformation activity of strain NMM.1 is much higher than that of model strain *L. plantarum* ATCC 14917<sup>T</sup> (Fig. 1B). When D3G was the sole carbon in the reaction mix, strain NMM.1 was highly efficient in transforming a large part of D3G in only 1 h (Fig. 2B). However, the D3G transformation efficiency of NMM.1 was affected by the environment pH and exhibited higher conversion efficiency under alkaline conditions (Fig. 2C). Under normal circumstances, the human intestine is alkaline, which undoubtedly provides a suitable environment for certain intestinal microorganisms with D3G transformation ability. By testing the D3G conversion effect of different location parts of bacteria, we found that *L. plantarum* released DON by absorbing D3G instead of conjugating the cell wall (Fig. 2D). The transcriptomic profile of *L. plantarum* NMM.1 exposed to 5 µg/mL of D3G for 1 h revealed the upregulation of the BP category and the downregulation of mainly the phosphotransferase activity, alcohol group as acceptor, according to the GO enrichment analysis (Fig. 4). The KEGG enrichment analysis showed that the downregulated genes were mainly enriched in the metabolic pathways of several extracellular sugars, including cellobiose, maltose, sucrose, and trehalose (Figs. 4 and 5). Therefore, in order to explore the relationship between carbohydrate metabolism and D3G conversion, we added different concentrations of glucose, fructose, cellobiose, maltose, sucrose, and trehalose to the reaction matrix of *L. plantarum* NMM.1 and found that cellobiose was highly efficient in inhibiting the release of DON by *L. plantarum* NMM.1 (Fig. 6).

Early reports showed that cellobiase from *Aspergillus* has the ability to hydrolyze D3G while its hydrolysing ability differs from that from different sources (Berthiller et al., 2011). Furthermore, studies have verified that a versatile family 3 glycoside hydrolase from *Bifidobacterium adolescentis* is highly hydrolytic toward D3G and the gene *BaBgl* encoding this glycoside hydrolase is not highly homologous in other species (Michlmayr et al., 2015). Based on previous research and our studies, we conclude that the conversion of D3G is related to cellobiose metabolism. Moreover, several *L. plantarum* strains have been reported possess tannase-activity and are able to hydrolyze tannin into glucose and gallic acid, a harmful and anti-nutritional compound, which illustrates that some *L. plantarum* strains are able to respond to these toxic compounds as well as use them as energy sources, thereby selectively stimulating their growth (Jimenez et al., 2014; Reveron et al., 2013; van den Nieuwboer et al., 2016). In our study, when D3G and cellobiose were used as the carbon sources of *L. plantarum* at the same time, the conversion of D3G was inhibited. Therefore, we infer that when D3G was the only carbon source, it was utilized by *L. plantarum* as a sugar with function similar to those of cellobiose; on the contrary, the utilization of D3G was inhibited when it was used concurrently with a better carbon source such as cellobiose. Generally, *L. plantarum* strains are able to ferment glucose, maltose, cellobiose, fructose and sucrose (Sanchez et al., 2019; Ucar et al., 2020). Cellobiose is a disaccharide that can be used as a food ingredient (Messinger et al., 2020). Studies have pointed out that cellobiose has a positive impact on porcine gut bacteria, including *Lactobacillus* spp., *Bifidobacterium* spp., *Roseburia* spp., *Clostridium cluster IV* spp., and *Enterobacteriaceae* (Heinritz et al., 2018). Therefore, cellobiose has the potential application *in vivo*. Except for ruminants, mammals do not have the ability to digest cellulose into cellobiose (Ciudad-Mulero et al., 2019; Kumar et al., 2020). Accordingly, using exogenous cellobiose to inhibit the conversion of D3G by *L. plantarum* in the gastrointestinal tract of animals, especially mammals may be a potential way to control free native DON release.

#### Declaration of Competing Interest

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

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochms.2022.100077>.

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