Transcriptome Analysis of Spleen in Chickens Administered β-glucan Derived From Yeast Cell Wall

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Research

Keywords: Transcriptome, spleen, β-glucan, immune function

Posted Date: November 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1074713/v1

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Abstract

**Background:** Our previous study shown that oral administration of a product contained yeast cell wall polysaccharides enhanced immune responses elicited by Newcastle disease virus and changed microbial community of cecum in chickens.

**Results:** The present study was design to investigate the potential molecular mechanism in relation to the immunomodulation of β-glucan in chickens. Using RNA-sequence (RNA-seq) technique, we identified 198 DEGs in spleen in chickens after oral administration of β-glucan. In addition, these DEGs were significantly enriched in 205 GO terms and 7 KEGG pathways.

**Conclusions:** β-glucan might regulate chicken immune system by regulating expression of genes involved in cognition, cytokines, binding, enzyme activities and multiple signaling pathway.

Background

Yeast cell wall polysaccharides, mainly composed of β-glucan and mannan-oligosaccharides (MOS), are derived from the cell wall of *Saccharomyces cerevisiae* [1]. They are extensively used as growth promoters, antimicrobial agents or immunomodulator in poultry for the purpose of improving production and health [2,3,4]. We have previously shown that oral administration of PW220, a product contained yeast cell wall polysaccharides, enhanced immune responses elicited by Newcastle disease virus and changed microbial community of cecum in chickens [5]. However, the underlying molecular mechanism remained obscure.

RNA sequencing (RNA-seq) is a high through-put technology that can be employed to detect quantitative gene expression and to analyze different expression profiles at the whole transcriptome level [6]. With the enhanced sensitivity and declining cost, RNA-seq has been widely used in livestock and poultry research [7,8]. Because the spleen is one of the dominating sites for priming of the primary immune responses, in the present study, transcriptome sequencing was performed to investigate the effect of β-glucan on gene expression in chicken spleen and to explore the potential signal transduction pathways.

Materials And Methods

**Reagents**

β-glucan with purity ≥ 70% derived from yeast cell wall was provided by Angel Yeast Co. LTD (Yichang, China). PrimeScript™RT Master Mix and SYBR ® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara Bio INC (Dalian, China).

**Birds and Experimental Design**

One-day-old White-feather silky chickens (male) were purchased from Sichuan Lihua Poultry Co., Ltd. (Chongqing, China) and randomly allocated into two groups. Group 1 was orally administered β-glucan at
a dose of 20 mg/kg of BW (the dose was chosen based on a preliminary experiments) in drinking water for 7 d before vaccination; group 2 was administered saline as a control. On the day following administration of β-glucan, each bird was received intraocular and intranasal immunization with a live vaccine of NDV at a dose of $2 \times 10^3$ EID, according to the manufacturer’s instruction. On 2 weeks post immunization, chickens were humanly killed by cervical dislocation and the whole spleen was immediately frozen and stored at −196°C in liquid nitrogen for sequence analysis.

**RNA extraction and cDNA Library Construction**

The total RNA of the spleen was extracted using TRIzol reagent (Thermo fisher Scientific). RNA was detected free of contamination and degradation using 1% agarose gel, and the RNA purity was estimated using a NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA). RNA concentration was measured using a Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The results showed that the RNA was intact and free of DNA contamination. The libraries were sequenced using Illumina HiSeq platform with a PE150 strategy.

**Transcriptome analysis**

Transcriptome sequencing, sequence assembly, and data analysis are provided by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The transcriptome analysis process was performed as follows: (a) mRNA was purified from total RNA using poly-T oligo-attached magnetic beads; Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). (b) First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse. Transcriptase (RNase H-); Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. (c) Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities; After adenylation of 3’ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. (d) In order to select cDNA fragments of preferentially 250~300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. TruSeq PE Cluster Kit v3-cBot-HS (Illumina) was employed to carry out the cluster of the index-coded samples. Then, the sequencing was executed on an Illumina Novaseq platform, and 150 bp paired-end reads were produced. Reference genome and gene model annotation files were downloaded from the genome website ([ftp://ftp.ensembl.org/pub/release-98/fasta/gallus_gallus](ftp://ftp.ensembl.org/pub/release-98/fasta/gallus_gallus)) and ([ftp://ftp.ensembl.org/pub/release-98/gtf/gallus_gallus/](ftp://ftp.ensembl.org/pub/release-98/gtf/gallus_gallus)). Using HISAT2 (v2.0.5) to build the index of the
reference genome and align paired-end clean reads with the reference genome. Feature Counts v1.5.0-p3 was used to count the reads numbers mapped to each gene, and then FPKM of each gene was calculated basing the length of the gene and its reads count. DESeq2 R package (1.16.1) was used to exporting the differential expression between the H with the control group. Genes with $P < 0.05$ and $|\log_2(\text{foldchange})| > 1$ were defined as differential expression genes (DEGs).

**Real-time Quantitative PCR Validation**

Two DEGs that were up-regulated and four DEGs that were down-regulated in the comparison H vs. Control were selected to validate the transcriptome sequencing results using RT-qPCR. PrimeScript™RT Master Mix (Takara, Dalian, China) was used to convert RNA into cDNA on a T100™ thermal cycler (Bio-Rad, USA). Information of primers were provided in Table S3. The Chicken $\beta$-actin was served as the internal control gene. RT-qPCR with SYBR®Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China) on selected genes was carried out on a Multiple Real-Time PCR System (AB, USA). A relative quantitative method ($2^{-\Delta\Delta CT}$) was employed to evaluate the quantitative variation. All samples were analyzed in triplicate.

**Results And Discussion**

**RNA sequencing data analysis**

To detect differences between H and Control, RNA-seq of splenic samples was performed using the Illumina sequencing platform. Four individual samples were included for each group, and they were marked as C1, C2, C3, C4 and H1, H2, H3, H4, respectively. As shown in supplementary materials Table1, RNA sequencing of the 8 spleen libraries produced 24.1G raw data. The C1, C2, C3, C4, H1, H2, H3 and H4 libraries contained 45,591,492, 47,424,782, 46,193,492, 54,403,376, 47,118,476, 45,899,262, 45,841,884 and 45,504,438 raw reads. After removing adaptor, ambiguous and low-quality sequences, 44,050,198, 45,449,884, 44,261,112, 52,097,846, 45,542,404, 44,566,290, 44,113,172, and 44,026,960 clean reads were remained. The percentage of clean reads among raw reads was greater than 96%, the sequencing reads from the eight libraries were aligned to a reference database, which consisted of the Gallus gallus genome, using HISAT2. More than 95% of the clean reads mapped to this database. In particular, 40921384 (92.9%), 41302100 (90.87%), 40771075 (92.11%), 46905784 (90.03%), 42444112 (93.2%), 40213444 (90.23%), 40922895 (92.77%) and 40971972 (93.06%) reads from the C1, C2, C3, C4, H1, H2, H3 and H4 libraries, respectively, uniquely mapped to the reference database.

**Differentially expressed genes**

As shown in Fig.1A, there were 198 differentially expressed genes in total, including 47 up-regulated and 151 down-regulated genes. Detailed description of DEG will be shown in supplementary material Table S2. The distribution of differentially expressed genes was depicted in the heatmap (Fig.1B). The clustered biological replicates of DEGs indicated good reproducibility of treatment.
Gene Ontology classification and KEGG enrichment analysis

According to the Gene Ontology (GO) classification system, the differentially expressed genes were classified into three main functional categories: biological process, cellular component and molecular function. Fig.2 displayed top 10 GO terms in three categories. Genes involved in the “humoral immune response”, “response to chemokine”, “antimicrobial humoral response”, “defense response to bacterium”, “antimicrobial humoral immune response mediated by antimicrobial peptide”, “defense response to Gram-negative bacterium”, “defense response to Gram-positive bacterium” were predominant in the biological process category. Moreover, a significant proportion of the genes were involved in “CCR chemokine receptor binding”, “chemokine receptor binding”, “lipopolysaccharide binding” in the category of molecular function. In addition, “mitochondrial respiratory chain complex I”, “NADH dehydrogenase complex”, “respiratory chain” were the predominant enriched terms in the cell components category.

Immunomodulatory effect of polysaccharides from yeast cell wall have been demonstrated effective to change immune response and altered gut microbiota composition in chickens [9,10]. In our previous study, a yeast cell wall product contained β-glucan and MOS was found effectively to enhance intestinal IgA response to NDV vaccination and modulated the cecum microbiota by oral route [5]. However, only a few reports analyzed the mRNA expression of spleen after oral administration of yeast polysaccharides. RNA-seq based transcriptome analysis is a tool that allows a deep understanding of complicated physiological pathways. β-glucan may exhibit its effects on the immune function of chicken by differentially expressed genes in relation to innate and adaptive immunity in lymphocyte. Several pattern recognition receptors target β-glucan were enriched on the surface of immune cells [11]. After recognition of β-glucan, these receptors stimulate tyrosine kinase and nuclear factor κB, which consequently induced secretion of proinflammatory cytokines and activate immune reaction [12,13,14,15]. Recently, transcriptome analysis was used to study the mechanism of a vegetable oil adjuvant in mice [16]. They found DEGs in spleen were enriched in immune response related GO terms such as “humoral immune response”, “antimicrobial humoral response” and “defense response to bacterium”, which were consistent with our results.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed genes was also performed. The results indicated that these genes were mainly classified into seven pathways included “Neuroactive ligand-receptor interaction”, “Gap junction”, “MAPK signaling pathway”, “ABC transporters”, “Biosynthesis of amino acids”, “Drug metabolism”, “Protein export”. These results indicated that β-glucan regulate immune response through multiple pathways (Fig.3). Similar results were also reported by others [17-18]

Interestingly, genes encoding cathelicidin including CATH3, CATH1, CATH2, and genes encoding beta-defensin such as AvBD6, AvBD7, AvBD1, and AvBD4 were significantly down-regulated in the comparison H vs. Control. Host Defense Peptides (HDPs) are important effector molecules of the innate immune system [19]: These peptides have been found in a wide range of animals from mammals to birds. The chicken has four cathelicidins, including CATH1, CATH2, CATH3 and CATHB1, which were able to
efficiently kill a variety of bacteria [20]. Avian beta-defensins (AvBDs), also known as gallinacins, are small cationic peptides having three cysteine disulfide bonds between their cysteine residues and play essential roles in the innate immune system [21]. The decreased expression of cathelicidins and beta-defensin might be explained by the feedback regulation, in which the population of *Bacteroides* and *Parabacteroides* were reduced by yeast cell wall polysaccharide [5]. Similar results were found in other studies. Yu reported that supplementation of yeast β-glucan in broiler chicken inhibited *Salmonella* infection and reduced HDPs expression by quantitative real-time PCR analysis [22].

**Confirmation of differentially expressed genes by qRT-PCR**

We verified the 6 DEGs which were up- or down-regulated using real-time quantitative PCR. The result showed that the RT-qPCR data were consistent with the RNA-seq data in general, indicating the reliability of the sequencing results (Fig.4).

In summary, this study identified 198 DEGs in spleen in chickens after oral administration of β-glucan. GO and KEGG pathway enrichment uncovered 205 significantly enriched GO terms and 7 KEGG pathways. β-glucan might regulate chicken immune system by changing expression of genes involved in cognition, cytokines, binding, enzyme activities and multiple signaling pathway.

**Declarations**

**Acknowledgements**

Not applicable.

**Authors' contributions**

LTC and SCB conceived and designed the experiments. JRZ, YWQ and WHL performed the experiments and analyzed the data. LTC and SCB wrote the paper. YM, JL and WX reviewed and edited the paper. All authors read and approved the final manuscript.

**Funding**

This research was supported by the National Natural Science Foundation of China (32002325), Chongqing Research Program of Basic and Frontier Technology (cstc2020jcy-jmsxmX0418), and Fundamental Research Funds for the Central Universities (SWU119056).

**Availability of data and material**

The raw sequence data have been submitted to NCBI SRA (Accession: SAMN16965324, SAMN16965325, SAMN16965329, SAMN16965330, SAMN16965351, SAMN16965352, SAMN16965353, SAMN16965354).

**Ethics approval and consent to participate**
The animal management and study protocol were evaluated and approved by the Southwest University Committee on Animal Care and Use. All animal behavior and care procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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Tables
Table 1. Sequences of primers for RT-qPCR

| Gene   | Primer sequence (5'-3')                                      | Product size (bp) |
|--------|-------------------------------------------------------------|-------------------|
| CATH-3 | 5'-GCTCCCTGCACAACCTCAACTTC-3' 5'-AGCCCCGTCTCCTTGAACCTC-3' | 97                |
| AvBD-6 | 5'-CTGTTGTCTCTTTTGTGGTGCTC-3' 5'-GTCCACTGCCACATGAC-3'      | 148               |
| AvBD-7 | 5'-TGTTGCAAGGTCAGCCTTCATTC-3' 5'-CCTCCTCCAGATCCTGCAG-3'   | 85                |
| PAX-3  | 5'-AGATGGAGGAAGCAGGCAGGAGG-3' 5'-ATGGAGGTGGGCTGATGGG-3'   | 134               |
| LYG2   | 5'-GCTGGGGTGACCCTTGGAATG-3' 5'-TCTCGTGCCCCGGCTGATG-3'    | 107               |
| RGS16  | 5'-TCCCCGCTCTACCTCGTCTGTG-3' 5'-GGACCTCTGATGCCGCTCT-3'   | 138               |

Table 2. List of top 10 molecular and cellular functions

| GO-ID   | GO-Term                              | P-value | Molecule |
|---------|--------------------------------------|---------|----------|
| GO:0050878 | regulation of body fluid levels     | 1.80E-04 | 14       |
| GO:0004857 | enzyme inhibitor activity            | 3.54E-03 | 14       |
| GO:0010951 | negative regulation of endopeptidase activity | 3.83E-04 | 12       |
| GO:0010466 | negative regulation of peptidase activity | 5.84E-04 | 12       |
| GO:0004866 | endopeptidase inhibitor activity     | 2.24E-05 | 12       |
| GO:0061135 | endopeptidase regulator activity     | 3.76E-05 | 12       |
| GO:0030414 | peptidase inhibitor activity         | 4.57E-05 | 12       |
| GO:0061134 | peptidase regulator activity         | 2.55E-04 | 12       |
| GO:0042742 | defense response to bacterium        | 1.62E-04 | 11       |
| GO:0098800 | inner mitochondrial membrane protein complex | 5.05E-03 | 8        |

Supplementary Files

The Supplementary Files are not available with this version.

Figures
Figure 1

Summary of RNA-Seq data. (A) List of differentially expressed genes, (B) Clustering map of the DEGs
Figure 2

Top10 enriched GO terms in biological process, cellular component and molecular function categories
Figure 3

KEGG pathway analysis
Figure 4

RT-qPCR confirmation of selected DEG candidates. Data are expressed as mean ± SE.