

Effects of yeast cell wall-derived mannan-oligosaccharides on jejunal gene expression in young broiler chickens

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ABSTRACT The use of mannan-oligosaccharides (MOS) as alternatives to antibiotic growth promoters (AGP) has gained in popularity in recent years due to regulatory restrictions of using AGP in food animal production. Benefits of MOS usage include improvement on animal performance, feed efficiency, and gastrointestinal health. The molecular mechanisms of these functions however are not clear. The goal of the current study was to use a transcriptomics approach to investigate the effects of MOS on the intestinal gene expression profile of young broilers and characterize biological gene pathways responsible for the actions of MOS. One hundred and twenty 1-d-old Cobb 500 broiler chicks were randomly divided into 2 groups and were fed either a standard wheat-soybean meal-based (control) diet or the same diet supplemented with 2.2 g/kg of MOS (Bio-Mos, Alltech, Nicholasville, KY) for 3 wk, followed by jejunal gene expression profiling analysis using chicken-specific Affymetrix microarrays. Results indicated that a total of 672 genes were dif-

ferentially expressed ($P < 0.01$ and fold change >1.2) in the jejunum by MOS supplementation. Association analysis indicated that differentially expressed genes are involved in diverse biological functions including energy production, cell death, and protein translation. Expression of 77 protein synthesis-related genes was differentially regulated by MOS in the jejunum. Further pathway analysis indicated that 15 genes related to oxidative phosphorylation were upregulated in the jejunum, and expression of genes important in cellular stress response, such as peroxiredoxin 1, superoxide dismutase 1, and thioredoxin, were also increased by MOS. Differential expression of genes associated with cellular immune processes, including lysozyme, lumican, β 2-microglobulin, apolipoprotein A-1, and fibronectin 1, were also observed in MOS-fed broilers. In summary, this study systematically identified biological functions and gene pathways that are important in mediating the biological effects of MOS in broilers.

Key words: mannan-oligosaccharides, chicken, nutrigenomics, gene expression

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INTRODUCTION

Mannan-oligosaccharides (MOS) are mannose-rich carbohydrates found in the yeast cell wall (Sentandreu and Northcote, 1968, 1969; Young et al., 1998). Currently, MOS products, particularly those derived from the cell wall of *Saccharomyces cerevisiae*, are extensively used as natural feed additives in livestock and poultry because of documented benefits in performance (Hooge, 2004; Rozeboom et al., 2005; Rosen, 2007) and gastrointestinal health (Spring et al., 2000; Sims et al., 2004; Baurhoo et al., 2007b; Yang et al., 2008; Baurhoo

et al., 2009) but without the resistance-related risks associated with the use of dietary antibiotics.

Several mechanisms have been proposed to explain the beneficial effects related with MOS. One popular model is based on the fact that mannose can block the colonization of intestinal pathogens, such as *Salmonella* spp. and *Escherichia coli*, which contain type 1 fimbriae with mannose-binding lectins (Kelly et al., 1994). Mannan-oligosaccharide-bound pathogens are prevented from attaching to intestinal mannose residues (Spring et al., 2000). Mannan-oligosaccharide has also been linked with improved gut health, indicated by increased villi length and the number of goblet cells and increased populations of beneficial bacteria such as lactobacilli and bifidobacteria in the guts of broilers and turkeys while reducing the populations of *Salmonella* and *Escherichia coli* (Sims et al., 2004; Baurhoo et al., 2007b; Brümmer et al., 2010). Another suggested mode

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of action of MOS is immune system modulation activity, which suggests that MOS can stimulate intestinal mucosal immunity, perhaps by acting as a nonpathogenic microbial antigen (Davis et al., 2004a,b). It was reported that dietary supplementation of 0.05% of MOS increased mucosal IgA secretions and humoral and cell-mediated immune responses of neonatal chicks (Gómez-Verduzco et al., 2009). The relationship between the effects of MOS as well the dominant mode of the action remains unclear due to our limited understanding of the underlying effects of MOS at a molecular level.

The emerging field of nutrigenomics has created unprecedented opportunities for increasing our understanding on how nutrients may modulate gene expression and has provided much novel information concerning the molecular mechanisms of dietary modulation of host immunity, physiology, and metabolism (Dawson, 2006; Kussmann et al., 2006; Crujeiras et al., 2008). The aim of the current study was to use this technology to evaluate the response of intestinal gene expression of chickens fed with MOS in an effort to identify functional pathways of genes responsible for the effects of MOS. Wheat-soybean meal (SBM)-based diets without exogenous enzyme supplementations were used in this study with an intent to create a challenge-like environment and to help accentuate the effects of MOS.

MATERIALS AND METHODS

Bird Handling and Diets

One hundred and twenty 1-d-old Cobb 500 broiler chicks were randomly divided into 2 groups and assigned to either a wheat-SBM control diet (control; Table 1) or the basal diet supplemented with 2.2 g/kg of MOS (Bio-Mos, Alltech, Nicholasville, KY). The chicks were housed in pens of 10 birds each and grown on the floor with recycled litter. Birds and feed were weighed at the start of the study and then weekly. After 3 wk of feeding, 7 chicks from each diet group (1 or 2 chicks from each pen) were randomly selected and euthanized using argon gas followed by cervical dislocation. Jejunum samples of 1 cm from the location of approximately 2 cm away from Merkel's diverticulum were snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation and microarray analysis. All animal care procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Performance results were subjected to ANOVA by using linear models of Statistix V.8 (Analytical Software, Tallahassee, FL). Mean differences were determined using Fisher's least significant difference test.

RNA Extraction

Tissues were homogenized with a Qiagen TissueRuptor (Qiagen, Valencia, CA). Total RNA isolation was performed using an RNeasy Mini kit (Qiagen). To remove contaminating DNA, on-column DNA digestion

with RNase-free DNase (Qiagen) was performed. Integrity and purity of isolated RNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and further confirmed with an Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA).

Microarray Procedures

Complementary RNA preparation, hybridization, and scanning were performed following the standard protocols recommended by Affymetrix (Santa Clara, CA). Briefly, purified RNA was used for biotin-labeled cRNA synthesis using the Affymetrix GeneChip Expression 3'-Amplification One-Cycle Target Labeling Kit (Affymetrix) according to the manufacturer's recommended procedures. Labeled cRNA was hybridized to Affymetrix chicken genome arrays (http://media.affymetrix.com/support/technical/datasheets/chicken_datasheet.pdf; GEO platform: GPL3213) for 16 h at 45°C , followed by washing, streptavidin-phycoerythrin staining, and finally scanning in a GeneChip Scanner 3000 7G (Affymetrix). Probe signal intensities were analyzed using an Affymetrix MAS5 algorithm scaled to the default trimmed mean signal intensity of 500. Gene expression data were obtained for each of 7 birds from control and MOS groups, respectively.

Table 1. Ingredients and composition of the basal diet¹

Item	%
Ingredient	
Wheat (hard)	63.95
Soybean meal (48%)	27.00
Corn oil	5.00
Limestone	1.42
Dicalcium phosphate	1.65
Salt	0.45
Vitamin-mineral premix ²	0.25
DL-Methionine	0.23
L-lysine	0.05
Total	100
Calculated nutrient composition	
ME, kcal/kg	3,120
CP, %	22
Ca, %	1.00
Available P, %	0.45
Lysine, %	1.10
Methionine, %	0.57
Met + Cys, %	0.90
Na, %	0.22

¹Basal diet was used as control. Equivalent wheat was replaced with mannan-oligosaccharide in mannan-oligosaccharide-supplemented diet.

²Supplied per kilogram of diet: vitamin A (retinyl acetate), 11,025 IU; vitamin D₃ (cholecalciferol), 3,528 IU; vitamin E (DL- α -tocopheryl acetate), 33 IU; vitamin K₃ (2-methyl-1,4-naphthoquinone), 0.91 mg; thiamine, 2 mg; riboflavin, 8 mg; niacin, 55 mg; Ca pantothenic acid, 18 mg; vitamin B₆ (pyridoxine), 5 mg; biotin, 0.221 mg; folic acid, 1 mg; choline, 478 mg; vitamin B₁₂ (cyanocobalamin), 28 μg ; iron (FeSO₄·H₂O), 80 mg; manganese (MnSO₄·H₂O), 60 mg; iodine (KIO₃), 0.35 mg; copper (CuSO₄·5H₂O), 20 mg; selenium (Na₂SeO₃), 0.15 mg; zinc (ZnSO₄·H₂O), 40 mg.

Microarray Data Analysis

GeneSpring GX 10.0 (Agilent) was used to validate and normalize microarray data and to perform statistical and gene expression pattern analyses. Briefly, normalization was done by first scaling the intensity of probe sets of the arrays to a mean target intensity of 500, followed by baseline transformation to median of all samples of this study. Background corrections were done by MAS5, based on its perfect match and mismatch probe design of the microarray. To minimize the possibility of misleading findings, probe sets with low signal intensity that were labeled as absent by the Affymetrix MAS5 algorithm across samples were excluded from further analysis. The differentially expressed genes between broilers fed with control and MOS-supplemented diets were filtered using the volcano plot method. These genes were defined by $P < 0.01$ and corresponding signal intensity fold change (FC) >1.2 or $FC < -1.2$.

Gene Annotation and Functional Analysis

To dissect the biological themes represented by differentially expressed genes, genes were first classified based on the terms of gene ontology (GO) using the PANTHER classification system (<http://www.pantherdb.org>), whereby genes were grouped into categories based on common biological properties. Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, Redwood City, CA) was then used to group annotated genes into networks, functions, and canonical pathways. Briefly, the data set containing gene identifiers and corresponding expression FC was uploaded into the application. Each identifier was mapped to its corresponding gene object in the Ingenuity Knowledge Base (IKB), which contains hundreds of canonical pathways that are constructed from those scientific findings (Zhan and Desiderio, 2010). A network analysis was performed whereby focus genes (imported genes that are eligible for generating interaction networks based on IKB) were overlaid onto a global molecular network developed from information contained in the IKB. Networks of focus genes were then algorithmically generated based on their connectivity. A functional analysis was performed to determine biological functions that were most significant to the genes in the data set, whereas the canonical pathway analysis identified pathways that were significant to the regulated genes. Fischer's exact test was used to determine the significance of the association between the genes and the given network, biological function, or canonical pathway.

Real-Time Quantitative PCR Confirmation

For validation of microarray data, 11 differentially expressed genes were chosen by function of interest and analyzed in real-time quantitative (RT-q) PCR analysis. The same total RNA used for microarray analysis

was also employed for RT-qPCR analysis. Each sample of total RNA (0.5 μg) was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The RT-qPCR was performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7500 real-time PCR system (Applied Biosystems). Primers (Table 2) were designed using Primer Express software v. 2.0 (Applied Biosystems) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Integrin β 1 (*ITGB1*) was used as a reference gene to account for any nonbiological variations that occurred in the process. The relative quantification was calculated as a ratio of the target gene to control gene using the $\Delta\Delta C_t$ method. Conditions for RT-qPCR were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a hold at 4°C. The RT-qPCR results were analyzed using the GLM procedure of the SAS version 9.1 statistical package (SAS Inst. Inc., Cary, NC). Values are presented as means \pm SEM, and differences between treatment means were considered significant at $P < 0.05$.

RESULTS

Effects of MOS Supplementation on the Growth Performance of Broilers

Compared with chicks fed with the control diet, chicks fed with the MOS-supplemented diet showed an improvement of approximately 4% in BW gain and 3% in gain-to-feed ratio (Table 3). These differences however are not statistically significant ($P > 0.05$), probably due to a relatively small sample size ($n = 6$) and large variations between chicks.

Gut Gene Expression Profiles Were Altered by Dietary MOS

Of the approximately 28,000 genes present on the gene-chip used in this study, about 50% were expressed in the jejunum of young broilers. Compared with the control birds, 672 were identified as differentially expressed (315 down-, 357 upregulated) by MOS supplementation. In addition, results indicated that gene expression changes (shown as FC of signal intensity) were relatively small, with only 6 genes showing an FC >2.0 or $FC < -2.0$ (Supplemental Table 1; available in the online version of this paper at ps.fass.org).

GO Annotation and Biological Processes Represented by MOS-Regulated Genes

To highlight the biological processes involved in MOS effects, the gene IDs corresponding to genes with changed expression patterns were first classified by the GO index using the PANTHER database (Thomas et

Table 2. Primer sequences used for real-time quantitative PCR¹

Gene	Forward 5'-3'	Reverse 5'-3'	Accession no.
<i>LYZ</i>	CCCAGGCTCCAGGAACCT	CACGCTCGCTGTTATGTCTGA	NM_205281
<i>FN1</i>	TGAAACCAGAGGCCATTACACA	CCGAATCTTATAGCCCGTAATGG	BU410331
<i>PRDX1</i>	TCAGGAAAGGCTTTTCATTGGA	TTGCCCATCTGGCATTACAG	BX930147
<i>ATP5G1</i>	TGTCTGTGTTTCAGCAGGCCT	TGGATAGGACACACCAGCTGG	BU142346
<i>LUM</i>	TGAGCAGCTTCTGTAAAGTTGTTG	TCCAGGCGTAAATGTGTGATCT	BX272380
<i>APOA1</i>	GAGGACATGGCTCCCTACTACAA	CAGAGCCTCGGTGTCCTTCA	NM_205525
<i>COL4A2</i>	GGCTCATGCTTGGCTCGTT	AACAAATATCCCCAGGGTTACAGTAG	CR385849
<i>NDUFV3</i>	TCTCTCAAATTCAGGCAGCC	TTCTTTCAGTGCCTTGGCG	BX950807
<i>COX7C</i>	CGAAGATGGACCTGGGAAGA	AACCGCCATTTGTTTTCCAC	CK607459
<i>SOD1</i>	TGTGCATGAATTTGGAGACAACA	TCCCCGACTCTTAGTTGCAGTAC	NM_205064
<i>TXN</i>	GCTGCTGGTGAGAAGCTTGTAGTA	TCCACACCATGTGGCAGAGA	NM_205453
<i>ITGB1</i>	TGGCCTTGACTTCTCGGATATAAC	TCAGGATTAAGTGAGCACCTGC	M14049

¹*APOA1* = apolipoprotein A-I; *LYZ* = lysozyme; *LUM* = lumican; *FN1* = fibronectin 1; *COL4A2* = collagen, type IV, α 2; *SOD1* = superoxide dismutase 1; *PRDX1* = peroxiredoxin 1; *TXN* = thioredoxin; *ATP5G1* = ATP synthase, H⁺ transporting, mitochondrial F0 complex, subunit C1; *COX7C* = cytochrome c oxidase subunit VIIc; *NDUFV3* = NADH dehydrogenase flavoprotein 3; *ITGB1* = integrin, β 1.

al., 2003). Of the 672 transcripts corresponding to genes regulated by MOS, 594 transcripts were grouped in one or multiple biological processes, whereas the other 78 transcripts (~12% of total) were labeled as unclassified because of a lack of functional annotations (Figure 1). As shown, a significant portion of the genes altered by MOS supplementation were related to metabolic processes (265 genes), which could be further categorized into genes involved in amino acid metabolism, carbohydrate metabolism, lipid and fatty acid metabolism, protein metabolism and modification, and nucleotide and nucleic acid metabolism (Supplemental Figure 1; available in the online version of this paper at ps.fass.org). Also, a large number of genes were involved in biological processes such as cell communication, development, adhesion, immune system functions, and stimulus response in the jejunum.

Function and Pathway Analysis

The IPA was used to further explore the biological functions and signal pathways of differentially expressed genes due to MOS. Of the 672 probe identifiers representing MOS-regulated genes, 557 were mapped to their corresponding genes in IKB, in which a total of 546 gene identifiers were eligible to proceed into pathway analysis. Based on the known relationships of genes in the IKB, network analysis identified that protein synthesis, cell cycle, carbohydrate metabolism, energy production, free radical scavenging, and cell death are among the top functions that the MOS-regulated genes are involved in (Table 4). The top 5

gene networks are summarized in Table 4, which includes network-associated genes, focus proteins (genes differentially expressed), biological functions, and the statistical score of each network. A function analysis which relates genes to known biological functions and physiological processes further confirmed the effects of MOS on protein synthesis (Figure 2), where as many as 77 related genes were differentially expressed in the jejunum (Supplemental Table 2; available in the online version of this paper at ps.fass.org) due to MOS supplementation. Results also showed that infectious disease and infectious mechanisms are among the biological functions that were associated with MOS-modulated genes in the intestine (Figure 2).

Six canonical pathways comprising oxidative phosphorylation, NFR2-mediated oxidative stress response, ephrin receptor signaling, maturity onset diabetes of young signaling, mitochondrial dysfunction, and clathrin-mediated endocytosis signaling were significantly altered in their overall gene expression profiles in response to MOS (Figure 3). Fifteen genes involved in oxidative phosphorylation and upregulated by MOS are listed in Table 5.

RT-qPCR Confirmation of Differentially Expressed Genes

Eleven genes in the categories of immune and defense response, cellular stress response, mitochondrial function and energy production were selected for RT-qPCR confirmation of microarray results. Lysozyme (*LYZ*), apolipoprotein A-I (*APOA1*), lumican (*LUM*), fibro-

Table 3. Effects of dietary treatments on the performance of young broilers (age 1 to 21 d)

Diet	Weight gain, ¹ g/bird	Feed intake, ¹ g/bird	Gain to feed, ¹ g/g
Control (wheat-soybean meal-based)	746.2	1,062.2	0.704
Control + MOS ²	777.6	1,072.4	0.726
SEM	11.8	23.7	0.011
<i>P</i> -value	0.08	0.77	0.18

¹Values presented are means from 6 groups of 10 birds.

²MOS = mannan-oligosaccharide.

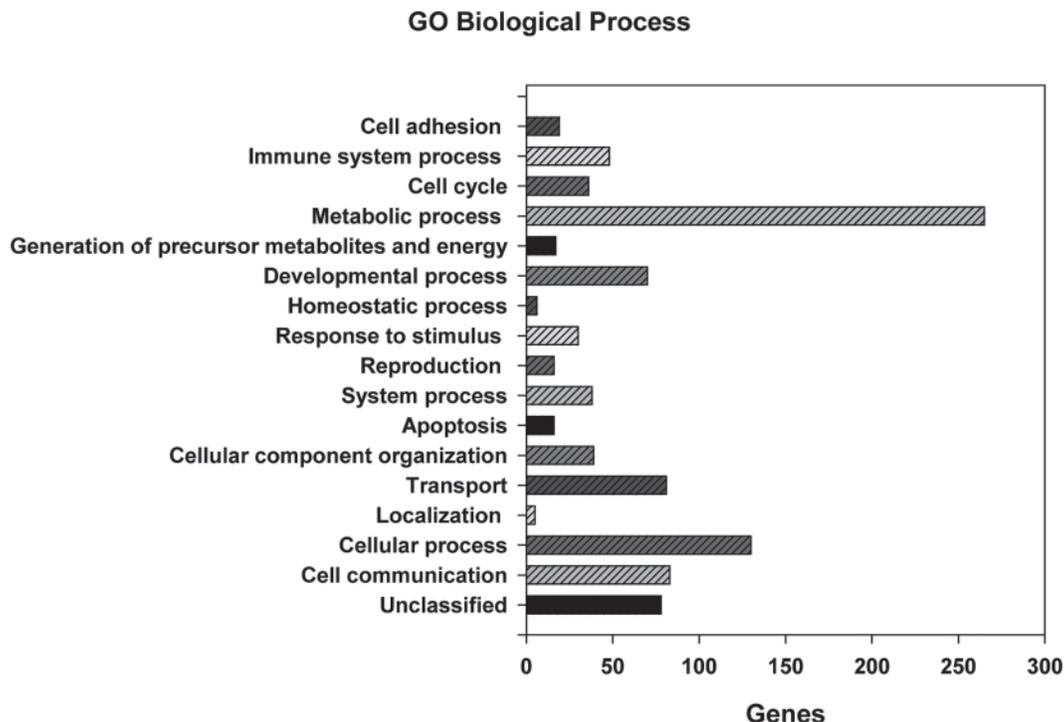


Figure 1. Gene ontology (GO) analysis of differentially expressed genes. The 672 genes significantly regulated by mannan-oligosaccharide (MOS) in broiler chicken diets were classified based on the GO terms using the PANTHER classification system (<http://www.pantherdb.org>) whereby genes were grouped into categories based on common biological properties.

nectin 1 (*FN1*), and collagen, type IV, $\alpha 2$ (*COL4A2*) are genes functionally related with cellular immune processes. As shown in Figure 4, expression of *LYZ*, *APOA1*, and *LUM* were significantly upregulated by MOS, whereas expression of *FN1* and *COL4A2* was reduced compared with control. Superoxide dismutase 1 (*SOD1*), peroxiredoxin 1 (*PRDX1*), and thioredoxin (*TXN*) are all involved in cell oxidative stress response, as defined by Ingenuity Pathway Analysis, and their upregulation in jejunum in response to MOS was also confirmed (Figure 4). ATP synthase, H⁺ transporting, mitochondrial F0 complex, subunit C1 (*ATP5G1*), cytochrome c oxidase subunit VIIc (*COX7C*), and NADH dehydrogenase flavoprotein 3 (*NDUFV3*) are key components of the mitochondrial respiratory chain and involved in ATP production. Increased expression of these genes in jejunum was also confirmed by RT-qPCR analysis (Figure 4).

DISCUSSION

The central role of improved gut functions due to MOS is quite obvious, as dietary supplementation with MOS has been frequently linked to improved gastrointestinal health (Baurhoo et al., 2007b), better feed efficiency (Hooge, 2004), and enhanced mucosal immunity (Shashidhara and Devegowda, 2003; Gómez-Verduzco et al., 2009). However, the molecular mechanisms underlying these effects are still not clear. The present study used microarray analysis to investigate the intestinal transcriptional changes after feeding young broil-

ers with a wheat-SBM diet supplemented with MOS and used pathway analysis to reveal possible pathways that are associated with its biological activities.

Gene ontology analysis of differentially regulated genes indicated that a wide range of biological themes may be associated with the effects of MOS. Several biological functions and signaling pathways stood out because of their statistical significance, the large number of genes involved, and their known biological relevance. For example, protein synthesis is one of the fundamental biological processes that was significantly overrepresented in genes differentially regulated by MOS. Proper regulation of protein synthesis is critical for cell growth, cell proliferation, and cell death. A direct link between modulation of protein synthesis by MOS and its nutritional role, however, is hard to define because of the wide range of potential impacts that may be affected.

In addition to its functions in digestion, nutrient transport, and endocrine and paracrine hormone production, the intestine represents the largest immune compartment of the body and is responsible for the prevention and control of mucosal infections and regulation of microbial colonization (Zeissig et al., 2007). The immunomodulating effects of yeast cell wall polysaccharides is popularly used to explain many of the benefits related to MOS (Kogan and Kocherm, 2007), such as the ability to stimulate cytokine production of macrophages (Majtán et al., 2005). In our study, analysis indicated that the expression of multiple genes involved in immune processes was changed by MOS in the jejunum.

Table 4. Signaling pathway networks that are involved with mannan-oligosaccharide (MOS)-regulated genes¹

ID	Molecules in network ^{2,3}	Score ²	Focus genes ²	Top functions ²
1	AMH, APOA1, BTG1, BTG2, Caveolin, DUSP10, EDEM2, EEF1D, EFNB1, FABP1, GAPDH, GLRX, HINFP, HNF4α dimer, HPCAL1, Jnk, NPAT, PDCD6, PRDX1, PTRF, RBM22, Rnr, RPL29, RPL36, RPS11, RPS16, RPS23, RPS15A, RPS3A, RTCD1, S100A6, TIMM17A, Trypsin, TXN, VDAC2	48	30	Protein synthesis, cell cycle, carbohydrate metabolism
2	Cbp/p300, CETN2, CKB, CLIC4, Creatine Kinase, Dynamin, EIF4G3, EIF5B, ERK1/2, G3BP1, GGPS1, HABP2, HAT1, HSBP1, Importin α, Importin β, MGAT3, MRPL23, MRPL53, MTA1, NUP43, NUP98, NUP160, NUP188, NUP214, PACSIN2, PHLPP1, RAN, SH3GL1, SH3GL3, Smad2/3, Tap, WASL, XPO7, XPO7	40	27	Molecular transport, protein trafficking, RNA trafficking
3	Alpha Actinin, BCCIP, CALD1, Calpain, CAPZA2, Collagen type I, DUSP6, FGF19, FGFR2, Filamin, growth factor receptor, Integrin, IPO13, IRAK4, MAGOH, MAP1A, Mapk, NOB1, PDE5A, PEPCK, PFKL, PTPRG, PXN, RG9MTD1, RPL5, RPL11, RPL15, RPL19, RPL23, RPS7, RPS15, RPS25, TLN1, Tropomyosin, UPF2	38	26	Protein synthesis, RNA posttranscriptional modification, cell cycle
4	Adaptor protein 2, adenosine-tetraphosphatase, AP1B1, AP2B1, ATP1A1, ATP5C1, ATP5G1, ATP5G3, ATP5H, ATP6V0A1, ATP6V0D1, ATP6V1G1, ATXN10, Bvr, CD74, Ctpb, EPCAM, ETV6, FLII, H⁺-transporting two-sector ATPase, MACF1, MKKS, MXD1, NFkB (complex), NUMB, peptidase, PHF12, Pias, SAP18, SDAD1, Sin3, SIN3A, TCIRG1, TGIF1, Vacuolar H⁺ ATPase	36	25	Energy production, nucleic acid metabolism, small molecule biochemistry
5	20s proteasome, 26s Proteasome, ACTR6, ARRD1, BCR, Calcineurin protein(s), CALCOCO2, CAPN1, DNAJA3, DNAJB12, FAM192A, Glutathione peroxidase, GPX7, Hsp70, Hsp90, Hsp22/Hsp40/Hsp90, MPG, NCS1, NFkB (family), NSMCE1, NSMCE2, NSMCE4A, POMP, PRNP, PSMA1, PSMA6, PSMD5, Ras, REXO2, SERTAD2, SOD1, SOD2, TMEM131, Ubiquitin, WWP2	34	24	Free radical scavenging, small molecule biochemistry, cell death

¹In total, 672 genes differentially regulated by MOS were applied for Core analysis, which interprets the data set in the context of biological processes, pathways, and molecular networks based on Ingenuity knowledge-base genes.

²Network score was based on a *P*-value calculation, which calculates the likelihood that the network-eligible molecules that are part of a network are found therein by random chance.

³Bolded genes are focus molecules that were changed by MOS and mapped to global molecular networks. The top 3 functions related to each network are listed.

For example, APOA1 is a major protein component of high-density lipoprotein in plasma. Besides its role in lipid metabolism, APOA1 may bind to lipopolysaccharide (endotoxin) which is a large molecule found in the outer membrane of gram-negative bacteria and neutralizes its toxicity (Ma et al., 2004). Increased expression of this gene (Figure 4), if it is reflected also on the protein level, may be beneficial in protecting animals from the toxicity caused by pathogenic bacteria in the gut. Similarly, increased expression of LUM (Figure 4), a gene that encodes a leucine-rich extracellular matrix glycoprotein, can promote the intestinal homeostasis by aiding innate immune and inflammatory responses in mice (Lohr et al., 2012). Lum^{-/-} mice were found hyporesponsive to lipopolysaccharide-induced septic shock, with poor induction of pro-inflammatory cytokines, TNF α , and interleukins 1 β and 6 in the serum (Wu et al., 2007). In the present study, MOS significantly upregulated the transcription of *LYZ* which is involved in mucosal immunity. Lysozyme may act as a first defense against bacteria through its hydrolase activity, which breaks down β -1,4 linkages in the peptidoglycan layer of bacterial cell walls, or through a catalytically-independent antimicrobial function (Ibrahim, 1998; Masschalck and Michiels, 2003). Bacteria

are either destroyed directly or are opsonized, enabling destruction through phagocytosis by phagocytes such as macrophages. Increased expression of these genes as well as other immune-enhancing proteins may at least partly explain why MOS is able to contribute similar effects in reducing intestinal population of pathogenic bacteria as growth-promoting antibiotics (Fairchild et al., 2001; Baurhoo et al., 2007a).

Another important observation of this study was the significant association of MOS with several mitochondrial pathways, including oxidative phosphorylation, oxidative stress response, and mitochondrial dysfunction (Figure 3). Oxidative phosphorylation is a pathway that uses energy released by the oxidation of nutrients to produce ATP. In this study, the expression of 15 genes that play key roles in the mitochondrial electron transport chain and ATP synthesis were increased by MOS (Table 5). Increased expression of these genes suggests that the energy production in the gut cells was increased in the birds fed with MOS. On the other hand, mitochondria play a central role in cellular oxidative stress, which is produced when the balance is disturbed between the production of reactive oxygen and the detoxification of reactive intermediates. Reactive intermediates such as peroxides and free radicals

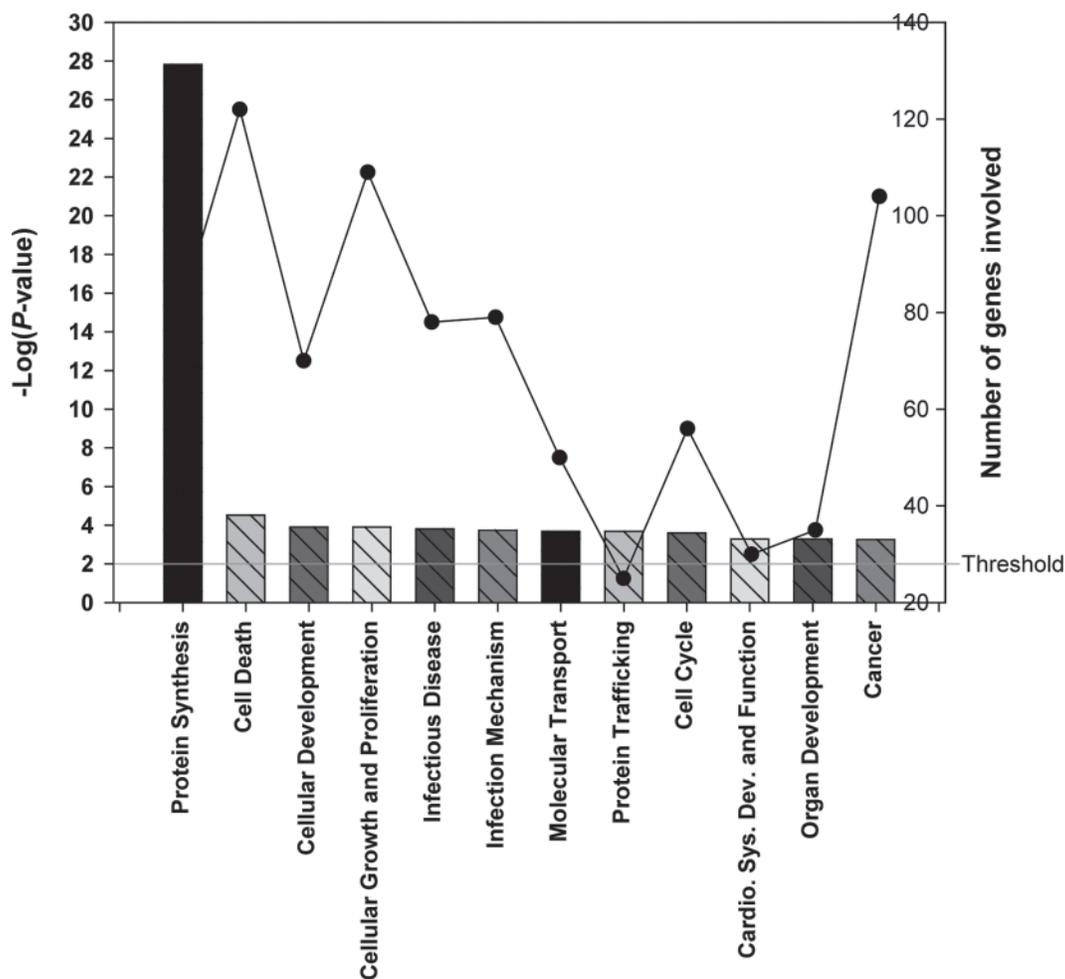


Figure 2. Top biological functions associated with mannan-oligosaccharide (MOS)-regulated genes in the gut. The significance of the biological functions to MOS-regulated genes was calculated based on Fischer's exact test and shown as bars of $-\log(P\text{-value})$. The values on the right axis are the number of regulated genes related to biological functions (line-connected dots). Only the top biofunctions are shown.

can cause injury to many cell components, including proteins, lipids, and DNA (Wei, 1998; Milei et al., 2007; Ren, 2007). The cellular defense response to oxidative stress includes induction of detoxifying enzymes and antioxidant enzymes. The current study indicates that

the intestinal expression of antioxidant genes, including *PRDX1*, *SOD1*, and *TXN*, was significantly induced in chicks fed with MOS, which suggests dietary addition of MOS improved the antioxidant status of broiler chickens.

Table 5. Select gene expression changes mediated by mannan-oligosaccharide supplementation¹

Gene title	Gene symbol	Fold change	Affymetrix ID
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1	<i>ATP5C1</i>	1.275	Gga.3279.2.S1_at
ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)	<i>ATP5G1</i>	1.488	Gga.1254.1.S1_a_at
ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)	<i>ATP5G3</i>	1.356	Gga.9188.1.S1_a_at
ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit d	<i>ATP5H</i>	1.296	Gga.2216.1.S1_a_at
ATPase, H ⁺ transporting, lysosomal 13 kDa, V1 subunit G1	<i>ATP6V1G1</i>	1.257	Gga.4824.1.S1_at
COX17 cytochrome c oxidase assembly homolog	<i>COX17</i>	1.635	Gga.6185.1.S1_a_at
Cytochrome c oxidase subunit VIc	<i>COX6C</i>	1.316	Gga.5304.1.S1_a_at
Cytochrome c oxidase subunit VIIc	<i>COX7C</i>	1.341	Gga.6171.1.S1_a_at
NADH dehydrogenase (ubiquinone) 1 α subcomplex, 1,	<i>NDUFA1</i>	1.213	Gga.5918.1.A1_a_at
NADH dehydrogenase (ubiquinone) 1 β subcomplex, 9,	<i>NDUFB9</i>	1.329	Gga.Affx.24938.1.S1_at
NADH dehydrogenase (ubiquinone) flavoprotein 3, 10 kDa	<i>NDUFV3</i>	1.293	Gga.5326.1.S1_at
Succinate dehydrogenase complex, subunit B, iron sulfur (Baurhoo et al.)	<i>SDHB</i>	1.22	Gga.4743.1.S1_at
Ubiquinol-cytochrome c reductase, complex III subunit X	<i>UQCRC10</i>	1.319	Gga.4600.1.S1_at
Ubiquinol-cytochrome c reductase core protein I	<i>UQCRC1</i>	1.24	Gga.4338.1.S1_at
Ubiquinol-cytochrome c reductase hinge protein-like	<i>UQCRL</i>	1.399	Gga.4648.2.S1_a_at

¹Transcripts regulated ($P < 0.01$, FC > 1.2, compared with control) by mannan-oligosaccharide and related to oxidative phosphorylation were included in this table.

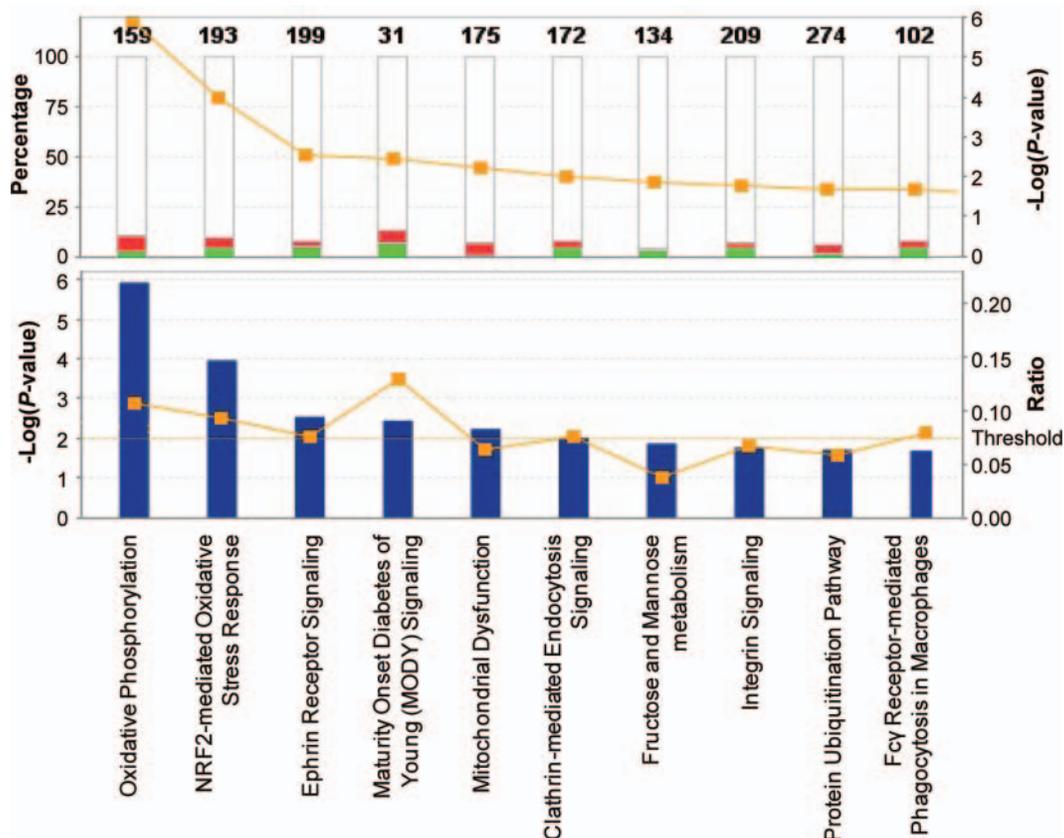


Figure 3. Significant canonical pathways associated to mannan-oligosaccharide (MOS)-regulated genes. The percentages of regulated genes (green = downregulation, red = upregulation, white = no change or overlap with the data set) in MOS-associated pathways are shown in the top panel, where line-connected dots indicate their significances (Fisher's exact test). Numbers at the top of each column are the total number of genes involved in each of the signaling pathways. The bar graph in the bottom panel shows the significance of each MOS-associated pathway; line-connected dots represent the ratios of differentially expressed genes in total number of genes that make up that pathway.

Mechanistically, altered mitochondrial activity may have a critical effect on animal performance, resulting in changes in metabolic balance and nutrient use efficiency (Elsasser et al., 2008). A direct correlation between high mitochondrial function and better feed efficiency has been reported in chickens (Bottje et al., 2002; Iqbal et al., 2005; Bottje et al., 2006; Bottje and Carstens, 2009). Likewise, upregulation of genes participating in mitochondrial electron transport and ATP synthesis may exhibit the improved feed efficiency observed in MOS-fed birds (Hooge, 2004; Rosen, 2007). In the current study, MOS-fed birds showed an improvement of approximately 3% on feed efficiency (gain-to-feed ratio) when compared with control. The difference is not statistically significant ($P = 0.18$) probably due to a relative small sample size and variations. A challenge-like condition (e.g., detrimental effects related with high contents of soluble nonstarch polysaccharides in wheat) in the current experiment may have contributed to increased variation.

In addition to pathways that directly relate to mitochondrial activities, clathrin-mediated endocytosis signaling may be another interesting pathway important to the effects of MOS (Figure 3). This pathway plays an important role in regulating the internalization of nutrients, pathogens, hormones, and other signaling

molecules from the plasma membrane into intracellular compartments (Yang et al., 1999). Hence, the regulation of this signaling cascade may have a critical effect on the gut functions. Further studies are required to investigate the effects of MOS on this pathway.

In conclusion, this study investigated for the first time the genome-wide transcriptional changes in broiler intestine after feeding with MOS-supplemented diets. We confirmed the immunomodulating activity of MOS at the transcriptional level and identified protein synthesis as the fundamental molecular biological function that was regulated by MOS. This study also showed that signaling pathways, especially pathways related to mitochondrial functions such as oxidative phosphorylation, NRF2-mediated oxidative stress response, and mitochondrial dysfunction, may be central processes in the overall functions of MOS. This study may be used as a foundation for other studies investigating MOS in chickens.

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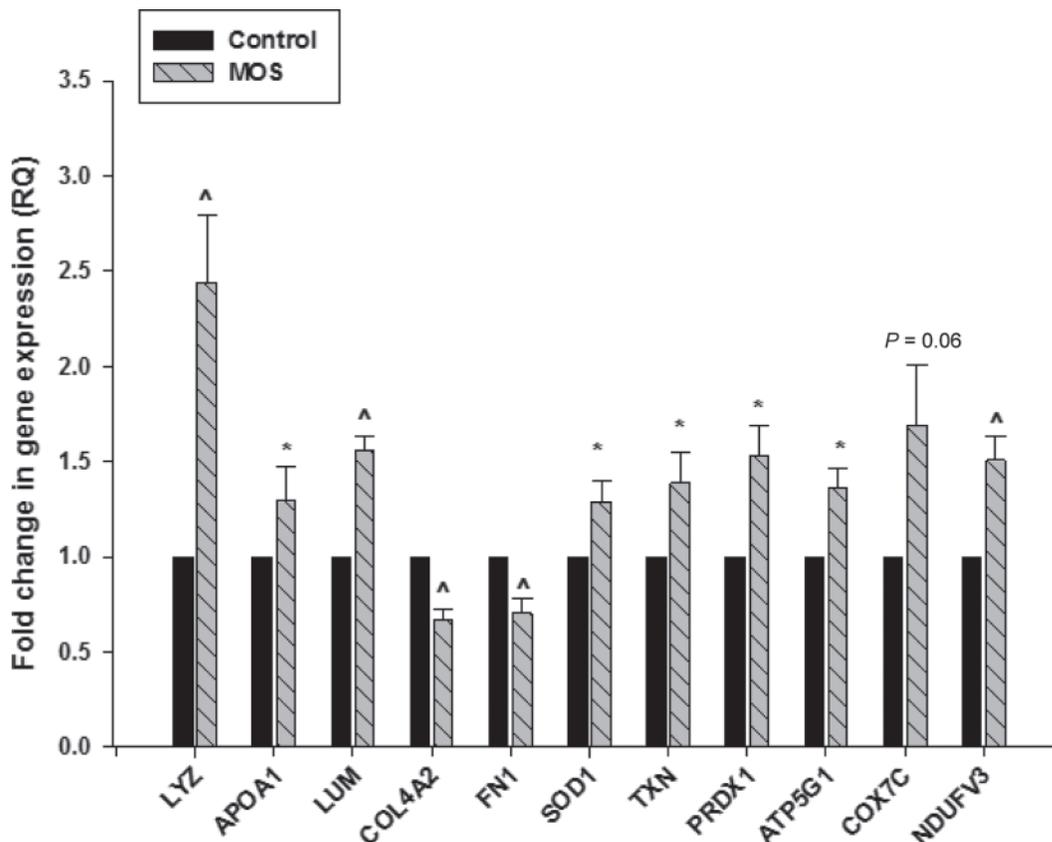


Figure 4. Real-time quantitative PCR validation of selected genes differentially expressed due to mannan-oligosaccharide (MOS). The RNA used for RT-qPCR was from the same birds as those used for microarray analysis. Selected genes were *LYZ* = lysozyme; *APOA1* = apolipoprotein A-I; *LUM* = lumican; *FN1* = fibronectin 1; *COL4A2* = collagen, type IV, α 2; *SOD1* = superoxide dismutase 1; *PRDX1* = peroxiredoxin 1; *TXN* = thioredoxin; *ATP5G1* = ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit C1; *COX7C* = cytochrome c oxidase subunit VIIc; *NDUFV3* = NADH dehydrogenase flavoprotein 3. Values represented are least squares means \pm SEM ($n = 7$). * $P \leq 0.05$ and ^ $P \leq 0.01$ relative to the control.

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