# The effect of in ovo administration of mannan oligosaccharide on small intestine development during the pre- and posthatch periods in chickens

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**ABSTRACT** Early intestinal development is essential for chicken embryos to fulfill their maximal growth potential. Mannan oligosaccharide (MOS) is known to improve gut morphology, function, and innate immunity; therefore, we hypothesized that its administration in the prehatch period to the sterile intestine of embryos would affect intestinal development and functionality without mediation of gut microflora. The MOS was administered by in ovo feeding procedure to embryos 3 d before hatch. the effects of MOS administration on intestinal morphology, activity of the brush-border enzymes amino peptidase (AP) and sucrase isomaltase (SI) and mRNA abundance of *AP*, *SI*, sodium-dependent glucose cotransporter 1 (*SGLT1*), peptide transporter 1 (*PepT1*), secreted mucin (*MUC2*), and toll-like receptors (*TLR2* and *TLR4*) were examined and compared with saline-injected and noninjected controls. Results show that on embryonic d 20 the only parameter affected was *MUC2* mRNA abundance, which exhibited a 3-fold increase in the MOS group versus controls. On day of hatch more parameters were affected: a 20 to 32% increase in villus area was found in the MOS group compared with controls; crypt depth and number of goblet cells per villus were higher by 20 and 50%, respectively, compared with the saline group; and AP and SI activities were higher by 44 and 36%, respectively, compared with the noninjected control. In addition, an increase in fold change mRNA abundance of *AP*, *SI*, and *TLR4* was observed in the MOS group compared with controls. However, on d 3 posthatch, a decrease in MOS effects was noted, indicating a temporally limited effect after administration of 1 dose. In ovo administration of MOS prehatch resulted in a hatching chick with more mature enterocytes and enhanced epithelial barrier and digestive and absorptive capacity at day of hatch. Results imply that the mechanism underlying the observed changes is not mediated through gut microflora but rather involves a direct effect of MOS on intestinal cells.

**Key words:** embryo, intestinal gene expression, mannan oligosaccharide, in ovo feeding, chicken

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## INTRODUCTION

The small intestine of the chicken goes through morphological, cellular, and molecular changes during the pre- and posthatch periods to accommodate the rapid transition in the prehatch embryo to external nutrient sources (Uni, 2006). Starting on embryonic day (**E**) 15, dramatic changes in intestinal relative weight, villi morphology, and expression and activity of brush-border (**BB**) enzymes and transporters prepare the embryo for exogenous feed ingestion (Uni et al., 2003). Intestinal development and maturation are known to play a major role in promoting growth of the newly hatched chick and to have a long-term effect on broiler performance (Uni et al., 1999; Sklan, 2001; Dibner and Richards, 2005). Nutrient digestion, absorption, assimilation, and incorporation into developing and growing tissues depend directly on the functional capabilities of the intestine and, therefore, any improvement in early intestinal maturation and digestive capacity has great importance.

Mannan oligosaccharide (**MOS**) is a mannan-based carbohydrate extracted from the outer cell wall of the yeast *Saccharomyces cerevisiae* (Spring et al., 2000). Several studies have demonstrated the benefits of adding MOS to broiler diets, such as improved gut morphology in features such as villus length and villus area (Iji et al., 2001; Baurhoo et al., 2007b; Solis de los Santos et al., 2007), growth performance characteristics such as BW, feed conversion rate, and apparent ME (Hooge, 2004; Rosen, 2007; Yang et al., 2007a,b,c). Adding MOS to the poultry diet also exhibited beneficial changes in mucin secretion and in number of


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goblet cells per villus (Baurhoo et al., 2007a,b; Solis de los Santos et al., 2007), in digestibility and BB enzyme activity (Yang et al., 2007a,b), and in gut immune responses (Newman, 1994; Kocher et al., 2004; Baurhoo et al., 2007b). Furthermore, MOS has been shown to alter the gut microflora (Fernandez et al., 2000; Baurhoo et al., 2007a) by reducing the number of pathogenic bacteria that colonize the gastrointestinal tract (Spring et al., 2000; Fernandez et al., 2002).

Although MOS effects are broadly studied, the specific mode of action underlying the beneficial effects of MOS remains unclear. It is suggested to involve several bacterium-related mechanisms, including pathogen exclusion through competitive binding to the mannose-specific type 1 fimbriae of certain pathogens such as *Escherichia coli* and *Campylobacter*, thereby altering the gut microflora (Spring et al., 2000; Baurhoo et al., 2007a; Yang et al., 2008a). Recently, other mechanisms have been suggested for the effects of indigestible oligosaccharides, including direct interaction of the oligosaccharides with carbohydrate receptors on intestinal epithelial cells and immune cells, and partial absorption of the oligosaccharides (Seifert and Watzl, 2007).

Based on these suggested mechanisms, we set out to test the hypothesis that administration of MOS before hatch into the sterile intestine of the chicken embryo (Yegani and Korver, 2008) would affect intestinal development and functionality without the mediation of gut microflora. Accordingly, MOS (Alltech Inc., Nicholasville, KY) was provided to broiler embryos using an in ovo feeding technique (Uni and Ferket, 2004). This technique enriches amniotic fluid with nutrients and therefore enables direct contact of the administrated substance with the small intestine and enterocytes following amnion fluid swallowing by the embryo. The effects of the administered MOS were compared with 2 control groups: saline (NaCl)-injected and noninjected broiler embryos. Effects on intestinal development and function were determined by examining gut morphology, activity of the digestive enzymes amino peptidase (**AP**) and sucrase isomaltase (**SI**), and mRNA abundance involved in BB digestion and absorption processes [sodium-dependent glucose cotransporter 1 (***SGLT1***), peptide transporter 1 (***PepT1***), AP, and SI, in addition to mRNA abundance of secreted mucin (mucin 2; ***MUC2***) and the toll-like receptors (**TLR**) *TLR2* and *TLR4*].

## MATERIALS AND METHODS

### Incubation Procedures, In Ovo Administration of MOS, and Growth

Fertile eggs (Cobb 500) were obtained from a maternal flock in a commercial hatchery (Brown Ltd., Hod-Hasharon, Israel) that was 41 wk in lay. The eggs were incubated under standard conditions at the Department of Animal Sciences of the Hebrew University (Rehovot). At E17, 300 eggs containing viable embryos were divided into 3 groups of 100 eggs each. Two groups were injected, using a 21-gauge needle, with sterile solutions (0.6 mL/egg) by in ovo feeding procedure (Uni and Ferket, 2004). The first group was injected with 0.1% (wt/vol) MOS (Alltech Inc.) in 0.9% (vol/vol) NaCl (referred to as the MOS group), and the second group was injected with 0.9% NaCl (referred to as the NaCl group). The third group was not injected (**NI**) and served as a negative control. Eggs were sprayed with 75% ethanol before and after the in ovo procedure and were placed in hatching trays. Hatchability (90–91%) was equal for all groups. After hatch, chicks were placed in the hatchery. Birds were kept in pens (45 birds/pen) for the 3-d trial period. Posthatch feed, which was formulated to meet National Research Council (1994) requirements as appropriate, was offered ad libitum, and water was freely available at all times during the trial period. Mortality was recorded as it occurred (negligible; data not shown). All experimental procedures followed established guidelines for animal care and handling and were approved by the Hebrew University Institutional Animal Care and Use Committee

### Tissue Sampling

Embryos or chicks from each group (MOS, NaCl, and NI) were killed on E18, E19, E20, day of hatch (**DOH**; within 2 h of clearing the shell), and d 3 posthatch. The small intestine was removed from each animal (excluding the duodenal section) and 2-cm segments were placed into 2 separate tubes: ileal segments (n = 8) were frozen in liquid nitrogen and stored at −80°C for mRNA abundance analysis and activity determination of 2 BB enzymes AP and SI, and jejunal segments (n = 4) were fixed in 4% (vol/vol) neutral-buffered formalin solution for histology (on DOH only). At hatch, cecal contents were collected from 8 embryos to determine bacterial presence via detection of bacterial genomic DNA.

### Morphological Examination

Intestinal segments from DOH (4 from each study group) were fixed in 4% (vol/vol) buffered formaldehyde, dehydrated, cleared, and embedded in paraffin. Serial sections were then cut at 2 μm, deparaffinized in xylene, dehydrated, and stained with hematoxylin and eosin. Ten villi and crypts were measured in each chicken from each group. The gastrointestinal morphological variables evaluated were villus height, villus surface area, intestinal muscle thickness, and crypt depth. The measurements from each animal were then averaged to estimate a mean value for each variable per treatment (n = 40). The villus height was measured from the top of the villus to the top of the lamina propria. Villus surface area was calculated by the formula $2\pi \times VH \times (VW/2)$, where VH is villus height and VW is villus width (Uni et al., 1999). Intestinal muscle thickness

was measured in the space between the base of the crypt and the edge of the muscle tissue. Crypt depth was measured from the base upward to the transition region between the crypt and the villus. Neutral goblet cells were detected by staining 2-μm sections with periodic acid-Schiff stain (**PAS**) as described previously (Smirnov et al., 2005). Following deparaffinization and dehydration, slides were incubated in 0.5% periodic acid for 5 min, washed and incubated with Schiff's reagent (Sigma Chemical Co., St. Louis, MO) for 20 min, and counterstained with hematoxylin for 5 min. The number of PAS-positive cells staining a red color along the villi was counted by light microscopy. All slides were analyzed under an Olympus Optical BX40F-3 microscope (Olympus, Center Valley, PA). Evaluation of morphological measurements was performed using Cell$^{B}$ 2.4 Olympus Biosystems software.

## BB Enzyme Activity

Enzyme activities were assayed in homogenized ileum tissue (100 mg of tissue/0.5 mL of water) using Ultraturrax T-25 (IKA, Staufen, Germany). The SI (EC 3.2.20) activity was assayed colorimetrically using maltose as a substrate (45 min incubation at 37°C; Dahlqvist, 1964; Palo et al., 1995; Uni et al., 1999) and was defined spectrophotometrically at 492 nm according to the manufacturer's instructions (kit no. TR15103; Thermo Electron, Louisville, CO). The AP (EC 3.4.11) activity was assayed by hydrolysis of L-leucine-p-nitroanilide for 15 min at 37°C. p-Nitroanilide was determined spectrophotometrically at 405 nm (Benajiba and Maroux, 1980; Uni et al., 1999) and calculated according to the manufacturer's instructions (Randox LAP kit, Spinreact S.A, Girona, Spain). Total protein was determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) for protein concentration following detergent solubilization (Bradford, 1976).

## Total RNA Extraction and cDNA Synthesis

Total RNA was isolated (Chomczynski and Sacchi, 1987) from the intestinal segments using TRI reagent (10 mL/g of tissue) according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Quantification of RNA was done spectrophotometrically (NanoDrop ND-1000 spectrophotometer, NanoDrop, Wilmington, DE). Reverse transcription was carried out using the EZ-First Strand cDNA Synthesis Kit for RT-PCR according to the manufacturer's protocol (Biological Industries, Beit Haemek, Israel). The reaction was performed using RNA at 1 μg/μL at 70°C for 10 min followed by 60 min at 42°C and 15 min at 70°C.

## Bacterial Genomic DNA Extraction

Bacterial genomic DNA was isolated from the cecal content using the Wizard Genomic DNA Purification Kit according to the manufacturer's protocol (Promega, Madison, WI). Bacterial genomic DNA presence and concentration were checked spectrophotometrically.

## Real-Time PCR

To assess the relative mRNA abundance of *AP*, *SI*, *SGLT1*, *PepT1*, *MUC2*, *TLR2*, and *TLR4* the genes were normalized on E20, DOH, and d 3 against an endogenous reference gene (glyceraldehyde 3-phosphate dehydrogenase; ***GAPDH***) using the comparative threshold cycle (**Ct**) method. To validate this method, serial dilutions (0.2, 0.04, 0.008, and 0.0016) were prepared from intestine cDNA for each day tested, and the efficiencies of all of the genes and *GAPDH* were compared by plotting Ct versus log(template). Gene-specific primers were used for SYBR Green detection according to the published cDNA sequences for each of the studied genes. Primers used for the real-time PCR were either designed with the aid of Primer Express 2.0 software (PerkinElmer, Waltham, MA) [*GAPDH* (NM_204305) GAPDHc-F: GTGAAAGTCGGAGTCAACGGA, GAPDHC-R: AAGGGATCATTGATGGCCAC; *SI* (NM_204305) SI-F: GCAACAAGACAAGCCATCGA, SI-R: AGCCAGTGTCCTGTGTGCTTT; *MUC2* (XM_421035) MUC2c-F: CCTGTGCAGACCAAGCAGAAA, MUC2C-R: CCTCTGAGTTTTTCAGCAAAGAACAC] or taken from previous work [*AP*, *PepT1*, *SGLT1* (Gilbert et al., 2008), *TLR2*, *TLR4* (Higgs et al., 2006)]. The PCR mixture consisted of 3 μL of diluted cDNA sample, 200 n*M* of each primer, and 10 μL of Platinum SYBR Green qPCR super mix-UDG (Invitrogen, Carlsbad, CA) in a final volume of 20 μL. The $R^2$ value and slope were calculated by linear regression for all genes tested. Amplification was carried out in a Stratagene Mx3000p cycler (Stratagene, Amsterdam, the Netherlands) according to the manufacturer's protocol. All PCR were performed in ABgene PCR plates closed with Absolute QPCR seals (Thermo Fisher Scientific Inc., Waltham, MA) under the following conditions: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min. In addition, a melting curve was determined according to the manufacturer's instructions. Each gene was amplified independently in triplicate within a single instrument run. Results were analyzed with the MxPRO QPCR Software (Stratagene). A dissociation curve analysis was run after each real-time experiment to confirm the presence of only 1 product. To control for false positives, a nontemplate control was run for each template and primer pair. Fold change was calculated relative to the control using the relative quantification mathematical model method (Pfaffl, 2001) including the efficiencies (calculated by MxPro softwere; Stratagene) for all of the experimental genes and *GAPDH* (internal control). The mRNA abundance was determined by analyzing the resultant Ct values for each sample (gene of interest), normalized to the level of *GAPDH* mRNA abundance for the same RNA sample.

### Statistical Analysis

Treatment-dependent changes were analyzed by ANOVA. Statistical differences among means were considered significant at $P \leq 0.05$. A posthoc test (Tukey-Kramer) was performed following ANOVA. JMP version 6.0 (SAS Institute, Cary, NC) was used for all analyses. Values are presented as means ± SEM.

## RESULTS

### Morphological Observation and Cecal Microflora

Significant effects were observed on gut structure development following 1 dose of MOS administration prehatch. The effects of the treatments on the morphometric parameters are summarized in Table 1. histological assessment of the jejunum sections on DOH showed larger villus height and area after MOS administration compared with both control groups. However, in the MOS group, crypt depth and number of goblet cells per villus were significantly higher compared with only the NaCl control group, whereas intestinal muscle thickness was significantly higher compared with only the NI control group. Villus height was more uniform following MOS treatment relative to both controls, in which a nonuniform pattern was seen (Figure 1). No bacterial DNA was found in the cecal contents at hatch, when hatchlings were sampled 2 h after clearing of the shell.

### BB Enzyme Activity

An increasing trend in activity was seen with age for both enzymes (see NI control in Figures 2 and 3). The AP activity (Figure 2) on E18 (24 h after MOS administration) was significantly higher than in the NI control, whereas on E19, E20, and d 3 no significant differences were found. On DOH (4 d after MOS administration), AP activity was significantly higher than in both of the controls. The SI activity showed a different activity pattern, where on E18, MOS-treated groups and NI controls showed significantly lower levels than the NaCl control. However, on DOH, SI activity was significantly higher in the MOS and NaCl groups than in the NI control. On E19, E20, and d 3, SI activity showed no significant differences between MOS and both control groups.

### mRNA Abundance

The Ct ratios (gene:*GAPDH*) for each of the genes in each group on the days tested are shown in Table 2; lower Ct ratio values indicate a higher abundance level. On E20, 72 h after MOS administration, the fold change mRNA abundance of *MUC2* increased in the MOS group compared with the controls (Figure 4), whereas no significant differences were observed for *AP* abundance. On DOH (4 d after administration), an increase in fold change abundance of *AP*, *SI*, and *TLR4* was noted (Figures 5A, 5B, and 6A, respectively). However, the *MUC2* Ct ratio showed no significant differences between the MOS group and controls, and *TLR2* mRNA abundance was significantly lower in the MOS group than in the NaCl control but with no significant difference compared with the NI control (Figure 6B). On d 3 (7 d after MOS administration), only *AP* mRNA abundance was significantly higher in the MOS group compared with both controls (Figure 5A), whereas no significant differences were seen in SI activity (Figure 5B) and transporters on that day. The *MUC2* mRNA abundance in the MOS group was significantly lower than in the NI controls, but not significantly different from the NaCl control (Figure 4). The same results were obtained for *TLR2* mRNA abundance, which decreased on d 3 (7 d after MOS administration; Figure 6B) compared with the NI control. Although expression was detected, no differences were found in the relative mRNA abundance of the BB transporters *PepT1* and *SGLT1* on any of the days examined (Table 2).

## DISCUSSION

We found that administration of 1 dose of MOS during the prenatal period to late-term embryos, when the intestine is under intensive process of development, leads to changes in gut morphology, functionality, and innate immunity, implying early maturation of the small intestine of the chick, independently from microflora presence. Results indicated that MOS administration before hatch affects mRNA abundance of *SI*, *AP*, *MUC2*, *TLR2*, and *TLR4* in different patterns. First to

**Table 1.** Morphological measurements of villi in the different treatments on day of hatch[1]

| Treatment[2] | Villus height (μm) | Villus area (μm) | Crypt depth (μm) | Goblet cells (n/villus) | Intestine muscle thickness (μm) |
|---|---|---|---|---|---|
| MOS | 395.8 ± 12.5[a] | 84,778.3 ± 4,030.5[a] | 59.8 ± 2.9[a] | 24.6 ± 1.2[a] | 115.1 ± 2.8[a] |
| NaCl | 322.6 ± 13.9[b] | 64,178.0 ± 3,700.8[b] | 49.2 ± 3.0[b] | 16.8 ± 0.8[b] | 104.8 ± 3.2[ab] |
| NI | 322.3 ± 11.4[b] | 71,241.3 ± 3,878.5[b] | 57.7 ± 2.9[ab] | 22.2 ± 1.2[a] | 101.3 ± 4.9[b] |

[a,b]Values within a column with different letters differ significantly ($P \leq 0.05$) between treatments.

[1]Values are means ± SEM.

[2]MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

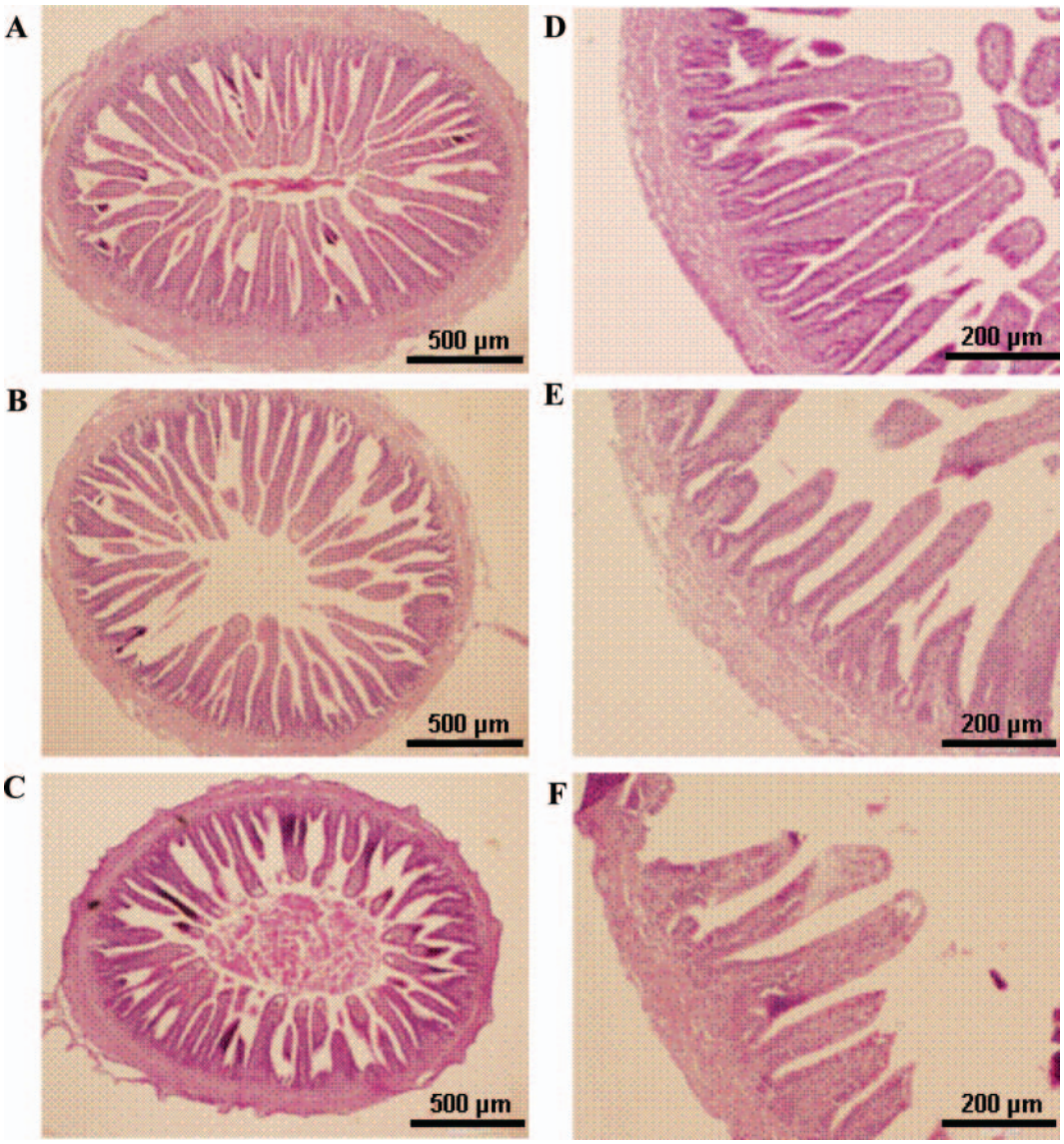


**Figure 1.** Representative light microscopic histological view of intestinal villi from the jejunum of broiler chicks on day of hatch in MOS (A, D), NaCl (B, E), and NI (C, F) treatment groups. Neutral goblet cells are stained red with periodic acid-Schiff stain. MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control). Color version available in the online PDF.

respond was the mucin-secretion system on E20 (72 h after administration), when a significant 3-fold increase in *MUC2* mRNA abundance was noted. Next to respond were the BB membrane enzyme genes, which exhibit at DOH (96 h postadministration) a 5-fold increase in *AP* mRNA and a 2-fold increase in *SI* mRNA. A response of immune-related genes in the gut was also found on DOH: *TLR4* mRNA abundance was significantly (2-fold) higher in the MOS group. Increased TLR4 and TLR2 are associated with an enhanced gastroepithelial barrier, which provides defense against pathogen invasion and infection (Chen et al., 2007). Accordingly, the MOS group exhibited enhancement of the innate line of defense against infectious organisms, which gives a clear advantage to the hatchlings on DOH.

The effect of MOS was also demonstrated on DOH in which greater villus height and area as well as intestinal muscle thickness were observed. Both controls showed a less well developed villus pattern (Figure 1) as indicated by the nonuniform villus length, a pattern compatible with a less mature intestine (Uni et al., 2003), whereas the MOS-treated group showed a more uniform villus length, indicating a more mature degree of development.

The early maturation of several BB enzymes following MOS treatment was evidenced on DOH by the 44% increase in AP biochemical activity and the 36% increase in SI activity compared with the NI group. These results reflect the actual digestive capacity of the enterocyte and indicate its higher maturation status in the MOS group.

However, the beneficial effect of 1 dose of MOS on intestinal development is temporally limited because on d 3 (7 d after MOS administration) a decrease in *MUC2*

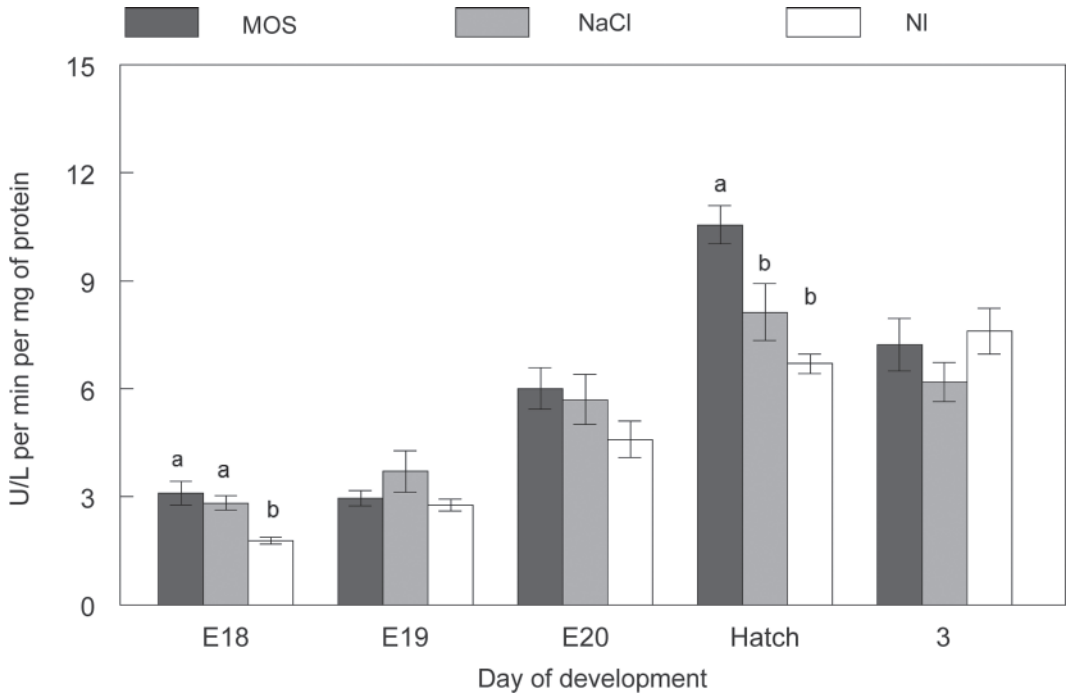


**Figure 2.** Activity of brush-border enzyme leucine amino peptidase in the small intestine of embryonic [embryonic d (E) 18, 19, and 20] and posthatch (day of hatch and 3 d of age) chicks. Values (n = 8) are means ± SEM. Columns with different letters differ significantly ($P \leq 0.05$) between treatments. MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

and *TLR2* mRNA abundance was observed in the MOS group compared with the NI group. In addition, no differences were found between the MOS group and controls in the BB enzyme *SI* on d 3 and, although *AP* mRNA abundance was still significantly higher in the MOS treatment than in the controls, it exhibited lower

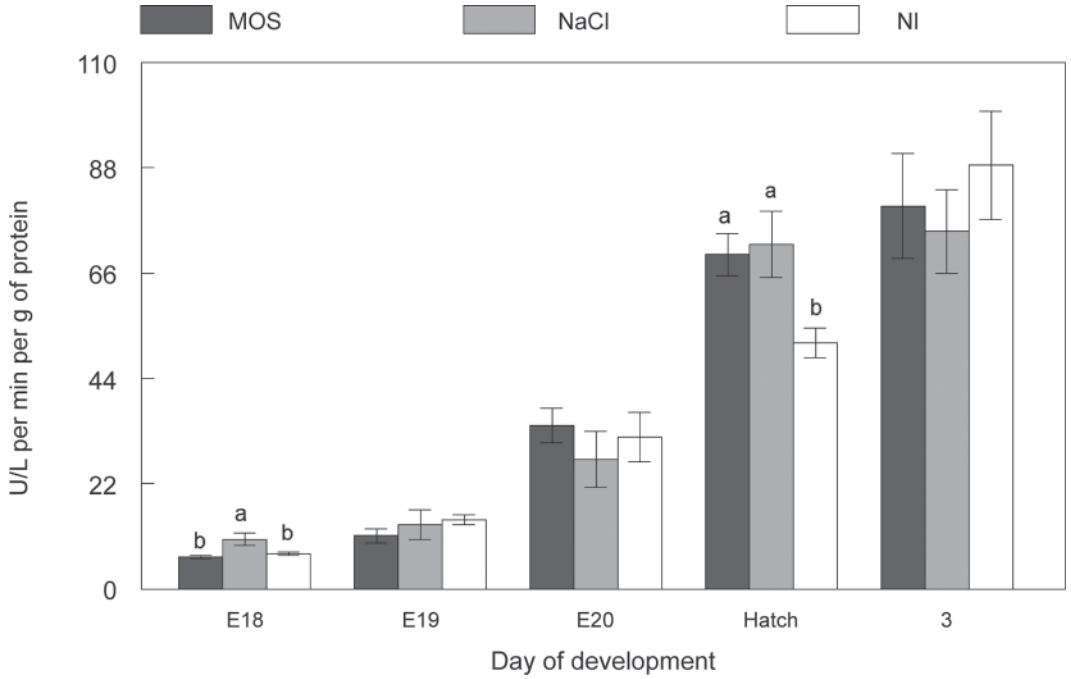


**Figure 3.** Activity of the brush-border enzyme sucrase isomaltase in the small intestine of embryonic [embryonic d (E) 18, 19, and 20] and posthatch (day of hatch and 3 d of age) chicks. Values (n = 8) are means ± SEM. Columns with different letters differ significantly ($P \leq 0.05$) between treatments. The bar for NI on E20 is the mean of E19 and day of hatch values. MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

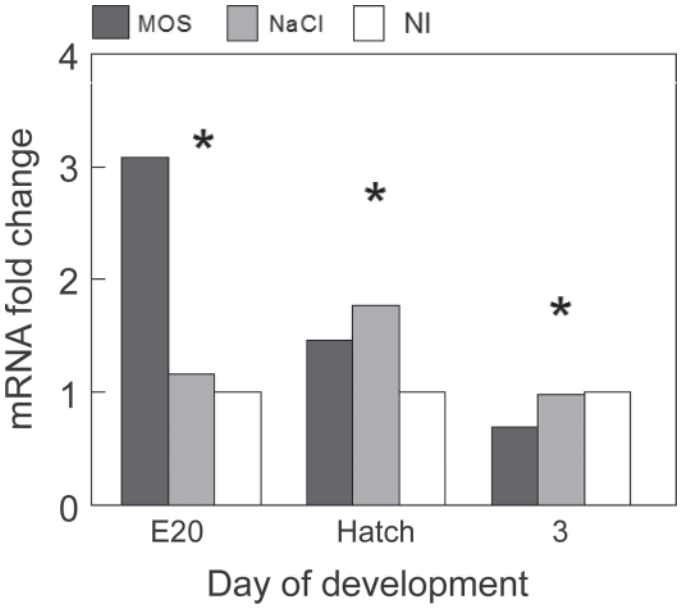


**Figure 4.** Effect of mannan oligosaccharide administration prehatch on mRNA abundance of mucin 2 (*MUC2*) in the ileum of birds at embryonic d (E) 20, day of hatch, and 3 d of age. Values (n = 8) are mean fold change. Asterisk indicates significantly different threshold cycle ratio (Table 2). MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

fold change differences than on DOH. This is probably attributable to the administration of a single MOS dose rather than a continuous administration regiment.

Taken together, these results demonstrate the positive effects of in ovo-administrated MOS on intestinal maturation and development from 24 h after in ovo administration until the first days posthatch. In this study we used a saline control group to determine whether the effects seen resulted from the medium solution (NaCl) or the MOS itself. Because saline solution comprises the ions $Na^+$ and $Cl^-$, which have many metabolic and nonmetabolic roles in the intestine and in general, it is reasonable to see significant effects after saline administration in ovo. Accordingly, the results show some effects of NaCl on gene expression of MUC2, TLR2, and TLR4 and on enzymatic activity.

The experimental model used in this research, in which we delivered the MOS supplement into the sterile intestine of the chicken embryo, enabled us to examine whether MOS has a direct effect on intestinal development in the absence of microflora. The positive effects of MOS on growth performance, gut development, and morphology are well documented (Spring et al., 2000; Baurhoo et al., 2007a,b, 2009; Solis de los Santos et al., 2007; Yang et al., 2008a,b,c); however, these effects have been attributed to changes in microbial ecology. Several modes of action have been suggested for the effects of dietary supplementation of MOS (Spring et al., 2000; Baurhoo et al., 2007a,b, 2009); all of them assume the involvement of intestinal bacteria. One suggested mode of action is pathogen exclusion by MOS: several studies have shown that MOS supplementation in chicken diets results in pathogenic exclusion from the

**Table 2.** Cycle threshold ratios (gene:*GAPDH*) for examined genes in the ileum of each treatment group on embryonic d 20 (E20), day of hatch, and 3 d of age[1]

| Gene[2] | Treatment[3] | E20 | Day of hatch | Day 3 |
|---|---|---|---|---|
| *AP* | MOS | 1.38 ± 0.031 | 1.30 ± 0.01[a] | 1.41 ± 0.01[a] |
| | NaCl | 1.40 ± 0.05 | 1.45 ± 0.01[b] | 1.50 ± 0.03[b] |
| | NI | 1.50 ± 0.03 | 1.47 ± 0.02[b] | 1.49 ± 0.01[b] |
| *SI* | MOS | 1.38 ± 0.02 | 1.21 ± 0.01[a] | 1.34 ± 0.01 |
| | NaCl | 1.40 ± 0.02 | 1.23 ± 0.01[ab] | 1.35 ± 0.02 |
| | NI | 1.42 ± 0.03 | 1.26 ± 0.02[b] | 1.32 ± 0.01 |
| *PepT1* | MOS | 1.40 ± 0.01 | 1.41 ± 0.02 | 1.39 ± 0.01 |
| | NaCl | 1.44 ± 0.03 | 1.40 ± 0.02 | 1.39 ± 0.01 |
| | NI | 1.47 ± 0.02 | 1.42 ± 0.02 | 1.37 ± 0.01 |
| *SGLT1* | MOS | 1.27 ± 0.02 | 1.23 ± 0.01 | 1.16 ± 0.01 |
| | NaCl | 1.27 ± 0.02 | 1.21 ± 0.01 | 1.15 ± 0.01 |
| | NI | 1.33 ± 0.02 | 1.22 ± 0.01 | 1.16 ± 0.02 |
| *MUC2* | MOS | 1.38 ± 0.04[a] | 1.61 ± 0.02[ab] | 1.54 ± 0.01[b] |
| | NaCl | 1.47 ± 0.03[b] | 1.56 ± 0.02[a] | 1.51 ± 0.02[ab] |
| | NI | 1.51 ± 0.02[b] | 1.64 ± 0.02[b] | 1.49 ± 0.01[a] |
| *TLR2* | MOS | 1.20 ± 0.02 | 1.47 ± 0.01[b] | 1.46 ± 0.01[b] |
| | NaCl | 1.23 ± 0.04 | 1.37 ± 0.01[a] | 1.43 ± 0.02[ab] |
| | NI | 1.28 ± 0.02 | 1.44 ± 0.01[b] | 1.40 ± 0.02[a] |
| *TLR4* | MOS | ND[4] | 1.64 ± 0.01[a] | 1.81 ± 0.01 |
| | NaCl | ND | 1.64 ± 0.01[a] | 1.80 ± 0.02 |
| | NI | ND | 1.70 ± 0.01[b] | 1.80 ± 0.02 |

[a,b]Values for the same gene on the same day with different letters differ significantly ($P \leq 0.05$) between treatments.

[1]Values (n = 8) are means ± SEM.

[2]*AP* = amino peptidase; *SI* = sucrase isomaltase; *PepT1* = peptide transporter 1; *SGLT1* = sodium-dependent glucose cotransporter 1; *MUC2* = mucin 2; *TLR2* = toll-like receptor 2; *TLR4* = toll-like receptor 4.

[3]MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

[4]ND = not detected.

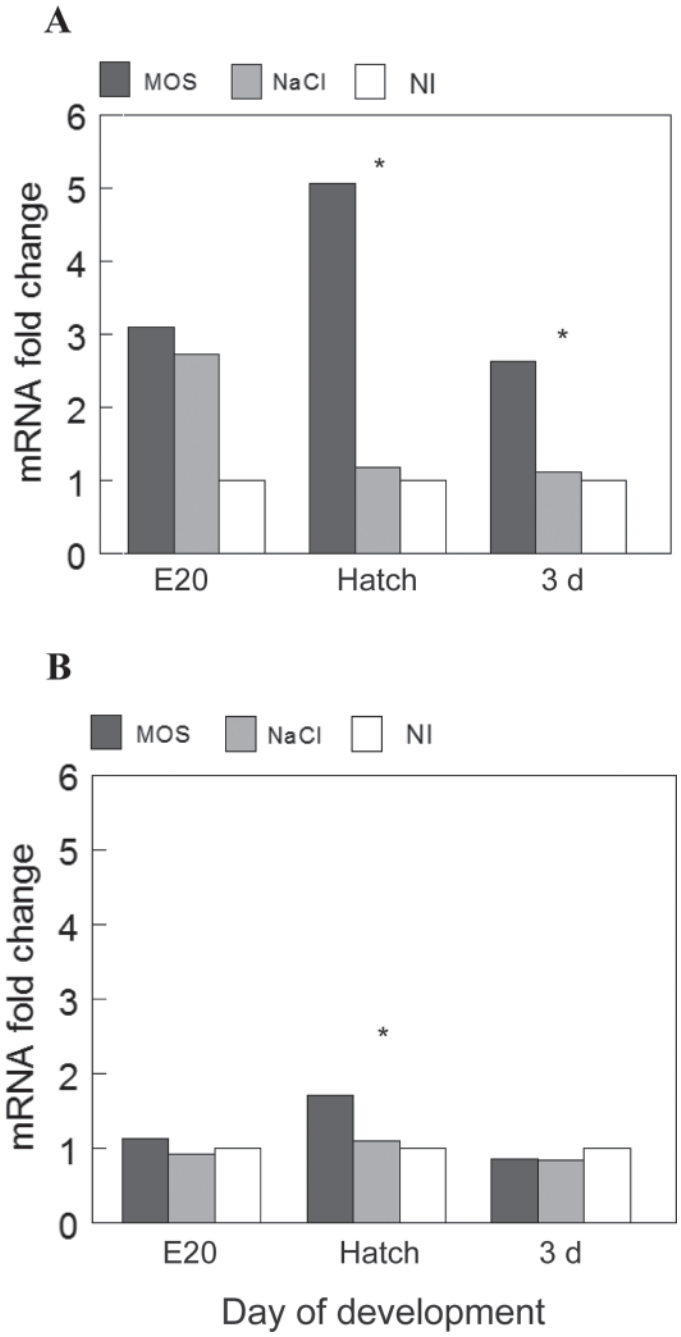


**Figure 5.** Effect of mannan oligosaccharide administration prehatch on mRNA abundance of (A) amino peptidase and (B) sucrase isomaltase in the ileum of birds at embryonic d (E) 20, day of hatch, and 3 d of age. Values (n = 8) are mean fold change. Asterisk indicates significantly different threshold cycle ratio (Table 2). MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

gut (Fernandez et al., 2000, 2002; Spring et al., 2000). Pathogen exclusion by MOS may occur through its mimicking of bacterial binding sites, such as the type-1 fimbrae, thereby blocking the oligosaccharide lectin of pathogens such that they are excluded without attachment to the host intestine; alternatively, it may occur through induction of innate immune mechanisms, including local inflammatory responses and synthesis and secretion of defensins that enhance host defense against pathogens (Kocher et al., 2004; Franklin et al., 2005; Nochta et al., 2009). An additional suggested mechanism is alteration of the gut microflora by MOS, resulting in differences in bacterial colonization and general bacterial load (Castillo et al., 2008; Yang et al., 2008c) and, in some cases, an increase in beneficial bacteria such as *Lactobacillus* and bifidobacteria, which are associated with increased villus length (Baurhoo et al.,

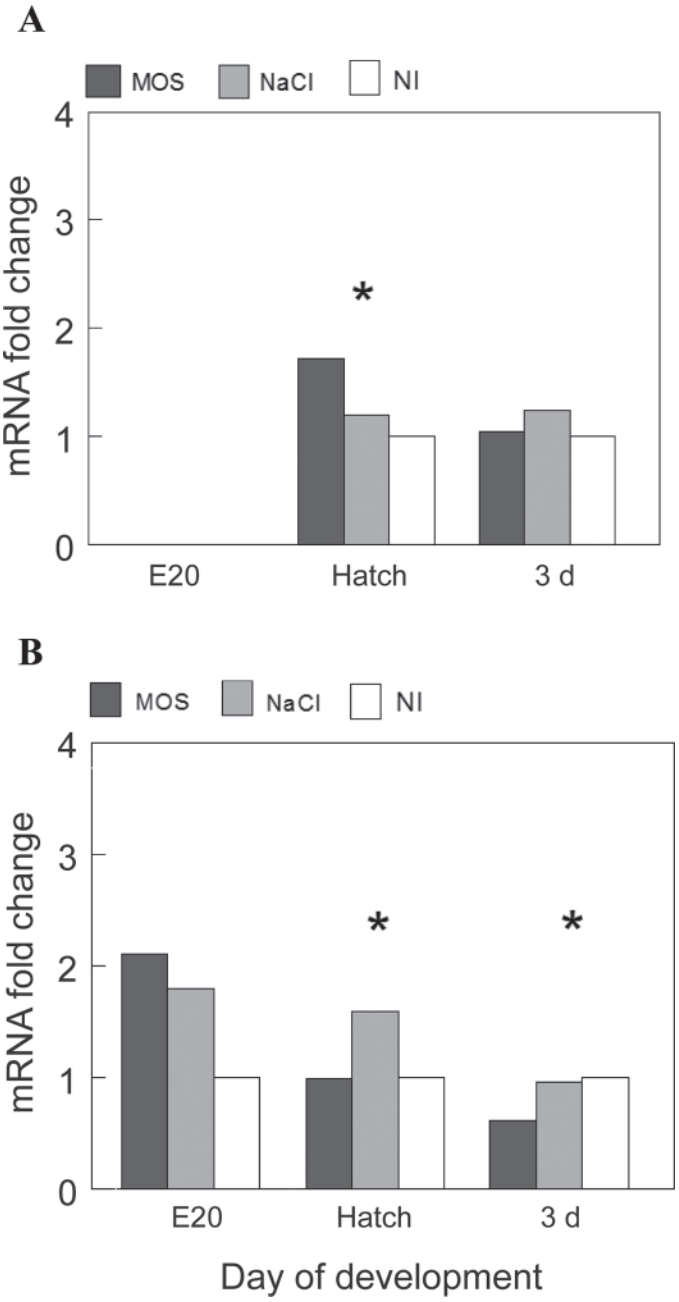


**Figure 6.** Effect of mannan oligosaccharide administration prehatch on mRNA abundance of toll-like receptors (A) *TLR4* and (B) *TLR2* in the ileum of birds at embryonic d (E) 20, day of hatch, and 3 d of age. Values (n = 8) are mean fold change. Asterisk indicates significantly different threshold cycle ratio (Table 2). MOS = injected with 0.1% (wt/vol) mannan oligosaccharide (Alltech Inc., Nicholasville, KY) in 0.9% (vol/vol) NaCl; NaCl = injected with 0.9% NaCl; NI = not injected (negative control).

2007b, 2009). Another bacterium-related MOS mechanism is competitive exclusion of pathogens. It has been suggested that an increase in the number of goblet cells following MOS supplementation (Baurhoo et al., 2007b, 2009; Solis de los Santos et al., 2007) causes the establishment of an intestinal population of lactobacilli and bifidobacteria (Baurhoo et al., 2007b, 2009), which has been associated with competitive exclusion of pathogens (Rolfe, 2000; van der Wielen et al., 2002). Excluding the possible effects of MOS on gut microflora because the embryo's intestine is considered sterile prehatch (Yegani and Korver, 2008) and based on our findings in which no bacterial DNA was found in cecal content on DOH, methodology applied enabled us to conclude that, in this study, the mechanism underlying the MOS-induced alterations involved a direct effect of MOS on the intestine rather than being mediated by intestinal microflora.

We propose direct binding of MOS to transmembranal receptors on the small intestine of the chicken and consequent induction of a response. Seifert and Watzl (2007) suggested that a direct effect of other oligosaccharides (inulin and oligofructose) may result from interaction of those with carbohydrate receptors on intestinal epithelial cells and immune cells, or possibly even partial absorption of the oligosaccharides, resulting in local and systemic responses. Several potential pathways could explain a direct effect of MOS on host responses. Among the receptors are several host transmembrane molecules, potentially including the TLR (Harris et al., 2006), mannose receptors (Netea et al., 2006), the galectin family receptors (Almkvist and Karlsson, 2004), and the transmembrane mucins (MUC1 and MUC4; Carraway et al., 2003). In addition, direct attachment of MOS to immune cells might occur through carbohydrate receptors located on immune cells. Evidence of such receptors, for example the C-type lectin-like receptors dectin-1 cluster, has been shown to be located on immune cells such as monocytes, macrophages, neutrophils, and microglia (Brown, 2006). Further research under germ-free conditions should include a molecular study of the pathways involved after the interaction of MOS with transmembrane receptors.

In conclusion, given the absence of bacteria in the embryonic intestine, this study shows a direct effect of MOS on intestinal maturation and development. The in ovo administration of MOS shows a short-term effect resulting in a hatching chick with more mature enterocytes in the small intestine and enhanced digestive capacity and epithelial barrier, which can, in turn, improve development and growth in the first days after hatch. The beneficial effects of MOS begin 72 h after in ovo administration and last at least until DOH.

## REFERENCES


Almkvist, J., and A. Karlsson. 2004. Galectins as inflammatory mediators. Glycoconj. J. 19:575–581.

Baurhoo, B., P. R. Ferket, and X. Zhao. 2009. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult. Sci. 88:2262–2272.

Baurhoo, B., A. Letellier, X. Zhao, and C. A. Ruiz-Feria. 2007a. Cecal populations of lactobacilli and bifidobacteria and *Escherichia coli* populations after in vivo *Escherichia coli* challenge in birds fed diets with purified lignin or mannanoligosaccharides. Poult. Sci. 86:2509–2516.

Baurhoo, B., L. Phillip, and C. A. Ruiz-Feria. 2007b. Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. Poult. Sci. 86:1070–1078.

Benajiba, A., and S. Maroux. 1980. Purification and characterization of an amino peptidase A from hog intestinal brush-border membrane. Eur. J. Biochem. 107:381–388.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Brown, G. D. 2006. Dectin-1: A signaling non-TLR pattern-recognition receptor. Nat. Rev. Immunol. 6:33–43.

Carraway, K. L., V. P. Ramsauer, B. Haq, and C. A. Carothers Carraway. 2003. Cell signaling through membrane mucins. Bioessays 25:66–71.

Castillo, M., S. M. Martin-Orue, J. A. Taylor-Pickard, J. F. Perez, and J. Gasa. 2008. Use of mannanoligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: Effects on microbiota and gut function. J. Anim. Sci. 86:94–101.

Chen, J., J. N. Rao, T. Zou, L. Liu, B. S. Marasa, L. Xiao, X. Zeng, D. J. Turner, and J. Y. Wang. 2007. Polyamines are required for expression of Toll-like receptor 2 modulating intestinal epithelial barrier integrity. Am. J. Physiol. Gastrointest. Liver Physiol. 293:G568–G576.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.

Dahlqvist, A. 1964. Method for assay of intestinal disaccharidases. Anal. Biochem. 7:18–25.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: History and mode of action. Poult. Sci. 84:634–643.

Fernandez, F., M. Hinton, and B. Van Gils. 2000. Evaluation of the effect of mannan-oligosaccharides on the competitive exclusion of *Salmonella* Enteritidis colonization in broiler chicks. Avian Pathol. 29:575–581.

Fernandez, F., M. Hinton, and B. Van Gils. 2002. Dietary mannanoligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella* Enteritidis colonization. Avian Pathol. 31:49–58.

Franklin, S. T., M. C. Newman, K. E. Newman, and K. I. Meek. 2005. Immune parameters of dry cows fed mannan oligosaccharide and subsequent transfer of immunity to calves. J. Dairy Sci. 88:766–775.

Gilbert, E. R., H. Li, D. A. Emmerson, K. E. Webb Jr., and E. A. Wong. 2008. Dietary protein quality and feed restriction influence abundance of nutrient transporter mRNA in the small intestine of broiler chicks. J. Nutr. 138:262–271.

Harris, G., R. KuoLee, and W. Chen. 2006. Role of Toll-like receptors in health and diseases of gastrointestinal tract. World J. Gastroenterol. 12:2149–2160.

Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk, and C. O'Farrelly. 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infect. Immun. 74:1692–1698.

Hooge, D. 2004. Meta-analysis of broiler chicken pen trials evaluating dietary mannan oligosaccharide, 1993–2003. Int. J. Poult. Sci. 3:163–174.

Iji, P. A., A. Saki, and D. R. Tivey. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. Br. Poult. Sci. 42:505–513.

Kocher, A., N. J. Rodgers, and M. Choct. 2004. Efficacy of alternatives to AGPS in broilers challenged with *Clostridium perfringens*. Pages 130–133 in Proc. Australian Poult. Sci. Symp, Sydney.

National Research Council. 1994. Nutrient Requirements of Poultry. National Academy Press, Washington, DC.

Netea, M. G., N. A. Gow, C. A. Munro, S. Bates, C. Collins, G. Ferwerda, R. P. Hobson, G. Bertram, H. B. Hughes, T. Jansen, L. Jacobs, E. T. Buurman, K. Gijzen, D. L. Williams, R. Torensma, A. McKinnon, D. M. MacCallum, F. C. Odds, J. W. Van der Meer, A. J. Brown, and B. J. Kullberg. 2006. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J. Clin. Invest. 116:1642–1650.

Newman, K. 1994. Mannan-oligosaccharides: Natural polymers with significant impact on the gastrointestinal microflora and the immune system. Pages 167–174 in Proc. Alltech's 10th Ann. Symp, Nottingham University Press, Nottingham, UK.

Nochta, I., T. Tuboly, V. Halas, and L. Babinszky. 2009. Effect of different levels of mannan-oligosaccharide supplementation on some immunological variables in weaned piglets. J. Anim. Physiol. Anim. Nutr. (Berl.) 93:496–504.

Palo, P. E., J. L. Sell, F. J. Piquer, L. Vilaseca, and M. F. Soto-Salanova. 1995. Effect of early nutrient restriction on broiler chickens. 2. Performance and digestive enzyme activities. Poult. Sci. 74:1470–1483.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45.

Rolfe, R. D. 2000. The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130:396S–402S.

Rosen, G. D. 2007. Holo-analysis of the efficacy of Bio-Mos in broiler nutrition. Br. Poult. Sci. 48:21–26.

Seifert, S., and B. Watzl. 2007. Inulin and oligofructose: Review of experimental data on immune modulation. J. Nutr. 137:2563S–2567S.

Sklan, D. 2001. Development of the digestive tract of poultry. World's Poult. Sci. J. 57:415–428.

Smirnov, A., R. Perez, E. Amit-Romach, D. Sklan, and Z. Uni. 2005. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J. Nutr. 135:187–192.

Solis de los Santos, F., A. M. Donoghue, M. B. Farnell, G. R. Huff, W. E. Huff, and D. J. Donoghue. 2007. Gastrointestinal maturation is accelerated in turkey poults supplemented with a mannan-oligosaccharide yeast extract (Alphamune). Poult. Sci. 86:921–930.

Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. 2000. The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. Poult. Sci. 79:205–211.

Uni, Z. 2006. Early development of small intestine function. Pages 29–42 in Avian Gut Function in Health and Disease. G. C. Perry, ed. CAB International, Oxon, UK.

Uni, Z., and P. R. Ferket. 2004. Methods for early nutrition and their potential. World's Poult. Sci. J. 60:101–111.

Uni, Z., Y. Noy, and D. Sklan. 1999. Posthatch development of small intestinal function in the poult. Poult. Sci. 78:215–222.

Uni, Z., E. Tako, O. Gal-Garber, and D. Sklan. 2003. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. Poult. Sci. 82:1747–1754.

van der Wielen, P. W., L. J. Lipman, F. van Knapen, and S. Biesterveld. 2002. Competitive exclusion of *Salmonella enterica* serovar Enteritidis by *Lactobacillus crispatus* and *Clostridium lactatifermentans* in a sequencing fed-batch culture. Appl. Environ. Microbiol. 68:555–559.

Yang, Y., P. A. Iji, and M. Choct. 2007a. Effects of different dietary levels of mannanoligosaccharide on growth performance and gut development of broiler chickens. Asian-australas. J. Anim. Sci. 20:1084–1091.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2007b. Effects of mannanoligosaccharide on growth performance, the development of gut microflora and gut function of broiler chicken raised on new litter. J. Appl. Poult. Res. 16:280–288.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2008a. Effects of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic *Escherichia coli* challenge. Br. Poult. Sci. 49:550–559.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2008b. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility and gut development of broilers given different cereal-based diets. J. Anim. Physiol. Anim. Nutr. (Berl.) 92:650–659.

Yang, Y., P. A. Iji, A. Kocher, E. Thomson, L. L. Mikkelsen, and M. Choct. 2008c. Effects of mannanoligosaccharide in broiler chicken diets on growth performance, energy utilization, nutrient digestibility and intestinal microflora. Br. Poult. Sci. 49:186–194.

Yegani, M., and D. R. Korver. 2008. Factors affecting intestinal health in poultry. Poult. Sci. 87:2052–2063.