Studies of Natural Feed Ingredients β-1,4-Mannobiose and Mannanase-Hydrolyzed Copra Meal

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Studies of Natural Feed Ingredients
β-1,4-Mannobiose and Mannanase-Hydrolyzed Copra Meal

（天然飼料添加物、β1,4 マンノビオースおよびマンナナーゼ加水分解コプラミールに関する研究）

January 2014

Masahisa Ibuki

Contents

Chapter I

General Introduction

Food safety is very important for our social life. Once they were threaten, we would not live normal social life. In order to prevent threat, several ways of approaching this problem are required. For example, we have to reduce "foodborne-incidents" with infected invisible things such as virus and micro-organism by proper way based on science technology such as bacteriology.

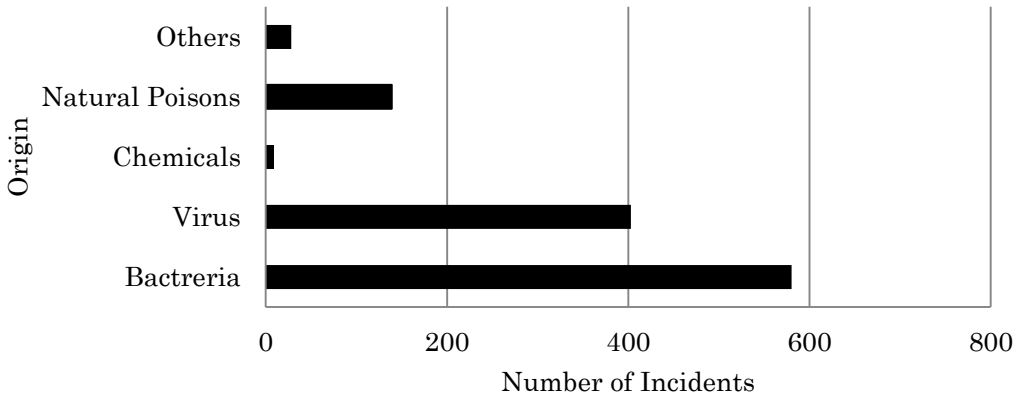


Figure 1 The Number of Foodborne Incidents
(2010 Japanese Government Bulletin HP)

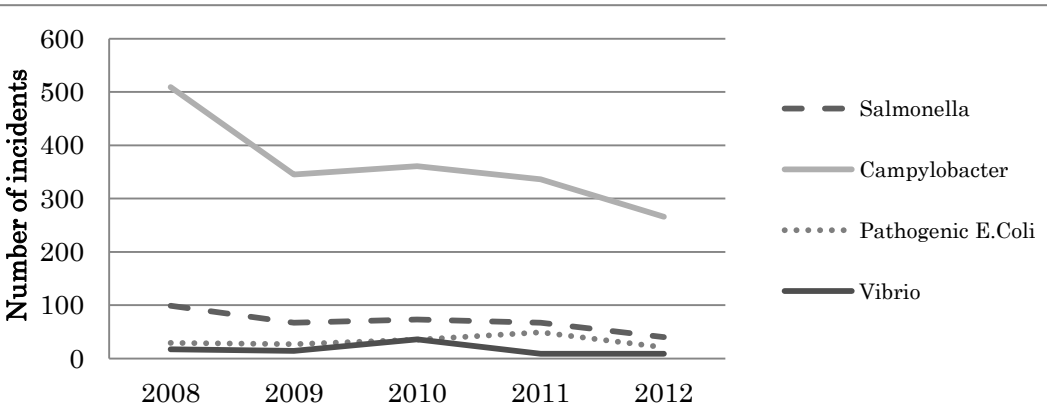


Figure 2 The number of Foodborne incidents on several year
(Health, Labour and Welfare Ministry of Japan HP)

Figure 1 shows the number of foodborne-illness incidents in Japan (Japanese Government Bulletin). The biggest number of them is origin from bacteria, indicating that safety measures against this origin is most important.

Most of foodborne-incidents by bacteria comes from *Salmonella*, pathogenic *E. coli* such as O-157, *Campylobacter* and *Vibrio*. **Figure 2** shows the number of incidents for foodborne-incidents from bacteria on recent years in Japan (Ministry of Health, Labor and Welfare, Japan HP, 2013). The number from *Campylobacter* is biggest one, and next one is from *Salmonella.* However, *Salmonella* incident is the most popular foodborne incident in the USA (**Figure 3**, Centers for Disease Control and Prevention). These observations suggest that adequate measures should be taken to prevent *Salmonella* incident worldwide.

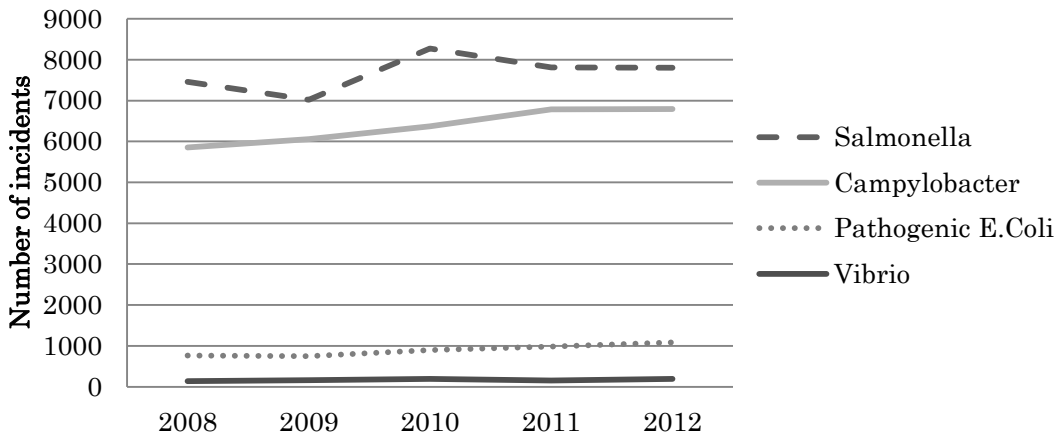


Figure 3 The number of Foodborne incidents on several year (CDC in USA)

Table 1 Bacteria infection of several foods that are designated by Wefare Ministry of Japan(2011) (Health, Labour and Welfare Ministry of Japan HP)

	Product name	Products No.	Results(Negative No.)			
			E. coli	Salmonella	Pathogenic E. coli	Campylobacter
Vegetable	Alfalfa	13	3	-	-	-
	Radish Sprouts	91	15	-	-	-
	Cutting Vegetable	150	12	-	-	-
	Cucumber	112	7	-	-	-
	Mitstba	58	20	1	-	-
	Sprout	103	41	-	-	-
	Lettuce	103	12	-	-	-
	Vegetable Pickles	158	16	-	-	-
Meat	Mince(Cow)	102	67	3	-	-
	Mince(Swine)	144	99	2	-	-
	Mince(Mixed)	103	72	3	-	-
	Mince(Chicken)	159	127	88	-	60
	Cow Lever(be cooked)	225	159	2	-	34
	Steak Meat(cutting)	52	21	-	-	-
	Seared Beaf	13	3	-	-	-
	Seared Chicken	33	29	1	-	4
	Horse Sashimi	78	8	-	-	-
	Loasted Beaf	108	3	-	-	-
Total		1805	714	100	0	98

Table 1, which shows bacteria infection of several foods in 2010, indicates that most of *Salmonella* infection comes from animal foods, especially in chicken. Host domestic animals are, in many case, infected during feeding period in a farm, and then they were tranfered to slaughter house and meat processing center with a bactreia. Sometimes contamination of meat occurred in the house and center, and then the contamination is increased by touching each other. To prevent the infection of pathogenic these bacterias from a stockbreeding, in general, three principles are proposed in a farm: “No invasion”, “No propagating”, and “No carrying out” . No invasion means that no contaminated feed with bacteria and no host animal such as mice in a farm. No propagating means that once some bactreria invase to a farm, no propagating will be done untill it become hamful for human. No carrying out means no carrying infected animal out from a farm. Thus, antibiotics have been applied widely in the human and domestic animal. In feed antibiotics have been used with the aim of not only curing infected animals but also promoting growth of animals. However, recently it is getting bigger that social issue for appearing drug-resistance strain of bacteria on several antibiotics. For example, NARMS (National Antimicrobial Resistance Monitoring System) Retail Meat Annual Report 2011 showed that 69% of pork chop, 55% of ground beef, 39% of chicken

breast and 81% of ground turkey had a drug resitance strain of bacteria. *Salmonella* and *Campylobacter* were found so many there. The Pew Charitable Trusts reported on their web site that sold antibiotics for animals in 2010 is more than 13,000 ton, and one for human is only about 3,500 ton in USA (Pew Charitable Trusts). European community banned using all antibiotics for animal feeding as purpose of growth promoting on 2005 (Dibner and Richard, 2005), and FDA ban using some antibiotics as same purpose as well (Ohshima, 2006). From above social situation, most important thing is to find out an effective and safety material alternative to antibiotics.

Mannose and mannose-based oligosaccharides have been extensively explored in poultry because of its inhibitory properties to the FimH adhesins present in enteric salmonellas (Fernandez *et al.*, 2002; Oyofo *et al.,* 1989a; Oyofo *et al.,* 1989b). Particularly mannose has more active-point to adhere with *Salmonella* comparing to Mannose-based oligosaccharides. However, mannose is very unstable in the intestine when compared to mannobiose. because it is degraded faster by intestinal bacterial flora than β 1,4 mannobiose (MNB) which is coming from mannanase hydrolyzed copra meal (MCM). (Morikoshi *et al.* 2003). Therefore it is possible that MNB and MCM can be used as an alternative to antibiotics.

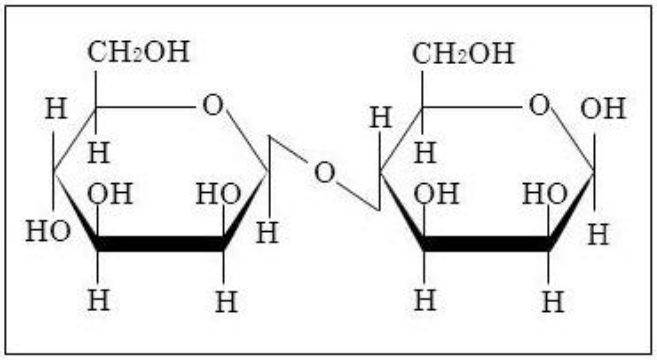


Figure 4 Structure of MNB

The structure of MNB is shown on **Figure 4**. MNB, one of the disaccharides, has an interesting structure: upper side is like a hydrophilic and under side like a hydrophobic.

Table2 Typical Content of Copra meal(%)

Crude Protein	20
Crude Fat	12
Crude fiber	8
Ash	6
Moisture	6
Glucide	18
Mannan	30

The flow chart of production of MCM is on **Figure 5**. Copra Meal (CM), de-fated or expellered coconuts as the by-product of oil, is generally granule powder form. Typical composition of CM is shown in **Table 2**. It contains about 20% of crude protein, 12% of crude fat, and 30% of mannan as raw material of MNB. MNB is produced from CM by mannanase treatment. The author designed the MCM including MNB as "MCM-B1" (11.4% MNB), "MCM-B2" (67.8% MNB), and "MNB" (99% MNB)

The aim of this study is that the the evaluation of the natural feed ingredients MNB and MCM as alternatives of antibiotics or growth promoters in poultry industry.

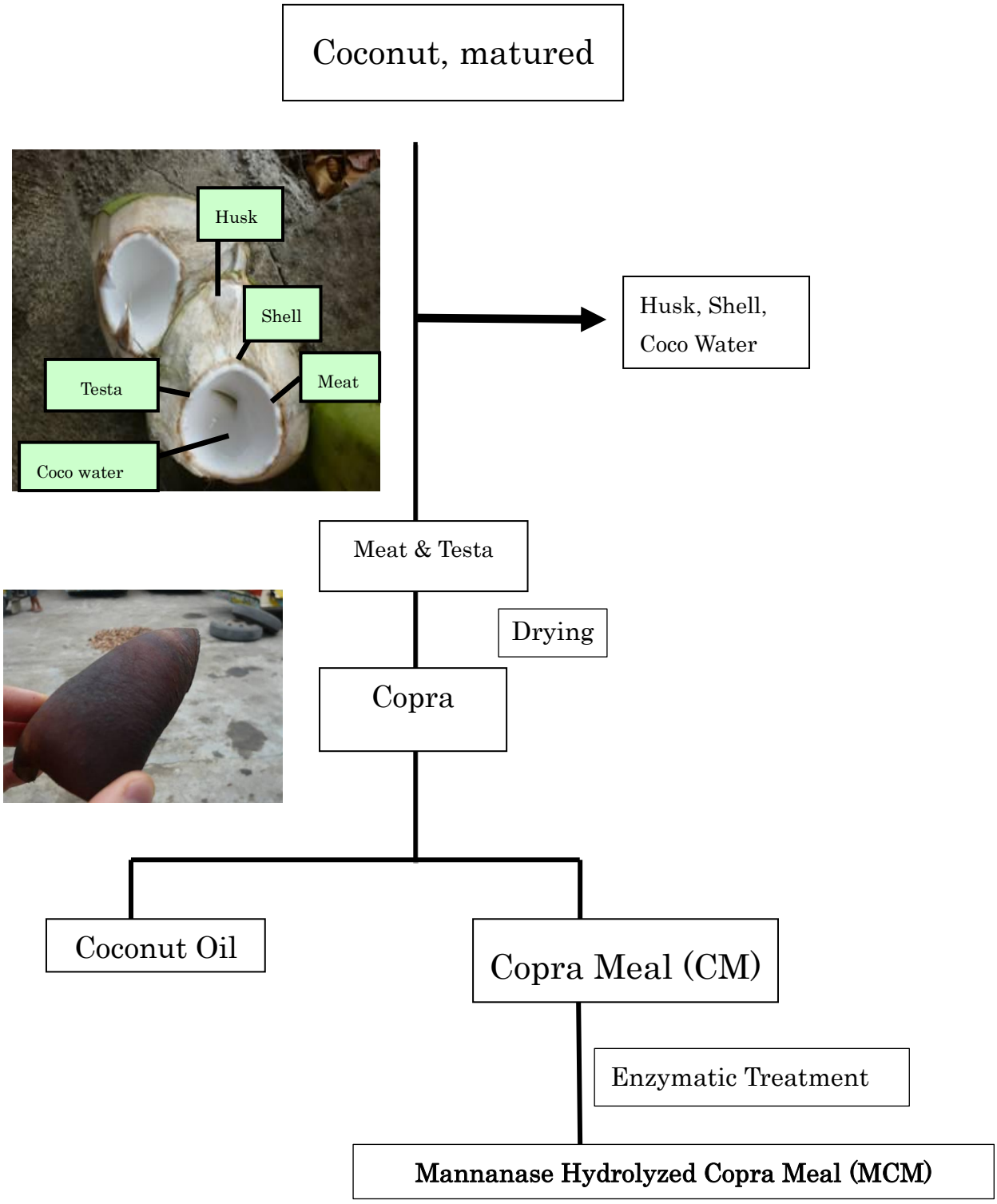


Figure 5 Production of MCM

Chapter Ⅱ

Effect of dietary β 1,4 mannobiose (MNB) in the prevention of *Salmonella enteritidis* infection in broilers

Introduction

Salmonella enterica serovar Enteritidis (SE) is widely distributed in commercial chicken flocks and high levels of cecal carriage and shedding may lead to broiler meat contamination. Recently, chicken consumption is a newly identified risk factor in SE infection in humans (Kimura *et al.*, 2004). The uses of oligosaccharides in domestic animal species including horses (Spearman, 2004), dairy cattle (Franklin *et al.,* 2005), pigs (LeMieux *et al.,* 2003) and poultry (Fernandez *et al.*, 2002) have been very popular in the last decade. In human research, oligosaccharides gained a significant level of interest because of its potential impact on nutritional immunology (Watzl *et al.*, 2005). A number of different oligosaccharides have emerged with variable effects in the general health and immune response to pathogens in both humans and animals. In poultry, oligosaccharides have been well documented as an alternative to antimicrobials in the reduction of SE, a major causative agent in enteritis outbreaks associated with the consumption of SE-contaminated broiler meats.

A range of oligosaccharides is used as "prebiotics" in both humans and animals. These are "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995; Gibson *et al.*, 1995). These carbohydrates are basically derived from various plants sources and can resist hydrolysis and digestion in the upper gastrointestinal tract but are hydrolyzed and fermented in the large intestines (Delzenne, 2003). Some of the oligosaccharide evaluated in food animals include: fructose-oligosaccharides (Chambers *et al.*, 1997; Bailey et al., 1991), galactose-oligosaccharides (Smiricky-Tjardes *et al.,* 2003a), sucrose-oligosaccharides (Orban *et al.*, 1997a; Orban *et al.*, 1997b), isomalto-oligosaccharides (Chung and Day, 2004), and mannan-

oligosaccharides (Franklin *et al.*, 2005; Spearman, 2004). Oligosaccharides are used alone or sometimes in combination with probiotics (Fukata *et al.*, 1999), growth promoting antimicrobials (Sims *et al.*, 2004; Parks *et al.*, 2001), polyunsaturated fatty acids (Bomba *et al.,* 2003), and micronutrients (David *et al.*, 2002).

A review of various animal studies suggests that continuous feeding of oligosaccharides can result to an increased production of short chain fatty acids (SCFA) such as acetate, lactate, propionate, and butyrate, leading to proliferation of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (Delzenne, 2003) in the distal intestines. A substantial population of *Bifidobacterium* and *Lactobacillus* is required for the formation of mucosal biofilm, which serves as a barrier in the gut mucosa against pathogenic insults (Kleessen and Blaut, 2005). It has been demonstrated that oligosaccharides, regardless of its chain length are readily fermentable but varied in amount and type of SCFA produced (Smiricky-Tjardes *et al.*, 2003b).

Mannose-containing oligosaccharide (MOS, mannanoligosaccharide) is one of the most popular and commercially available prebiotics for poultry use (Ferket, 2004). MOS exerts various health benefits in two of the economically important food animal species – poultry and swine. MOS depresses the proliferation of *Clostridium perfringens*, the causative agent of necrotic enteritis in broilers which remains as the most important Gram-positive bacterium in intensive commercial broiler operations following the banning of antimicrobial growth promotants (Denev *et al.*, 2005). In birds vaccinated with Infectious Bursal Disease (Gumboro) and Newcastle Disease Virus (NDV, Fowl plague), the feeding of MOS improves the vaccinal response, improving the resistance of birds to field challenge with these pathogens. The nutritional status of the bird is also influenced by the addition of MOS in the diet, exemplified by morphological changes in the intestinal villi increasing the surface area for nutrient resorption. In the presence of bacterial (*S. Typhimurium*) and protozoal (*Eimeria spp.*) organisms, MOS can effectively maintain the targeted feed conversion rate (FCR), therefore reducing the

impact on performance during a disease challenge (Rios *et al.*, 2005). In birds fed diets contaminated with aflatoxin, MOS can increase adsorption and degradation of aflatoxins (Zaghini *et al.*, 2005), therefore reducing liver pathology and enteritis. In breeder flocks, MOS feeding improves production efficiency parameters, vaccine response, and; passive transfer of antibodies to the progeny (Shashidhara and Devegowda, 2003).

Mannose and mannose-based oligosaccharides have been extensively explored in poultry because of its inhibitory properties to the FimH adhesions present in enteric salmonellas (Fernandez *et al.*, 2002; Oyofo *et al.,* 1989a; Oyofo *et al.,* 1989b). MNB is a disaccharide and a potential inhibitor of FimH adhesion. Unlike D-Mannose, its specific functional health benefits to both humans and animals have not been described. MNB is found in wood and plant seeds (Twaddle *et al.*, 2003) and linkage-specific glycosidases are required for its production (Kremnicky *et al.,* 1996). Potentially useful FimH receptor-active manno-oligosaccharides of the α- linkage group have been described using enzymes derived from *Penicillum citrinum, Aspergillus phoenicis*, and almond (Maitin *et al.,* 2004). In addition to its potential use as an anti-adhesive against poultry pathogens, mannobiose may modulate the immune response of birds to SE infection, however, its immunological function has not been documented in poultry.

The objectives of this chapter were 1) to determine if MNB supplemented feed is effective in the prevention of SE infection in growing broilers by characterization of the kinetics of SE infection within a three-week observation time post-challenge, 2) to determine the response of chicken fed with the different diets on the secretary IgA, and 3) to be able to correlate the resolution of the disease by analyzing histological changes in the cecal mucosa and cecal tonsils.

Materials and Methods

Source of feed additive

The feed supplements MNB , and D-Mannose derived from copra meal by

enzymatic digestion were provided by Fuji Oil, Ltd. (Osaka, Japan). The feed supplements contains 11.4% MNB. The other one, on the other hand, contains 10% D-mannose. Since mannobiose and mannose are the major components of the crude feed additives, these feed supplements shall be designated as MCM-B1 (MNB as main component) and MAN (D-mannose as main component), respectively.

Experimental diets

The supplements were directly mixed in the broiler starter/grower crumble formulation resulting to a final mixture of 0.1% MCM-B1 or 0.1% MAN. The starter or grower crumble feeds used in this chapter were without antibiotics or anticoccidial drug (**Table 3**). All feeds used in this chapter were prepared at the Arkell Research Station, University of Guelph, Guelph, Ontario. The feeds used tested negative to *Salmonella spp.*

Animals

One hundred twenty 1-day-old chicks (Ross x Ross) were obtained from a local commercial hatchery and were screened for *Salmonella spp*. by cloacal swabbing prior to placement. Animals were housed at the Animal Isolation Unit, University of Guelph, The animal experiment was conducted in accordance to the animal care guidelines and with the approval of the Animal Care Committee, University of Guelph.

Bacterial culture

SE PT4 SA992212 resistant to novobiocin, a gift from Dr. Cornelius Poppe (Health Canada) and originally isolated from chickens was used in this chapter. The bacterial culture was retrieved from a bacterial stock and grown in Brain Heart Infusion Agar (BD Diagnostic System, Oakville, ON, Canada) overnight at 37 ℃ with shaking. The overnight culture was adjusted to the desired colony forming units (CFU) per ml by calorimetric technique (Biomerìeux Vitek, Inc. Hazelwood, MO, USA) and confirmed by plating on Brilliant Green Agar

(BGA, BD Diagnostic System, Oakville, ON, Canada). The methods described in the FDA Bacteriological Analytical Manual (Wallace *et al.* 1995) were used to confirm SE.

Table 3 Nutrient compositions of the different diets.

Ingredients	Starter (g/kg)	Grower (g/kg)	Finisher (g/kg)
Corn	494.3	596.4	638.3
Soymeal Hi-Pro	276.7	276.0	229.0
Wheat	75.0	75.0	75.0
Shorts/wheat Midds	58.9	-	-
Canola meal	50.0	-	-
Dicalcium phosphate	16.2	12.5	10.3
Calcium carbonate	14.9	14.6	13.3
Fat	-	12.7	21.1
Pellet binder	6.3	6.3	6.3
Salt	4.1	4.2	4.2
Liquid methionine (MHA)	1.8	1.6	1.2
Broiler trace mineral ultra	1.0	-	0.5
Liquid choline chloride	0.8	0.6	0.4
Lysine pure	-	0.1	0.4
Total	1000.0	1000.0	1000.0

Note: The regular diet contained all the listed ingredients. The treatment diets contained all these plus 0.1% (w/w) MCM-B1 or MAN.

Animal experiments

In this trial, the purpose of feeding MCM-B1 and MAN during the first two weeks of the growing period was to determine if a 2-week feeding can reduce the susceptibility of growing birds until they are marketed. Three groups of 20 chicks were placed in wire cages with unlimited access to feed and water. Treatment diets were fed for 2 weeks. Prior to inoculation, feeds and

water were withdrawn overnight. After this period, birds were then orally inoculated with 2 x 10^7 cfu/ml of SE. SE fecal shedding was monitored by faecal collection and culture every two days starting on the first day up to 19 days post-infection (p.i.). Post mortem was conducted in 3 to 5 birds per group prior to infection, and at days 1, 7, 14 and 23 days p.i. Various organs were collected for SE enumeration. The ceca and cecal tonsils were selected for histopathological examination. Bile and cecal contents were collected for SE-specific IgA assay.

Bacterial count

Overnight faecal droppings were collected in sterilized aluminum foil (12 x 12") randomly placed in three different locations in trays underneath the wire flooring and aliquoted for the different bacteriological enumeration. Twenty five grams of faeces from each collection site was mixed with 225 ml of tetrathionate brilliant green (TBG, BD Diagnostic Systems, Oakville, ON, Canada) broth supplemented with 0.02 µg/ml novobiocin (BD Diagnostic Systems, Oakville, ON, Canada) and thoroughly mixed in a kitchen blender (Osterizer®) for 5 minutes. Samples were incubated overnight at 41 ℃. Cultures were serially diluted 1:10 from 10^2 to 10^7 in 1% buffered peptone water (BD Diagnostic Systems, Oakville, ON, Canada) and plated in duplicates on BGA with 20 mg of novobiocin. Plates were incubated aerobically at 37 ℃ overnight. For bacterial enumeration in liver, spleen and cecal contents, pre-weighed 10 ml culture tubes (Simport®, Fisher Scientific, Neopan, ON, Canada) were used to collect samples during necropsy, stored on ice, 9 parts TBG with novobiocin added, and homogenized for one minute using Polytron® homogenizer (Brinkman Instruments, Inc., Missisauga, ON, Canada). For the enumeration of SE from samples of the cecal wall, samples were suspended in 10 ml of phosphate buffered saline (PBS, Gibco®, Invitrogen Canada Inc., Burlington, ON, Canada), stored on ice and washed 3x with PBS for removal of adherent bacteria and debris prior to homogenization. Succeeding incubation steps were similar to the procedure described for the enumeration of feces, but

Salmonella Shigella Agar (SS Agar, BD Diagnostic Systems, Oakville, ON, Canada) was used instead of BGA for plating.

Enzyme-linked immunosorbent assay

Bile, and cecal contents were analyzed for SE-specific IgA antibodies using SE whole cell antigen. For antigen preparation, bacterial stock previously stored at -80 ℃ in 20% glycerol was thawed and a loopful was plated on BGA. After an overnight incubation at 37 ℃, a colony was inoculated in 500 ml of BHI and incubated at 37 ℃ for 18 hours with shaking. The broth culture was centrifuged at 2,500 *x g* at 4 ℃ for 5 minutes. The supernatant was discarded and the pellet was washed 3 times with ice-cold PBS. The pellet was inactivated with 3.8% buffered formalin overnight at room temperature. After inactivation, the pellet was harvested by centrifugation and washed 3 times with ice-cold Milli-Q water to remove the formalin. The final pellet was frozen overnight at -80 ℃ and freeze dried for 24 hours. The SE whole cells were used as a coating antigen.

For bile collection, the liver was removed and the bile was aseptically aspirated from the gall bladder using a 3 ml syringe with gauge 18 needle. For cecal contents, the left side of the ceca was squeezed from the distal blunt end to the proximal end and was incised above the cecal tonsils. Approximately 0.5 to 1 g of the cecal content was placed in pre-weighed sterile 2 ml microcentrifuge tubes. The contents were weighed and added with an equal amount (1:1) of complete™ Protease Inhibitor Cocktail Tablet (Roche Diagnostics Canada), diluted according to manufacturer's suggestions. The bile and cecal samples were stored as above until use. Test samples were thawed at room temperature and diluted to 1:100 with Tris Buffered Saline (TBS) with 1% Bovine Serum Albumin (BSA) prior to analyzes.

For SE specific IgA analysis, 96 well plates (Corning Costar Corp., Cambridge, MA, USA) were coated with 100 microliters (ml) of SE whole cell antigen (10 mg/ml) in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 ℃ and washed four times with Tris Buffered Saline with Tween

20 (TBST, pH 8) in a plate washer (Immunowash® Bio-Rad, Hercules, Canada). Blocking of non-specific binding sites was carried out with 150 ml of 1% BSA in TBS (pH 8) for one hour and washed four times. One hundred microliters of test samples: bile (1:100) and cecal contents (1:100) diluted in sample diluent (TBS + 1% BSA) was incubated into the designated wells for 1 hour, followed by four washes of TBST. The plates were incubated for one hour using with 100 ml of Rabbit Antichicken IgA conjugated Horseradish Persoxidase (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted 60,000 times. Plates were subsequently washed 6 times with TBST. All incubations were carried out at 37℃ with shaking. For color development, 50 ml of 3, 3', 5, 5' Tetramethyl benzidine (TMB) Liquid Substrate System (Sigma-Aldrich, St. Louis, MO,USA) was incubated for 1 hour and the enzymatic reaction was stopped using 50 ml of 0.5 M H_2S0_4. Plates were read in an ELISA reader (Bio-Rad®) with an absorbance set to 450 nm. All samples were tested in triplicates and the SE specific antibodies were arbitrarily expressed as the average absorbance of the three replicates.

Histopathology

At 23 days p.i., sections of the liver, spleen, bursa of Fabricius, cecal tonsils and ceca (cross-section) were fixed in 10% buffered formalin for 24 hours, sectioned at 4 mm thickness, and embedded in paraffin. Slides were stained using routine Hematoxylin and Eosin (H & E) stain and Giemsa stain and then examined under light microscope [Nikon® Digital Camera DXM 1200F microscope (Melville, New York) equipped with Nikon Eclipse® E8000 Camera]. A scoring system for the cecal tonsils was applied. The quantification of intraepithelial lymphocytes was based on the protocol described by Sheela *et al.*, (2003). Typical enteric *Salmonella* paratyphoid lesions were evaluated and were judged on the basis of inflammation, hyperplasia of mucous gland cells, intraepithelial mononuclear cells in the intestinal villi flanking the cecal tonsils, and population of lamina proprial cells (Gast, 2003). The presence of granuloma was also recorded (Desmidt *et al.*, 1998a). In each of the three to

five birds necropsied, five different intestinal villi flanking the cecal tonsils were selected at random and the following parameters were evaluated: a) Inflammation: (3)-severe, multifocal coalescing areas of hemorrhages and edema, (2)-moderate, focal to multifocal areas of hemorrhages and edema, (1)-mild, focal areas of hemorrhages and edema. b) Mucous gland cells: (3)-severe, generalized hyperplasia and hypertrophy of mucous gland cells, (2)-moderate, focal to multifocal areas with hyperplastic and hypertrophied mucous gland cells, mostly confined in hemorrhagic and edematous areas, (1)-mild, focal areas of hyperplastic and hypertrophic mucous gland. c) Intraepithelial mononuclear cells: counted from 5 different microscopic fields at 40x magnification: (3)-Increased, presence of 35 to 50 cells per microcopic field, (2)-Moderate, presence of 20 to 34 cells per microscopic field, (1) Mild, presence of less than 20 cells per microscopic field. d) Lamina proprial cell population: (3)-increased, lamina propria was densely infiltrated with mononuclear cells consisting of mature lymphocytes, plasma cells, and macrophages (2)-moderate, lamina propria was moderately infiltrated with mononuclear cells (1)-mild, lamina propria was mildly infiltrated with mononuclear cells. The slides stained with H & E were used in the evaluation of parameters a, b, and d. Giemsa stained slides were used in the enumeration of intraepithelial mononuclear cells.

Statistical analyzes

Statistical differences between treatment groups were determined by one way analysis of variance (ANOVA). The means derived from the quantification of bacteria from faecal samples and organs, IgA and IgG absorbances were analyzed on each sampling schedule and were further separated for significance with an all pairwise multiple comparison applying the Tukey- Kramer test ($P < 0.05$, Graphpad Instat 3®, California, USA). Histopatho- logical scores,where applicable were analyzed using Kruskall-Wallis test for non-parametric ANOVA ($P < 0.05$) using the same software.

Results and Discussion

SE shedding and bacterial count in organs

The body weights are summarized in **Figure 6.** The objective of this trial is not to ascertain dietary effects on broiler weight gain but this information may provide economic justification of the cost of the additive and baseline insights for future studies (*i.e.* egg layer and broiler breeder performances). MAN-fed birds exhibited the heaviest final body weights that averaged to 1746 grams followed by the control birds with an average weight of 1672 grams. The MCM-B1-fed birds exhibited heavier body weight gain at days 21 and 28 yet averaged to a final body weight of 1561 grams. The weight gain was influenced by age "effect" ($p = 0.0001$), and not as a result of diet and age interaction ($p = 0.1546$), or diet effect ($p = 0.1329$). This is similar to the observations of Juskiewicz *et al.*, (2003), in MOS-fed turkeys, where no significant influence on weight gain and growth rate observed during the first four weeks of life. The dose and duration of feeding may also affect the over-all influence of the diet (Zdunczyk *et al.*, 2005), as was observed in the weights of MCM-B1-fed birds after the end of the two week of feeding but not at end-point.

The faecal shedding in birds fed MCM-B1 (6.36 $\log_{10}$CFU, $P \leq 0.001$) and MAN (6.48 $\log_{10}$CFU, $P \leq 0.01$) at 24 hours post-infection (p.i.) were slight lower compared with the control (8.38 $\log_{10}$CFU) (**Figure 7**), but thereafter, the MAN- and MCM-B1-fed birds exhibited different shedding patterns towards the resolution of the infection. SE shedding peaked at 4 day p.i. then decreased gradually in both groups but a marked decrease in shedding observed in birds fed MCM-B1 as early as 7 days p.i. (2.25 $\log_{10}$CFU, $P < 0.001$), and thereafter until 19 days p.i. (< 0.10 $\log_{10}$CFU, $P < 0.001$). In contrast, birds fed MAN displayed a more protracted SE shedding, having significantly reduced SE levels at 19 days p.i. (3.68 $\log_{10}$CFU, $P < 0.05$).

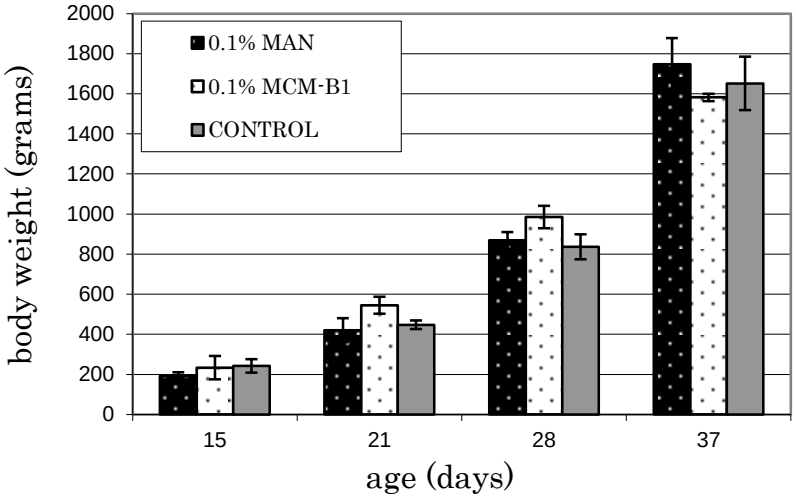


Figure 6 Change of average body weight from prevention trial comparing three different diets. Vertical bars represent standard error. n=5. Diet effect: $p = 0.1329$, age effect: $p = 0.0001$, diet and age interaction: $p = 0.1546$.

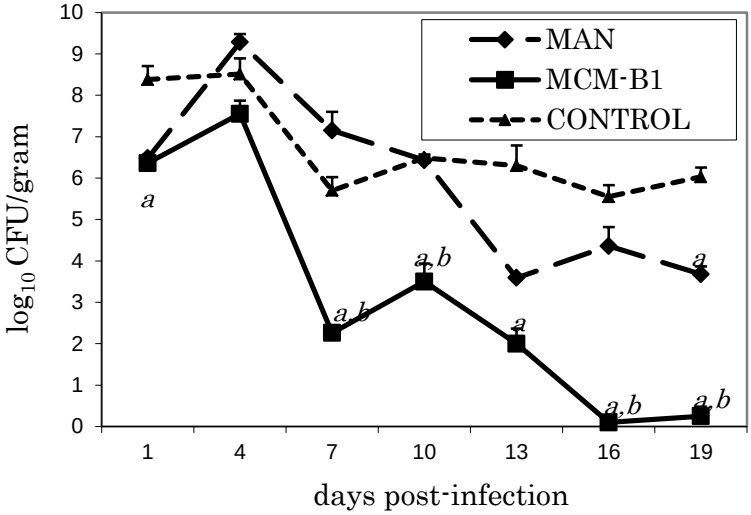


Figure 7 Faecal *Salmonella Enteritidis* counts from prevention trial
Mean SE $\log_{10}$ CFU/gram ± S.E.M.(n=5). a- significant differences with control. b- significant differences with Mannose (MAN) ($P < 0.05$, Tukey Kramer Test, Graphpad Instat 3™)

To obtain a more accurate estimate of the intestinal SE, at each necropsy, the cecal SE levels were analyzed (**Table 4**). In MCM-B1-fed birds, the cecal contents analyzed at various time points reflected the fecal shedding.

Interestingly, at 14 days p.i., the cecal levels were slightly higher than the faecal shedding at 13 days p.i. (4.77 $\log_{10}$ CFU). By 23 days p.i., the CFU level in the ceca was below the detection limit in all birds necropsied (< 0.10 $\log_{10}$CFU, P≤0.001) and reflected the levels of SE in the faeces (< 0.10 $\log_{10}$CFU). MAN-fed birds on the other hand, have ower mean SE cecal carriage at day 23 (2.89 $\log_{10}$CFU) compared with the control (6.34 $\log_{10}$CFU), but these levels were not statistically significant. The SE levels in the liver was reduced in birds fed MAN (P< 0.05) and MCM-B1 (P< 0.05) starting at 7 days p.i. and thereafter, with mean SE not exceeding 2.5 $\log_{10}$CFU compared with the control (ranged from 4.89 to 6.71 $\log_{10}$CFU) (**Table 4**).

Table 4 The effect of dietary MCM-B1 or MAN provided for two weeks after hatching on the numbers of *Salmonella Enteritidis* in the liver and cecal contents of broilers infected at 15d of age[1].

	Days post-infection			
	1	7	14	23
Liver				
MAN	4.19 ± 0.43 (5/5)[a]	< 0.1 (0/5)[a]	1.15 ± 0.66(2/5)[a]	2.46 ± 0.05(5/5)[a]
MCM-B1	5.61 ± 0.38 (3/5)	1.64 ± 0.71 (2/5)[a]	1.61 ± 0.48 (2/5)[a]	1.76 ± 0.54 (5/5)[a]
CONT.	5.52 ± 0.17 (3/5)	4.89 ± 0.29 (5/5)	5.90 ± 0.21 (5/5)	6.71 ± 0.51 (5/5)
Cecal contents				
MAN	5.78 ± 0.22 (5/5)	7.09 ± 0.82 (5/5)	3.73 ± 1.21 (2/5)	2.89 ± 1.10 (2/5)
MCM-B1	7.74 ± 0.23 (5/5)	5.39 ± 0.71 (5/5)	4.77 ± 0.55 (5/5)	< 0.1 (0/5)[a]
CONT.	7.50 ± 0.47 (5/5)	5.85 ± 0.75 (5/5)	4.99 ± 0.69 (3/5)	6.34 ± 1.98 (3/5)

[1]Chicks were inoculated with 2×10^7 cfu with *Salmonella enteritidis* at 15d of age.

Mean SE $\log_{10}$ CFU/gram ± standard error of the mean (S.E.M.), (n=5).

[a]significant differences with control. (P< 0.05, Tukey Kramer Test, Graphpad Instat 3™).

The protracted nature of SE clearance in the ceca of MAN fed birds was similar to the findings of Fernandez *et al* (2002) in birds MOS, where significant reduction in SE cecal carriage was observed in the later stages of infection. The colonization of SE in the liver was reduced in MAN- and MCM-B1-fed birds indicating a restrained systemic dissemination of the organism. The spleen results were eliminated from the table as positive spleens were detected occasionally in all experimental groups. The exact influence of MAN and the mannose-oligosaccharides in the systemic colonization of pathogens is yet to be ascertained. Newman (1994) proposed that mannose stimulates the secretion of mannose-binding proteins. Mannose-binding protein secretion can bind to bacteria resulting to the trigger of the complement cascade pathway of the bird immune system.

In various investigations of the effects of oligosaccharide feeding on the immune system, IgA production was extensively explored as IgA is an important component of mucosal defense against enteric pathogens and maintenance of the integrity of the mucosal biofilm (Bollinger *et al.*, 2003). In turkeys fed mannan- oligosaccharides, bile IgA levels were increased (Savage *et al.*, 1996), while in mice models, derivatives of mannan-oligosaccharides such as glucomannan (Kudoh *et al.,* 1999), and water soluble konjak mannan (Lim *et al.*, 1997) have been reported to modulate IgA production. However, information has been lacking regarding carbohydrate feeding and the production of pathogen specific IgA response and if the same immunomodulatory effects are achieved in the presence of an enteric infection. IgA is required to abate infection by interacting with pathogens in the lumen allowing their exclusion from the gut and prevention of further colonization (Brandtzaeg *et al.*, 1987). SE-specific IgA, in particular has been demonstrated to block the penetration of SE in intestinal cells *in vitro* (Sugita-Konishi *et al.*, 2000). In this present study, simultaneous detection of cecal and bile IgA antibodies were applied at various time points to characterize the dynamics of SE-specific IgA production in response to SE infection. The cecal IgA levels in MAN, MCM-B1, and control groups peaked at 14 dpi. (**Figure**

8a). A waning IgA levels in the cecal contents were detected in the control birds but the IgA levels persisted until 23 days p.i. in birds fed MAN and MCM-B1. In the bile (**Figure 8b**), an increasing trend of SE IgA levels were detected and peaked at 23 days in MAN- and MCM-B1-fed birds. In contrast, a transitory increase (day 7) in bile IgA were detected in the control birds with levels tapering off at 14 and 23 days p.i. The high and persistent SE IgA response of MCM-B1- and MAN-fed birds paralleled the decline in SE shedding and cecal carriage at the later stages of infection indicating an influence of the diet on IgA production and SE clearance. Other aspects of mucosal immunity associated with oligosaccharide feeding have been extensively reviewed (Gibson *et al.* 2005; Kleessen & Blaunt 2005; Schley & Field 2002; Watzl *et al.* 2005) and different immunomodulatory mechanisms have been hypothesized including: 1) selective increase/decrease in specific bacteria which modulate cytokine and antibody production; 2) increased in intestinal SCFA production to G-coupled protein receptors on leukocytes, and; 3) interaction with carbohydrate lectins. However at this point, the exact immunological mechanisms underlying the production of IgA in oligosaccharide-fed animals are not well defined.

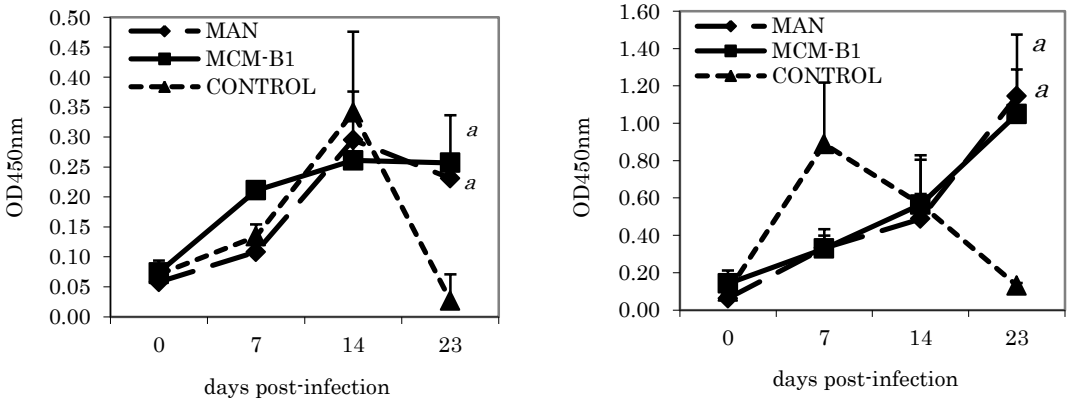


Figure 8 Specific IgA levels in cecal and bile contents. Mean absorbance of cecal IgA (a), and bile IgA (b). Vertical bars represent standard error of the mean (n=5). a – significant difference with the control ($P < 0.05$, Graphpad Instat 3™).

Histological scores

Information on the histological changes associated with the feeding of MAN/MCM-B1 in the face of an infection has been lacking. It is worthwhile to mention here that MAN/MCM-B1 and SE are both recognized by the mucosal immune cells as foreign (Iqbal *et al.,* 2005) and at this stage of infection, the effects of either MAN/MCM-B1 or SE can not be delineated. However, the purpose of the histological evaluation was to compare the effects of the diet on mucosal damage and repair, inflammation, and activity of the gut associated lymphoid tissue (GALT) towards the resolution of SE infection. This data provides insights and possible correlation to the bacterial isolations and serological analyzes. The histological scores are summarized in **Table 5**.

Table 5 Histopathological examination at 23 days p.i. from prevention trial.

	MCM-B1	MAN	CONTROL
Parameters			
Inflammation	Mild (0.00)*	Mild (0.33)*	Moderate (2.66)
Mucous gland cell hyperplasia	Mild (1.00)*	Mild (1.00)*	Moderate(2.00)
Intraepithelial mononuclear cells	Increased (2.00)*	Moderate (2.00)*	Mild (1.00)
Lamina proprial cell population	Increased (2.66)*	Moderate (2.00)	Moderate(1.66)
Other lesions:			
Necrosis of villous tips	Mild	Mild	Mild to moderate
Granuloma (cecal tonsils)	None	Yes	Yes

Values in parentheses indicate average histopathological scores. * significant difference with the control ($P < 0.01$, Kruskall Wallis for non-parametric analysis, Graphpad Instat®).

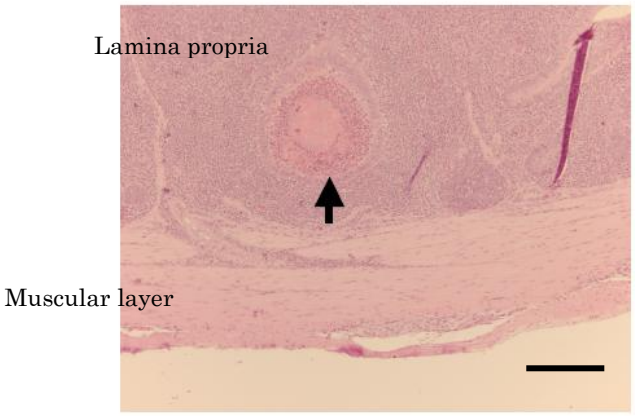
A lower inflammation score characterized by a mild focal hemorrhage and edema and mild mucous gland cell hyperplasia were observed in birds fed

MCM-B1 and MAN indicating a subtle inflammatory change and apparent clearance of the offending organism compared with the control where a multifocal moderate to severe hemorrhages were observed. Chronic granulomatous lesions in the cecal tonsils was found in f the control and MAN fed birds, a common finding in SE phage type 4 infection in birds (Desmidt *et al.*, 1998a) indicating a persistent infection and uncontrolled colonization (**Figure 9**). A significantly increased number of intraepithelial mononuclear cells (mature lymphocytes and macrophages) were observed in the lining epithelium of birds fed MCM-B1 and accompanied by an increased number of lamina proprial (lp) cells. The MAN-fed birds exhibited a moderate increase in intraepithelial mononuclear cell population but insignificant population of lp cells (**Table 5**). This moderate to increased population of lp cells in MCM-B1 and MAN-fed birds confirmed the IgA levels detected by ELISA. The histological changes exhibited by birds fed MCM-B1 demonstrated increase mucosal protection to SE, exhibited by the presence of mild focal hemorrhages (**Figure 10a**). Moderate to severe multifocal hemorrhages with early formation of granulomas were observed in birds fed MAN and regular diet (**Figure 10 b,c**). Improved cell mediated immune responses (*i.e.* presence of mature lymphocytes) may explain the clinical recovery to SE (cecal carriage and fecal SE). Robust lymphoid response in the gut mucosa exhibited by turkeys fed dietary MOS (Sims *et al.*, 2004), and mice models fed various types of non-digestible oligosaccharides (Hosono *et al.*, 2003; Lim *et al.*, 1997), but the exact changes occurring in the event of an infection in most dietary interventions has not been described elsewhere limiting the interpretation of the histological changes.

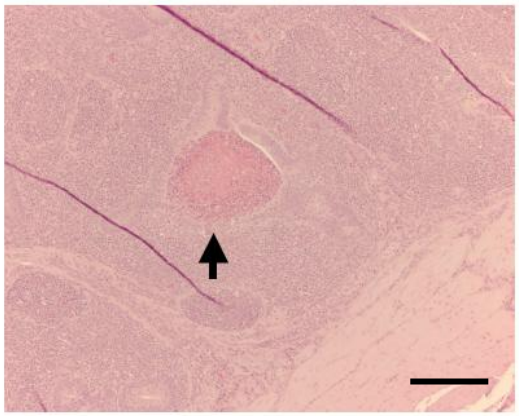
In conclusion, this chapter indicates that feeding MCM-B1 during the first two weeks of growing reduces the susceptibility of birds to SE by influencing antibody responses, and integrity of the gut mucosa. When MCM-B1 is fed in the face of an infection, 2 weeks of feeding can significantly reduce the cecal carriage and shedding of broiler birds but up to 3 weeks of feeding may be necessary for MAN to bring the SE to a significantly low level.

MCM including MNB is therefore safe, practical, and economical alternative to antimicrobials for the reduction of SE in broilers.

(a)



(b)



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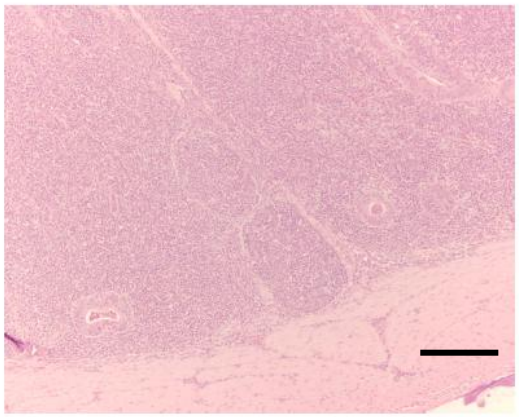
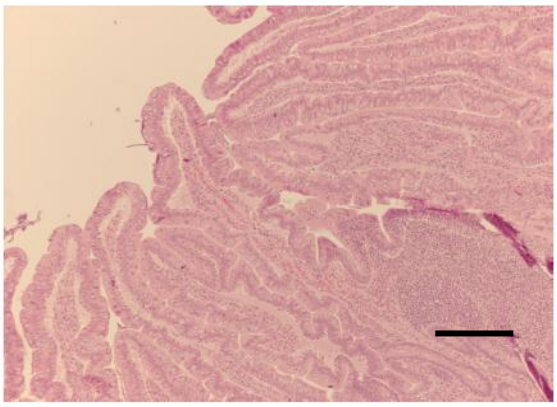
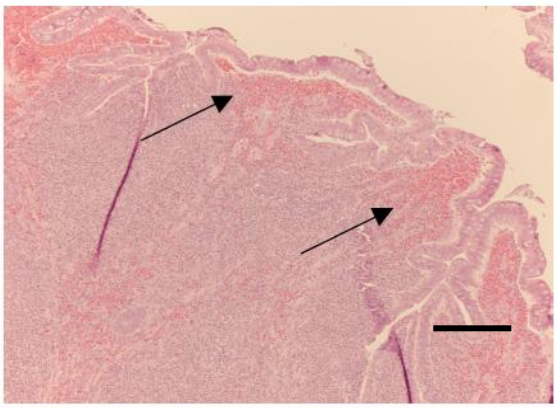


Figure 9 Chronic granulomatous inflammation in the cecal tonsils (black arrows).
Control bird (a), MAN-fed bird (b), MCM-B1-fed bird (c). The scale bar is 100 μm.

(a)



(b)



(c)

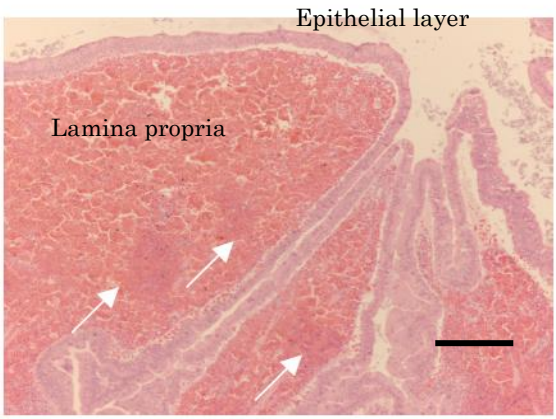


Figure 10 Histopathological examination at 23 days p.i. of cecal tonsils.
Villi flanking the cecal tonsils of birds fed MCM-B1 (a), MAN (b), and control (c). Black arrows indicate hemorrhagic lesions, White arrows in (c) indicates early granulomas. The scale bar is 100 µm.

Chapter Ⅲ

Analysis of gut immune-modulating activity of β 1,4 mannobiose (MNB) using microarray and real-time RT-PCR

Introduction

Normal functioning of the immune system is crucial to overall health, and diet is one of the major exogenous factor modulating individual immunocompetence. Enteric diseases are an important concern to the poultry industry because of lost productivity, increased mortality, and the associated contamination of poultry products for human consumption. With increasing concerns about antibiotic resistance, the ban on sub-therapeutic antibiotic usage in Europe and the potential for a ban in the United States, there is increasing interest in finding alternatives to antibiotics for poultry production. The gastrointestinal tract is the largest immune system; it contains 80% of all antibody-producing cells and produces antibodies more efficiently than any other part of body (Helgeland and Brandtzaeg, 1999). The chicken intestine has a organized scattered immune cells called gut-associated lymphoid tissues (GALT), comprised of Peyer's patches, (PP), lamina propria lymphocyte (LP), intraepithelial lymphocytes (IEL) and cecal tonsil (CT) (Shira *et al.*, 2005).

The intestinal microbiota, epithelium, and immune system provide resistance to enteric pathogens. Recent data suggest that resistance is not solely due to the sum of the components, but that cross-talk between these components is also involved in modulating this resistance. Inhibition of pathogens by the intestinal microbiota has been called bacterial antagonism, bacterial interference, barrier effect, colonization resistance, and competitive exclusion (Patterson and Burkholder, 2003). Probiotics and prebiotics are components present in foods, or that can be incorporated in to foods, which beneficially modulate gut immune responses. Recently, probiotics and prebiotics have attracted a great deal of notice for their effects on health promotion via changes in human-intestinal microbiota. Prebiotics are expected to exert beneficial effects on the prevention of pathogenic bacteria growth, the

production of antimicrobial agents, and the stimulation of mucosal barrier function in the initial developmental stage and immunomodulating effects in the late stage (Novak and Katz, 2006). No information, however, is available on how host organisms recognize ingested prebiotics in the process of expressing the immunomodulating effects and subsequent events.

The dominant prebiotics are fructo-oligosaccharide products (FOS, oligo-fructose, inulin). However, trans- galacto-oligosaccharides, gluco-oligosaccharides, glycol-oligosacchriades, lactulose, lactitol, maltooligo-saccharides, xylo-oligosaccharides, stachyose, raffinose, and sucrose thermal oligosaccharides have also been investigated (Collins and Gibson, 1999; Patterson *et al.*, 1997). Mannan oligosaccharides (MOS) are one of the most popular commercially available prebiotics for poultry use (Ferket, 2004). Although MOS have been used in the same manner as the prebiotics listed above, they do not selectively enrich for beneficial bacterial populations. Instead, they are thought to act by binding and removing pathogens from the intestinal tract and by stimulation of the intestinal immune system (Spring *et al.*, 2000; Shashidhara and Devegowda, 2003).

We have previously demonstrated that dietary supplementation of MCM including the disaccharide MNB could prevent SE infection in broilers, by increasing IgA production and improving SE clearance (Agunos *et al.*, 2007). However, the mechanism of action of MNB has yet to be elucidated. It is thus important to clarify the mechanisms of the immunomodulating effects of MNB in the host intestine in order to understand how host intestinal immune systems are modulated by food components. DNA microarray technology allows the analysis of the expression of many genes simultaneously, in order to further examine the effect of MCM including MNB on the intestine, as well as to identify potential novel biomarkers associated with its physiological function.

The objectives of this chapter were to confirm the immunomodulatory effects of MCM including MNB in chickens and carry out a comprehensive analysis to determine the effects of MNB on gene expression in the intestine and mucosal immune system by microarray and quantitative real-time

RT-PCR.

Materials and Methods

Animals and experimental design

One-day-old chicks (Ross x Ross) were obtained from a local commercial hatchery. Chicks were randomly divided into two groups (n = 12) and housed at the Animal Isolation Unit (University of Guelph, Guelph, Ontario) in raised wire cages with free access to feed and water. Foil was placed under the wire cages to collect droppings. The starter or grower crumble feeds used in this chapter did not contain antibiotics or anticoccidial drugs and were prepared at the University of Guelph, Guelph, Ontario. All experiments were approved by the University of Guelph Animal Care Committee and carried out in accordance with the Canadian Council of Animal Care Guide to the Care and Use of Experimental Animals.

Supplementation with MNB

MNB (MCM-B2) was derived from copra meal by enzymatic digestion, and was provided by Fuji Oil, Ltd. (Osaka, Japan). The supplement contained 67.8% MNB (w/w), with the remainder composed of mannose (8.9%), arabinose (0.4%), galactose (2.6%), glucose (5%), fructose (2.3%), sucrose (0.2%), protein (9.7%) and ash (3.1%) (Data provided from Fuji R&D). MCM-B2 was administered three times per week via oral gavage for four weeks, at the following doses: 3, 8, 15, and 18 mg/chick during weeks 1, 2, 3, and 4, respectively. MCM-B2 dose was based on previously reported supplementation of chicks with 0.1% MCM-B1 in feed (Agunos *et al.*, 2007). Negative control animals received the same volume of vehicle (sterile water) by oral gavage.

Body weight and histopathology

Body weights were recorded weekly. On day 14 and 28, half of the animals in each group were humanely euthanized, and weights of spleen and bursa of Fabricius were recorded. Results were expressed as the percent organ weight

relative to body weight for each animal. Samples of ileum, jejunum, cecum, spleen, thymus and bursa of Fabricius were fixed in 10% formalin followed by staining with hematoxylin and eosin (H&E).

Collection and processing of fecal samples

Fecal samples were taken weekly from each group by collecting droppings from five random locations beneath each cage, and were stored at -80°C until analysis. Samples were weighed, diluted 1:3 with PBS and mixed thoroughly. Clarified supernatant was obtained by centrifuging at 4000 rpm for 20 min at 4°C, and was stored at -80°C until IgA concentrations were measured (Tress *et al.*, 2006).

IgA enzyme-linked immunosorbent assay

Fecal total IgA quantification was carried out using a Chicken IgA ELISA Quantitation Set according to the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, 96-well plates (Corning Inc., Corning, NY) were coated with 100 µL of anti-chicken IgA capture antibody, diluted 1:100 in 0.05 M sodium carbonate buffer, pH 9.6, and incubated for 1 hr at 37°C. Plates were then washed with TBS containing 0.05% Tween-20 (TBST) and blocked for 30 min with TBS containing 1% (w/v) BSA (Thermo Fisher Scientific, Inc., Waltham, MA). IgA standards and samples were diluted in TBST containing 1% (w/v) BSA, and 100 µL/well was added to the plates and incubated for 1 hr at 37°C. Plates were washed and then incubated for 1 hr at 37°C with HRP-conjugated chicken IgA detection antibody, diluted 1:50000 in PBST containing BSA. Detection was carried out using 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, St. Louis, MO). The reaction was stopped with 2 M sulfuric acid (Thermo Fisher Scientific) and absorbances read at 450 nm.

Microarray and RT-PCR analysis

On day 28 birds were euthanized and the ileum was removed and stored in RNA*later*® (Applied Biosystems, Austin, TX) at -20°C until use. Total RNA was isolated from ileum samples using QIAGEN RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Inc., Mississauga, Ontario). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA).

For microarray analysis, all GeneChips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). Biotinylated complimentary RNA (cRNA) was prepared from 1.0 µg of total RNA using the MessageAmp II-Biotin Enhanced Single Round aRNA Amplification kit (Applied Biosystems, Austin, TX). Ten µg of labeled cRNA was hybridized to the GeneChip® Chicken Genome Array (Affymetrix, Santa Clara, CA) for 16 hours at 45°C as described in the Affymetrix Technical Analysis Manual. GeneChips were stained with Streptavidin-Phycoerythrin, followed by an antibody solution and a second Streptavidin-Phycoerythrin solution, with all liquid handling performed by a GeneChip Fluidics Station 450, and were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix). Probe signal intensities were generated using GCOS1.3 (Affymetrix) using default values for the Statistical Expression algorithm parameters and a Target Signal of 150 for all probe sets and a Normalization Value of 1. Gene level data was generated using the RMA preprocessor in GeneSpring GX 7.3.1 (Agilent Technologies Inc.). Data were then transformed (measurements less than 0.01 set to 0.01) and normalized per chip to the 50th percentile, and per gene to control samples. Fold change and t-tests were then run using GeneSpring GX.

First-strand cDNA synthesis was carried out using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. Real-time PCR was carried out using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) on a MyiQ™ Single Color Real-Time PCR Detection

System (Bio-Rad Laboratories, Inc.) using the following conditions: denaturation 15 s at 95°C, annealing 15 s at 56 °C, and extension 30 s at 72 °C. Primers for chicken GAPDH, interferon regulatory factor (IRF)-1, IRF-7, major histocompatibility complex (MHC) beta 1 doman (BLB-1), MHC class I glycoprotein (BF-2), tumor necrosis factor (TNF) (ligand) superfamily member 15 (TNFSF15), and Toll like receptor (TLR) 3 **(Table 6)** were designed using Primer3 v.0.4.0 (Rozen and Skaletsky, 2000) and synthesized by the University of Guelph Laboratory Services Molecular

Table 6 Chicken primers used for real-time RT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)	Accession number
GAPDH	CAA CAT CAA ATG GGC AGA TG	AGC TGA GGG AGC TGA GAT GA	130	NM_204305
IRF-1	GGA GGA GTC AGC AGA ACG AC	CGC AGG ACG AGA GGT CTA AG	131	NM_205415
IRF-7	TCA GGG TGT TTT GCA CAG AG	ACC AGC TTC ACC AGG ATG AG	142	NM_205372
BLB-1	ATG CAG TGG ATA CGT TCT GC	GGT AGA AGC CCG TCA CGT AG	153	NM_001044694
BF-2	ATC GTG GTT GGT GTT GGA TT	GTG TGG ACT GTT GGC TCC TT	168	NM_001031338
TNFSF15	AAG CCA AGA GCA CAC CTG AC	CAG GTA TCA CCA GTG CGT TG	148	NM_001024578
TLR3	GAT CCA TGG TGC AGG AAG TT	GCA CAG GGG GCA CTT TAC TA	250	NM_001011691

Biology Section (Guelph, ON). Results are expressed as relative mRNA levels compared to GAPDH.

Statistical analysis

Statistical analyzes to compare MCM-B2-treated and control groups were carried out using Student's t test. Statistical significance was determined using the GraphPad Prism statistical software (San Diego, CA, USA). A P-value of less than 0.05 was defined as significant.

Results

Body weights were recorded weekly, and are summarized in **Figure 11**. No significant difference was observed between the weight gain in chicks fed the control diet and those supplemented with 0.1% MCM-B2 over 4 weeks. Likewise, no significant difference was observed in the relative organ weights of spleen **(Figure 12A)** and bursa of Fabricius **(Figure 12B)** between the two groups on day 14 or day 28. No significant histopathological changes were observed in any of the tissues examined (ileum, jejunum, caecum, spleen and bursa of Fabricius) from either control or MNB-treated animals (data not shown).

Fecal samples were collected weekly, and the concentrations of fecal total IgA levels were measured by ELISA **(Figure 13)**. While total IgA levels continued to increase throughout the duration of the trial in both groups, MNB-supplemented animals showed higher ($P < 0.05$) concentrations of fecal IgA at all time points when compared to animals fed the control diet, further supporting the immunomodulatory role of MNB.

Analysis of microarray data revealed that the ileum expression of 171 of the 38,535 genes studied was significantly influenced by the administration of MNB for four weeks. Affected genes were investigated for their involvement in biological functions and classified based on known biological functions according to Gene Ontology **(Table 7)**. Up- and down-regulated genes are summarized in **Tables 8 and 9**. The identified up- or down-regulated genes were found to be related to a number of different cellular functions **(Table 8)**. Among the genes that were significantly up-regulated by MCM-B2 administration, 30 were directly related to immune response and host defense, including MHC class I and II, TNFSF15, interferon (IFN) and IFN regulatory factors (IRF-1, IRF-7, IFIT5 and IFITM1), as well as genes involved in pathogen recognition (TLR3) and innate immunity and host defense (lysozyme and Mx protein). However, several genes classified as participating in other cellular functions, such as signal transduction and physiological processes and cellular metabolism, are also indirectly related to immune responses and were found to

be up-regulated by MCM-B2. These genes included cysteine-rich angiogenic inducer 61 (CYR61), signal transducer and activator of transcription 1 (STAT1), complement component 1, radical S-adenosyl methionine domain containing 2 (RSAD2), and CD69. As a result, the number of up-regulated genes directly or indirectly related to immune responses was 37 out of 171, or 22% of significantly influenced genes.

In order to validate the microarray data, real-time quantitative RT-PCR was carried out on six genes selected as candidate biomarkers of MNB activity. BLB-1 BF-2, IRF-1, IRF-7, TNFSF15 and TLR3 were all up-regulated ($P < 0.05$) (**Figure 14**) in the ileum of MCM-B2-treated birds, consistent with the microarray data. Their up-regulation observed here, as well as the importance of the genes in host defense and immune responses would suggest that these genes may serve as markers of the physiological and immunomodulating activities of MNB.

Discussion

Mannose-containing oligosaccharides are one of the most common commercially available prebiotics for poultry use, however, little is known about their effect on the intestinal immune system. We previously observed that chickens fed MCM including MNB had increased IgA production and improved *S. enteritidis* clearance (Agunos *et al.*, 2007), suggesting that it may act as an immunomodulatory agent to enhance innate and adaptive immune responses. In the present study, we evaluated the effect of the administration of MCM including MNB alone, without bacterial challenge, in order to elucidate the role of MNB on the intestinal immune system, and carry out a comprehensive evaluation of the effect of MNB on gene expression in the intestine by microarray analysis.

Table 7 Function-based classifications of genes for which expression in ileum were significantly influenced by administration of MCM-B2 for 28 days.

Function	Number of genes[a]	
	up-regulated	down-regulated
Immune response	30	0
Physiological processes and metabolism	16	25
Signal transduction	6	1
Protein complex and signal translation	3	0
Catalytic activity	7	14
Nucleotide binding	16	4
Others	24	21
Unknown	2	2
Total	104	67

[a] Number of genes significantly influenced by MCM-B2 administration ($P < 0.05$)

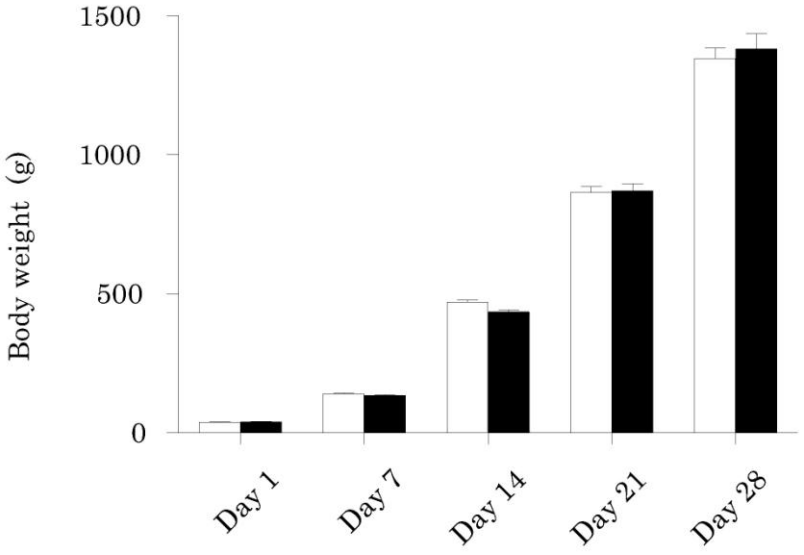


Figure 11 Change in mean body weights of chicks fed control diet (open bars) or supplemented with MCM-B2 (shaded bars) for 28 days. Bars represent means ± SEM of n = 12 (days 1, 7 and 14) or n = 6 (days 21 and 28) animals.

No significant changes were observed in body or organ weight between

MCM-B2-treated and untreated birds. This is consistent with reports by Agunos *et al.* (2007) and Juskiewicz *et al.* (2003), in which supplementation with mannose-containing oligosaccharides had no noticeable effect on weight gain.

Secretory IgA plays an important role in protecting against infection in the intestinal immune system, and its secretion was previously shown to be increased by dietary MNB in chickens infected with SE (Agunos *et al.*, 2007). Here we found that administration of MNB induced a similar increase in fecal IgA levels. This is in line with previous reports of increased IgA production in mice fed dietary FOS (Hosono *et al.*, 2003; Nakamura *et al.*, 2004), where it was observed that FOS could alter the microbial environment of the gut, and up-regulate both mucosal immune responses for protective immunity, but also systemic immune responses (Hosono *et al.*, 2003).

Among the genes that were significantly up-regulated by MNB administration, 37 (22% of all affected genes) were directly or indirectly related to immune responses. This suggests that one of the major physiological roles of MNB administration is immunomodulation in the small intestine.

Four out of the five represented genes relating to antigen presentation (both MHC I and II) and processing were up-regulated. The MHC molecule plays an important role in the regulation of the immune response by communicating among different cellular components of the immune system (Lamont, 1998). Expression of MHC class I antigen on the cell surface requires expression of multiple genes and peptide transporters associated with antigen processing, including (TAP-1) and TAP-2 (Guo *et al.*, 2002), which was found to be up-regulated here by MCM-B2, suggesting MNB may act as an essential factor for expression of MHC molecule on cell surfaces. Studies have demonstrated an association between the chicken MHC and antibody production against variety of antigens (Dunnington *et al.*, 1992; Weigend *et al.*, 2001) and Zhou and Lamont (2003) reported the genomic region bearing MHC class I and II genes had significant effects on antibody response kinetics to SRBC and *Brucella abortus* and antibody levels to SE vaccination.

Of the ten represented genes associated with host defense against organisms, MCM-B2 administration up-regulated all ten, including lysozyme, an antimicrobial protein important in innate defense which functions to protect against microbial attack in the gastrointestinal tract (Ganz, 2004), and TNFSF15, which is primarily expressed in spleen, liver, intestinal epithelial lymphocytes (IEL), peripheral blood lymphocytes and bursa (Park *et al.*, 2007), and is involved in the differentiation, proliferation and apoptosis of immune cells (Collette *et al.*, 2003). Like human TNFSF15, it has been reported that chicken TNFSF15 also possess cytotoxic activity against tumor cells and it has been reported that TNFSF15 transcripts were primarily expressed in $CD4^+$ intraepithelial lymphocytes in the duodenum and jejunum following *E. maxima* infection in chickens (Park *et al.*, 2007).

Interferons (IFN) are important cytokines which regulate antiviral, cell growth, immune-modulation and anti-tumor functions, and several IFN-related genes were found here to be up-regulated by MCM-B2, including the transcription factors IRF-1 and IRF-7. Type I interferons (IFN-α and -ß) are typically produced upon viral infection and affect the release of pro-inflammatory cytokines and nitric oxide by dendritic cells and macrophages (Bogdan, 2000). IFN and MHC II also play an important role in the expression of secretory IgA in the lamina propria, and increases in both MHC and IFN-related genes and IgA production have been observed in mice (Hosono *et al.*, 2003; Nakamura *et al.*, 2004; Fukasawa *et al.*, 2007) , and may in part explain the concomitant increase in fecal IgA levels and MHC and IFN-related gene expression observed in the present study. IRF-1 regulates the expression of several genes involved in both innate and acquired immunity. Induced by STAT1 (Saha *et al.*, 2009), it also regulates IFN-α/ß, IFN-γ, inducible nitric oxide synthase (iNOS), MHC class I molecule and beta 2 microglobulin. On the other hand, IRF-1 is induced by IFN-α/ß, IFN-γ and interleukin (IL)-12 therefore IRF-1 seems to be positioned at the intersection of different pathways leading to a Th1 response and host defense against intracellular microorganisms (Miyamoto *et al.*, 1988; Galon *et al.*, 1999). Furthermore,

Fantuzzi *et al.* (2001) reported that IRF-1 is an important regulator of IL-18 production and participates in the production of IFN-γ. IRF-7 is the master regulator of type-1 IFN-dependent immune responses, and is essential for the induction of IFN-α/ß genes via the virus-activated MyD88-independent pathway and the TLR-activated MyD88-dependent pathway (Honda *et al.*, 2005). The potent activity of IFN-α/ß against viral infections requires the expression of IFN-inducible protective genes including 2–5 oligoadenylate synthetase (OAS) and MxA protein, both of which were up-regulated by MCM-B2, and confer cellular resistance, inhibit viral replication and impede viral dissemination, as well as exerting other immunomodulatory effects (Sen and Ransohoff, 1993; van den Broek *et al.*, 1995; Hefti *et al.*, 1999) .

Interestingly, some of the up-regulated genes observed here have also been found to be up-regulated in response to infection with Marek's disease virus (MDV) *in vitro*, including interferon inducible protein, lymphocyte antigen 6 complex locus E (LY6E), and macrophage inflammatory protein (MIP) (Morgan *et al.*, 2001). Likewise, RSAD2, an IFN-inducible antiviral protein (Chin and Cresswell, 2001) was also up-regulated, further supporting the potential induction of anti-viral responses by MNB administration.

Of these up-regulated genes, 6 were chosen for validation by RT-PCR,as possible biomarkers of MNB activity. The up-regulation of BLB-1, BF-2, IRF-1, IRF-7, TNFSF15, and TLR3 observed both by microarray and RT-PCR, along with the significant up-regulation of a number of genes involved in immune response and host-defense would suggest that MCM-B2 administration may exert a combination effect on the modulation of the intestinal immune system. These results are further supported by the increased production of IgA observed here, as well as previous observations that MCM including MNB could prevent SE infection and improve integrity of the gut mucosa in MCM including MNB-fed chickens, indicating that MNB is a potent modulator of intestinal immune responses, and further work will be required to fully elucidate the mechanism of action of MNB in modulating intestinal immune responses.

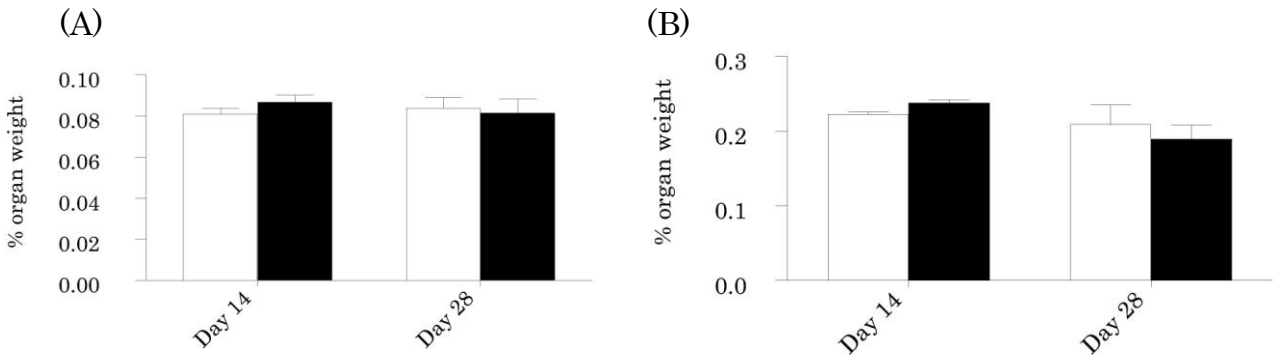


Figure 12 Weights of spleen (A) and bursa of Fabricius (B) in chicks fed control diet (open bars) or supplemented with MCM-B2 (shaded bars) for 14 or 28 days. Results are expressed as percent organ weight relative to body weight of each animal. Bars represent means ± SEM of n = 6 animals.

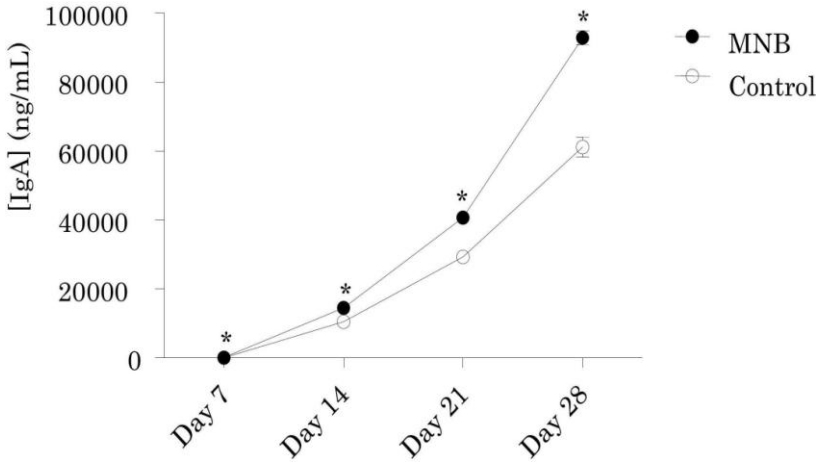


Figure 13 Fecal IgA concentrations from chicks fed control diet or supplemented with MCM-B2 for 28 days. Data points represent means ± SEM of n = 12 (days 7 and 14) or n = 6 (days 21 and 28) animals. *, $P < 0.05$.

Table 8 Genes significantly up-regulated by MNB administration.

Functional Grouping	Affymetrix ID	Gene Description	Fold change
Immune function			
Antigen presentation and processing	GGA.9239.1.S1_S_AT	similar to MHC rfp-y class i alpha chain	2.66
	GGA.417.1.S1_AT	**MHC class I glycoprotein (BF-2)[a]**	**1.83**
	GGA.5137.1.S1_X_AT	MHC class II antigen b-l beta	3.54
	GGA.517.1.S1_X_AT	**MHC class II beta 1 domain (BLB-1)**	**4.51**
Defense against organisms	GGA.131.1.S1_AT	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (MX1)	2.99
	GGA.5153.2.S1_AT	MHC class I antigen (YFV)	1.73
	GGA.418.1.S1_X_AT	major histocompatibility complex class I glycoprotein (BF2)	1.78
	GGA.12614.1.S1_AT	**tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15)**	**1.54**
	GGA.11252.1.S1_AT	similar to Small inducible cytokine A19 precursor (CCL19) (Macrophage inflammatory protein 3 beta) (MIP-3-beta) (EBI1-ligand chemokine) (ELC) (Beta chemokine exodus-3) (CK beta-11)	1.99
	GGA.3357.2.S1_A_AT	lysozyme G like 2	1.67
	GGA.536.1.S1_A_AT	2'-5'-oligoadenylate synthetase-like (OASL)	3.19
	GGA.518.1.S1_X_AT	MHC class II antigen B-F minor heavy chain (BLB1)	2.00
	GGA.9103.1.S1_AT	similar to Lysozyme G (1,4-beta-N-acetylmuramidase)	4.96
	GGAAFFX.25059.1.S1_AT	similar to Lysozyme G (1,4-beta-N-acetylmuramidase)	6.31
Interferon-related	**GGA.791.1.S1_AT**	**interferon regulatory factor 1 (IRF-1)**	**1.51**
	GGA.1087.1.S1_AT	**interferon regulatory factor 7 (IRF-7)**	**1.53**
	GGA.16457.1.S1_S_AT	interferon induced with helicase C domain 1 (IFIH1)	2.16
	GGAAFFX.21915.1.S1_AT	similar to interferon-induced protein with tetratricopeptide repeats 5 (IFIT-5)	2.22
	GGA.8227.1.S1_S_AT	putative isg12-1 protein/similar to interferon induced transmembrane protein 1 (IFITM1)	2.50
	GGA.6201.1.S1_AT	putative isg12-2 protein/similar to interferon,	2.64

		alpha-inducible protein 6	
	GGAAFFX.23449.1.S1_AT	interferon induced with helicase C domain 1	
Other immune functions	GGA.2865.1.S1_AT	transporter associated with antigen processing 2 (TAP2)	1.52
	GGA.4225.1.S1_AT	leukocyte cell-derived chemotaxin 2 (LECT2)	1.54
	GGAAFFX.8585.3.S1_S_AT	**toll-like receptor 3 (TLR3)**	**1.61**
	GGAAFFX.26422.2.S1_AT	similar to immune associated nucleotide 6; immune associated nucleotide	1.62
	GGAAFFX.20099.1.S1_AT	heat shock protein 25 (HSP25)	1.70
	GGA.985.1.S1_S_AT	heat shock protein 25	1.72
	GGA.16267.1.S1_S_AT	similar to class i alpha chain	1.86
	GGA.380.1.S1_AT	IgA h-immunoglobulin alpha heavy chain [chickens, mrna, 1866 nt]	1.96
	GGA.1171.1.S1_AT	lymphocyte antigen 6 complex locus E (LY6E)	3.57
Protein complex and signal transduction	GGAAFFX.9499.1.S1_AT	general transcription factor IIE, polypeptide 1, alpha 56kDa (GTF2E1)	1.56
	GGAAFFX.10104.2.S1_S_AT	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit D2 (ATP6V0D2)	1.76
	GGAAFFX.13119.1.S1_S_AT	ribosomal protein S14 (RPS14)	1.52
Nucleotide binding	GGA.17642.1.S1_AT	ring finger protein 213 (RNF213)	1.62
	GGA.11337.1.S1_AT	similar to Torsin B precursor (Torsin family 1 member B) (FKSG18 protein)	1.59
	GGAAFFX.25830.1.S1_AT	K(lysine) acetyltransferase 2A (KAT2A)	1.64
	GGA.13972.1.S1_S_AT	lupus brain antigen 1 (LBA1)	1.58
	GGAAFFX.8378.1.S1_S_AT	similar to immune associated nucleotide 6; immune associated nucleotide	1.66
	GGAAFFX.22137.1.S1_AT	PX domain containing serine/threonine kinase (PXK)	1.54
	GGA.4870.3.S1_A_AT	actin, alpha, cardiac muscle 1 (ATCTC1)	1.81
	GGA.9292.1.S1_AT	eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2)	1.55
	GGA.8244.1.S1_AT	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial (CMPK2)	3.29
	GGAAFFX.22996.1.S1_AT	similar to hypothetical protein flj20035	4.08
	GGA.9486.1.S1_S_AT	La ribonucleoprotein domain family, member 5 (LARP5)	1.66

	GGAAFFX.6546.1.S1_AT	similar to hect domain and RLD 5; cyclin-E binding protein 1	2.75
	GGAAFFX.11512.1.S1_AT	TRAF-type zinc finger domain containing 1 (TRAFD1)	1.51
	GGAAFFX.23767.1.S1_AT	lupus brain antigen 1 (LBA1)	1.99
	GGAAFFX.6052.1.S1_S_AT	hypothetical LOC422427	4.92
	GGAAFFX.10498.1.S1_AT	radical S-adenosyl methionine domain containing 2 (RSAD2)	5.11
Signal transduction	GGA.2200.1.S1_AT	cysteine-rich, angiogenic inducer, 61 (CYR61)	1.99
	GGA.701.1.S1_AT	leukocyte ribonuclease A-2 (RSFR)	1.55
	GGA.729.1.S1_AT	mature avidin	2.24
	GGA.11597.1.S1_S_AT	signal transducer and activator of transcription 1, 91kDa (RCJMB04_17i9)	1.93
	GGAAFFX.22313.1.S1_S_AT	signal transducer and activator of transcription 1	1.88
	GGA.1182.1.S1_S_AT	Mov10, Moloney leukemia virus 10, homolog (mouse) (RCJMB04_17i9)	1.64
Physiological processes and cellular metabolisim	GGAAFFX.9256.1.S1_S_AT	complement component 1, s subcomponent (C1S)	1.51
	GGAAFFX.7658.1.S1_AT	poly (ADP-ribose) polymerase family, member 9 (PARP9)	1.54
	GGAAFFX.24484.2.S1_S_AT	complement component 1, r subcomponent (C1R)	1.73
	GGAAFFX.20966.1.S1_S_AT	similar to hect domain and RLD 5; cyclin-E binding protein 1	2.36
	GGAAFFX.20602.1.S1_AT	zinc finger CCCH-type, antiviral 1 (RCJMB04_23i8)	1.58
	GGA.990.1.S1_AT	hypothetical protein LOC770777	3.75
	GGA.13502.1.S1_AT	PHD finger protein 11 (PHF11)	1.67
	GGAAFFX.6883.4.S1_S_AT	hook homolog 1 (Drosophila) (HOOK1)	1.59
	GGAAFFX.26344.1.S1_AT	zinc finger, NFX1-type containing 1 (ZNFX1)	2.12
	GGA.1111.1.S1_A_AT	ubiquitin specific peptidase 18 (USP18)	2.92
	GGAAFFX.13227.1.S1_S_AT	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit D2	1.53
	GGA.11554.1.S1_AT	asparagine-linked glycosylation 2 homolog (S. cerevisiae, alpha-1,3-mannosyltransferase) (ALG2)	1.68
	GGA.3236.1.S1_AT	PHD finger protein 11 (PHF11)	1.54
	GGA.10666.1.S1_AT	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G3	1.55
	GGAAFFX.21777.1.S1_S_AT	intestinal zipper protein	2.27

	GGAAFFX.6058.1.S1_AT	aminoadipate aminotransferase (AADAT)	1.59
Sugar-binding	GGA.4900.6.S1_X_AT	CD69 molecule /// similar to C-type lectin-like receptor	1.57
Catalytic activity	GGA.12925.1.S1_AT	structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1)	1.64
	GGAAFFX.10187.1.S1_AT	dpy-19-like 4 (C. elegans) (DPY19L4)	1.52
	GGAAFFX.25763.1.S1_AT	tripartite motif-containing 25 (TRIM25)	1.63
	GGA.1746.1.S1_S_AT	similar to kinesin, putative	2.58
	GGA.12561.1.A1_AT	dermatan sulfate epimerase (DSE)	2.07
	GGAAFFX.1838.1.S1_AT	motilin (MLN)	1.73
	GGAAFFX.5967.1.S1_AT	sterile alpha motif domain containing 9-like (SAMD9L)	10.02
Others	GGA.12409.1.S1_S_AT	similar to gag/env fusion protein	2.06
	GGAAFFX.4249.1.S1_X_AT	WAP four-disulfide core domain 2 (WFDC2)	1.93
	GGA.10351.1.S1_S_AT	chromosome 19 open reading frame 12 /// similar to RIKEN cDNA 1600014C10	1.90
	GGA.10903.1.S1_AT	finished cdna, clone chest789c2	1.91
	GGA.11753.1.S1_AT	finished cdna, clone chest393a1	2.92
	GGA.12098.1.S1_A_AT	hypothetical gene supported by BX933262; BX935476; CR406074	1.59
	GGA.12360.1.S1_AT	transmembrane protein 213 (TMEM213)	1.61
	GGA.13146.1.S1_AT	epithelial stromal interaction 1 (breast) (EPSTI1)	2.17
	GGA.13280.1.S1_AT	hypothetical protein LOC768499	1.50
	GGA.14299.1.S1_AT	finished cdna, clone chest149k6	4.44
	GGA.1442.1.S1_AT	transcribed locus	1.73
	GGA.15728.1.S1_AT	RCSD domain containing 1	1.66
	GGA.16084.1.S1_AT	similar to promyelocytic leukemia protein	1.63
	GGA.16635.1.S1_AT	sperm associated antigen 4 (SPAG4)	1.88
	GGA.16860.1.S1_AT	hypothetical gene supported by CR391572	1.57
	GGA.3263.1.S1_AT	hypothetical gene supported by cr391572	3.26
	GGA.4590.1.S2_AT	finished cdna, clone chest75j21	1.65
	GGA.4832.1.S1_AT	hypothetical protein LOC770612	1.82
	GGA.6433.1.S1_AT	claudin 2 (CLDN2)	1.60
	GGA.6770.2.S1_A_AT	loc419325	1.53
	GGA.9930.1.S1_AT	finished cdna, clone chest927g24	2.27

	GGAAFFX.10735.1.S1_AT	MIT, microtubule interacting and transport, domain containing 1 (MITD1)	1.56
	GGAAFFX.20778.1.S1_AT	coiled-coil domain containing 107 (CCDC107)	1.91
	GGAAFFX.21332.1.S1_S_AT	similar to hypothetical protein flj20035	6.59
Unknown	AY013303.CDS3.S1_S_AT	loc417860	2.96
	GGA.14508.1.S1_AT	DnaJ (Hsp40) homolog, subfamily B, member 5 (DNAJB5)	1.58

[a] Bold text indicates genes chosen for further analysis by real-time RT-PCR

Table 9 Genes significantly down-regulated by MNB administration.

Functional Grouping	Affymetrix ID	Gene Description	Fold change
Physiological processes and cellular metabolisim	GGAAFFX.22947.1.S1_S_AT	sucrase-isomaltase (alpha-glucosidase) (SI)	-2.15
	GGA.4248.1.S1_AT	lipoprotein lipase (LPL)	-1.63
	GGA.8817.1.S1_S_AT	farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	-1.64
	GGAAFFX.26670.1.S1_AT	solute carrier family 5 (sodium/glucose cotransporter) (SLC5A11)	-1.50
	GGA.10771.1.S1_S_AT	membrane metallo-endopeptidase (MME)	-1.89
	GGA.4447.1.S1_AT	phosphoenolpyruvate carboxykinase 1 (soluble) (PCK1)	-1.64
	GGA.11459.1.S1_AT	hexokinase domain containing 1 (HKDC1)	-1.52
	GGAAFFX.711.1.A1_AT	ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (ST3GAL4)	-1.63
	GGA.8851.1.S1_A_AT	isopentenyl-diphosphate delta isomerase 1 (IDI1)	-1.60
	GGAAFFX.8317.3.S1_AT	dopa decarboxylase (aromatic L-amino acid decarboxylase) (DDC)	-1.54
	GGAAFFX.23557.10.S1_S_AT	solute carrier family 4, sodium bicarbonate cotransporter, member 7 (SLC4A7)	-1.79
	GGAAFFX.3997.1.S1_S_AT	ADP-ribosyltransferase 1 /// GPI-anchored ADP-ribosyltransferase (ART1 /// ART7B)	-1.64
	GGAAFFX.10596.2.S1_S_AT	BMX non-receptor tyrosine kinase (BMX)	-1.88
	GGAAFFX.11774.1.S1_AT	serine/threonine kinase 38 like (STK38L)	-1.64
	GGAAFFX.8887.1.S1_S_AT	vanin 1 /// vanin 1 (RCJMB04_35g11 /// VNN1)	-1.76
	GGA.2896.1.S1_AT	carbonyl reductase 1 (CBR1)	-1.58

	GGAAFFX.7796.1.S1_AT	solute carrier family 39 (zinc transporter), member 8 (SLC39A8)	-1.50
	GGAAFFX.20374.1.S1_AT	succinate-CoA ligase, GDP-forming, beta subunit (SUCLG2)	-4.33
	GGA.4990.1.S1_AT	collagen, type IX, alpha 1 (COL9A1)	-1.62
	GGAAFFX.25502.1.S1_S_AT	solute carrier family 2 (facilitated glucose/fructose transporter), member 5 (SLC2A5)	-1.65
	GGAAFFX.7519.2.S1_S_AT	solute carrier family 6 (proline IMINO transporter), member 20 (SLC6A20)	-4.37
	GGAAFFX.6121.1.S1_S_AT	asparagine synthetase (ASNS)	-1.54
	GGAAFFX.24270.1.S1_AT	dermatan sulfate epimerase-like (DSEL)	-1.50
	GGAAFFX.25206.4.S1_S_AT	folate hydrolase (prostate-specific membrane antigen) 1 (FOLH1)	-2.03
	GGAAFFX.9792.1.S1_AT	similar to methionine adenosyltransferase ii, alpha	-1.52
Signal transduction	GGA.13328.1.S1_AT	similar to nuclear hormone receptor nor-1 (neuron-derived orphan receptor 1) (mitogen induced nuclear orphan receptor)	-1.99
Nucleotide binding	GGA.5413.1.S1_AT	DNAJ (Hsp40) homolog, subfamily C, member 15 (DNAJC15)	-1.51
	GGA.6183.1.S1_AT	regenerating islet-derived family, member 4 (REG4)	-1.63
	GGA.4939.1.S1_S_AT	fatty acid binding protein 4, adipocyte (FABP4)	-4.46
	GGA.10658.1.S1_AT	similar to kiaa2019 protein	-1.53
Catalytic activity	GGA.9991.1.S1_AT	sorbitol dehydrogenase (SORD)	-1.52
	GGA.1388.1.S1_S_AT	alcohol dehydrogenase 1B (class I), beta polypeptide (ADH1B)	-2.04
	GGAAFFX.5988.1.S1_S_AT	sucrase-isomaltase (alpha-glucosidase) (SI)	-1.65
	GGAAFFX.4985.1.S1_AT	similar to apical early endosomal glycoprotein precursor	-1.79
	GGA.8880.2.S1_S_AT	membrane metallo-endopeptidase (MME)	-1.68
	GGAAFFX.24663.2.S1_S_AT	similar to huntingtin-interacting protein-1 protein interactor; vestrogen-related receptor beta like 1	-1.51
	GGA.14230.1.S1_AT	dermatan sulfate epimerase-like (DSEL)	-1.60
	GGA.14579.1.S1_AT	cubilin (intrinsic factor-cobalamin receptor) (CUBN)	-1.66
	GGA.11227.1.S1_AT	bone marrow stromal cell antigen 1 (BST1)	-1.55
	GGA.19409.1.S1_AT	collagen, type IV, alpha 2 (COL4A2)	-1.50

	GGAAFFX.8477.1.S1_AT	solute carrier family 5 (sodium/glucose cotransporter), member 12 (SLC5A12)	-2.62
	GGA.4115.1.S1_AT	alcohol dehydrogenase 1B (class I), beta polypeptide /// alcohol dehydrogenase 1C (class I), gamma polypeptide (ADH1B /// ADH1C)	-1.63
	GGAAFFX.23840.1.S1_S_AT	alcohol dehydrogenase 1C (class I), gamma polypeptide (ADH1C)	-1.50
	GGA.3415.1.S1_AT	similar to alcohol dehydrogenase adh-f	-1.81
Others	GGA.4195.1.A1_AT	Similar to BWK-1	-2.22
	GGA.6324.1.S1_AT	retinol binding protein 2, cellular (RBP2)	-2.61
	GGA.9386.1.S1_AT	retinol binding protein 7, cellular (RBP7)	-1.61
	GGA.10858.1.S1_S_AT	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1)	-2.15
	GGA.12648.1.S1_AT	six transmembrane epithelial antigen of the prostate 1 (STEAP1)	-1.57
	GGA.16172.1.S1_S_AT	hypothetical gene supported by CR385555	-1.91
	GGA.16552.2.S1_A_AT	hypothetical gene supported by CR387685	-1.59
	GGAAFFX.11018.1.S1_S_AT	matrix metallopeptidase 1 (interstitial collagenase) (MMP1)	-1.63
	GGA.10034.2.S1_A_AT	Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2)	-1.54
	GGA.11602.1.S1_AT	transcribed locus	-1.54
	GGA.16043.1.S1_S_AT	CSRP2 binding protein (CSRP2BP)	-1.58
	GGA.1725.2.S1_A_AT	finished cdna, clone chest592m12	-1.58
	GGA.17737.1.S1_AT	finished cdna, clone chest1025a6	-1.66
	GGA.19101.1.S1_AT	finished cdna, clone chest926n8	-1.69
	GGA.19476.1.S1_AT	finished cdna, clone chest883k3	-1.62
	GGA.2890.1.S1_AT	finished cdna, clone chest1021c11	-2.18
	GGA.6292.1.S1_A_AT	transmembrane 4 L six family member 4 (TM4SF4)	-1.81
	GGA.6666.1.S1_AT	similar to serine protease inhibitor Kazal type 9	-1.63
	GGA.8082.1.S1_AT	RNA binding motif protein 45 (RBM45)	-1.51
	GGA.8540.1.S1_AT	finished cdna, clone chest879k19	-1.68
	GGAAFFX.9703.1.S1_AT	hypothetical protein LOC770012	-1.54
Unknown	GGAAFFX.1602.1.A1_X_AT		-1.51
	GGA.9024.1.S1_AT	chromosome 8 open reading frame 22	-1.51

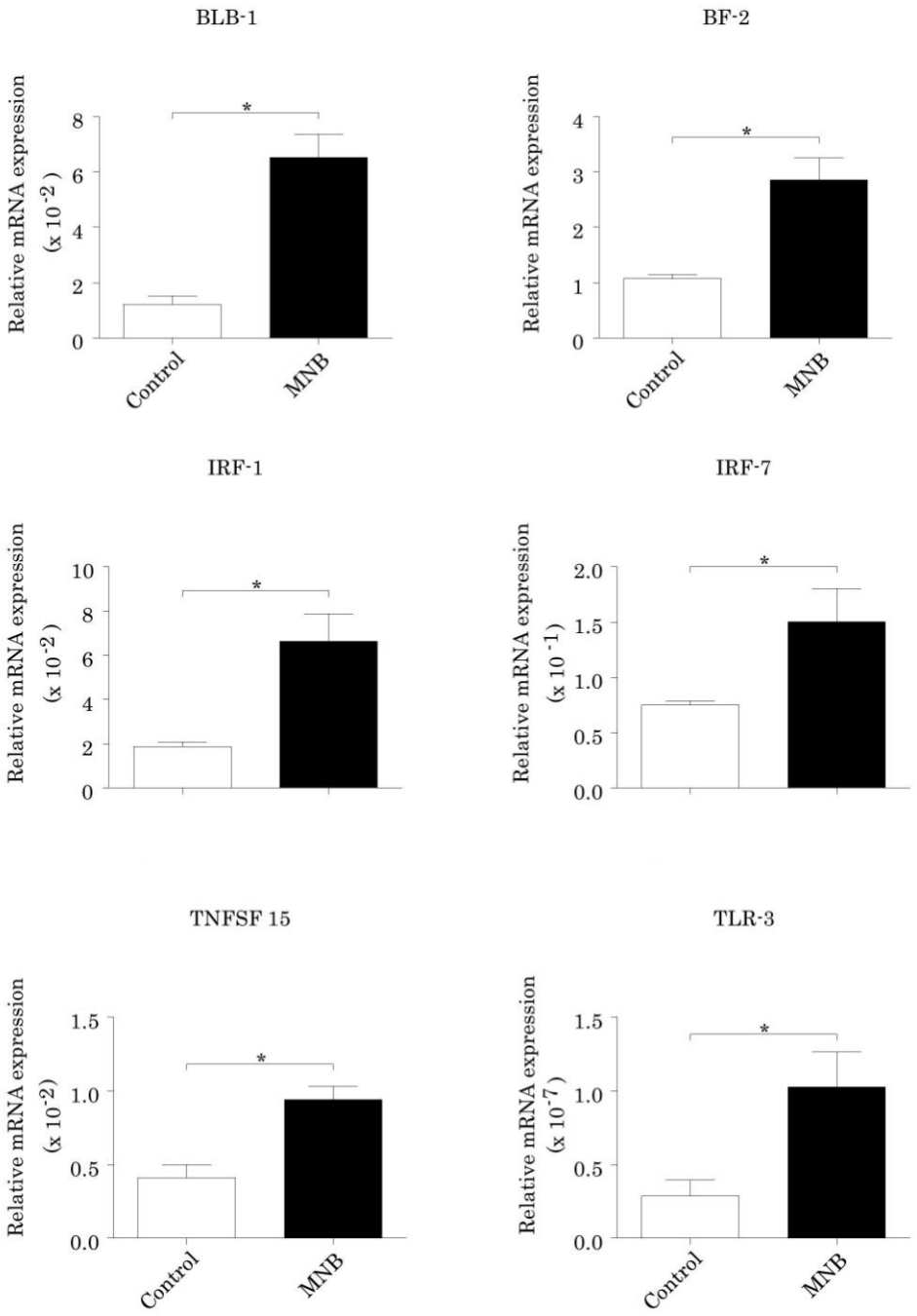


Figure 14 Relative mRNA levels of B-LB1, BF-2, IRF-1, IRF-7, TNFSF 15 and TLR3 in the ilea of chicks fed control diet (open bars) or supplemented with 0.1% MCM-B2 (shaded bars) for 28 days. Bars represent means ± SEM of n = 6 animals. *, $P < 0.05$.

Chapter Ⅳ

β 1,4 mannobiose (MNB) enhances *Salmonella*-killing activity and activates innate immune responses in chicken macrophages

Introduction

Salmonella enteritidis (SE) is a foodborne-illness pathogen that negatively affects both animal and human health. SE can infect poultry, and is one of the most common causes of food poisoning in the United States. Bans or reductions in antibiotic use have necessitated the identification of novel compounds for the control of SE (Malek *et al.*, 2004). Recent trends in food animal health research suggest an increased awareness and need of 'natural' dietary additives versus chemicals or antibiotics for the purpose of improving animal health and performance (Al-Batshan *et al.*, 2001). Food-derived compounds that can stimulate or enhance innate immune responses to pathogens are therefore an attractive alternative to antibiotics.

Macrophages are a central arm of the innate immune defense system against intracellular pathogens. Stimulated macrophages undergo a process of activation involving an increase in size and motility, enhanced phagocytic potential, bactericidal, and tumoricidal activity (Bliss *et al.*, 2005). Among the antimicrobial mechanisms associated with macrophages, the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as H_2O_2 and NO, plays a major role in intracellular bacterial killing (Lim *et al.*, 2004). Despite this array of antimicrobial activity of macrophages, some intracellular bacteria, including *Salmonella*, *Leishmania* and *Mycobacteria*, are able to survive within the phagosome. Activated macrophages, therefore, depend on a complex array of oxygen-dependent antimicrobial molecules to inhibit or kill intracellular *Salmonella*. An early phase of *Salmonella* killing is mediated through contribution of the NADPH phagocyte oxidase (phox, NOX-1) and subsequent sustained bacteriostatic effect, which is dependent on inducible nitric oxide synthase (iNOS), responsible for the production of NO (Vazquez-Torres *et al.*, 1999). In mice, a single dominant gene termed natural

resistance associated macrophage protein 1 (NRAMP1) controls innate resistance to such pathogens (Alter-Koltunoff *et al.*, 2008). The NRAMP1 gene is exclusively expressed in monocytes/macrophages and its importance in killing the intraphagosomal pathogens was reported both *in vitro* and *in vivo* (Vidal *et al.*, 1993; White *et al.*, 2005).

Prebiotics, including mannan oligosaccharides (MOS), one of the most popular commercially available prebiotics for poultry use (Ferket, 2004), have shown considerable promise for modulating innate immune responses in the gut and preventing bacterial infection. We previously demonstrated that MCM including MNB could act as an immunomdulatory agent *in vivo*, preventing SE infection in broilers by increasing IgA production and improving SE clearance (Agunos *et al.*, 2007). The objectives of the present study were to examine the *in vitro* innate immunomodulating effects of MNB using the chicken macrophage cell line MQ-NCSU. MNB-induced phagocytic and *Salmonella*-killing activity, production of antimicrobial effectors, and expression of key genes involved in antimicrobial and innate host defense mechanisms were examined in order to elucidate the mechanism of MNB-mediated *Salmonella* prevention *in vivo*.

Materials and Methods

Preparation of MNB

Crude MNB was prepared from Philippine coconut flour. Coconut flour was defatted with 2 g/g hexane, and then suspended in 10 mL/g distilled water at 80°C for 2 hrs. The solid was collected by centrifugation at 10,000 × *g* for 20 min, then suspended in 10 mL/g distilled water at room temperature for 10 min, and again collected by centrifugation. These steps were repeated, and the solid was hydrolyzed with ß-mannanase (Shin-Nihon Chemical Co Ltd., Aichi, Japan). Following centrifugation at 10,000 × *g* for 20 min, the supernatant was lyophilized and dissolved in deionized water at 20% (w/v). This crude sample was used for further purification. To obtain highly purified MNB, 5 ml of 20% (w/v) crude MNB solution was applied to a Bio-Gel P2 fine gel filtration column (25 Φ × 1 m; Bio-Rad Laboratories, Hercules CA, USA). The sample was

eluted with distilled water at 0.5 ml/min, and 4-ml fractions were collected. Saccharide concentrations were measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956) and mannobiose-containing fractions were combined and lyophilized. According to this method, 99% pure MNB was obtained and its purity and structure were confirmed by HPLC, MADLI-TOF-MS and NMR spectroscopy (data not shown).

Cells and culture conditions

MQ-NCSU cells, a macrophage-like cell line originally derived from spleen cells of leghorn pullets challenged with the JM/102W strain of Marek's disease virus (Qureshi *et al.*, 1990), were a gift from North Carolina State University. Cells were cultured as described previously by Kramer *et al.* (2003b). Briefly, cells were grown in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 5% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO), 8% FBS (**PAA Laboratories Inc.,** Etobicoke, ON), 10% chicken serum (Sigma-Aldrich), 50 IU/ml penicillin, 50 µg/ml penicillin-streptomycin (Invitrogen Corp.), and 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich) at 41°C in a 5% humidified CO_2 incubator.

Salmonella enterica serotype *Enteritidis* PT4 was inoculated into Brain Heart Infusion (BHI) broth (DIFCO/Beckton Dickinson, Sparks, MD) and grown overnight at 37°C with shaking. Overnight cultures were then diluted in RPMI 1640 medium and incubated for 3 hrs, to yield approximately 1 x 10^8 colony forming units (CFU)/ml. Total yield was confirmed by plating and colony counting on Brilliant Green Agar (BGA) (DIFCO/Beckton Dickinson).

Phagocytosis assay

Phagocytosis assays were carried out using a Vybrant™ Phagocytosis Assay Kit according to the manufacturer's instructions (Molecular Probes Inc., Eugene, OR). Briefly, MQ-NCSU cells were seeded at a density of 1 x 10^5 cells/well in 96-well black polystyrene plates (Corning Inc., Corning, NY), and allowed to adhere for 1 hr. MNB was added at the indicated concentrations, and

baseline levels of uptake were measured using untreated cells. Cells were also stimulated with SE LPS (0.5 μg/mL) (Sigma-Aldrich) as a positive control. Culture medium was removed and fluorescein-labeled *E. coli* BioParticles® (Molecular Probes) were added. Cells were incubated for 2 hrs, following which medium and BioParticles were removed and 200 µL/well of trypan blue solution (1.25 mg/mL) was added. After 10 min at 41°C the trypan blue solution was removed and the plate was read in a fluorescence plate reader using 480 nm excitation and 520 nm emission. Results are expressed as percent phagocytosis relative to untreated cells.

Salmonella-killing activity of chicken macrophages

The effect of MNB on *Salmonella*-killing activity was measured using a viable count assay of SE in macrophages stimulated with MNB, modified from protocols described by Kramer *et al.* (2003b) and Chadfield and Hinton (2004). MQ-NCSU cells were seeded at 5 x 10^5 cells/well in 24-well culture plates (Corning Inc.) and treated for 2 hrs with MNB at the indicated concentrations. Medium was removed and SE was added at a multiplicity of infection (MOI) of 100, and incubated for 30 min at 37°C to allow bacterial adhesion and colonization. Gentamicin (100 µg/mL) (Life Technologies Corp., Carlsbad, CA, USA) was then added to kill extracellular SE. Cells were washed twice with PBS containing 5% FBS, and 1 mL/well of RPMI 1640 supplemented with 10% FBS, 10% chicken serum and 10 µg/mL gentamicin was added to prevent re-infection with SE and to prevent growth of SE in the medium derived from the release of SE from dead cells. The number of intracellular bacteria at indicated time points post infection was determined by lysing cells with 1 mL of PBS containing 0.2% (w/v) saponin (Sigma-Aldrich) or 200 µg of 0.1% Triton X-100 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then plating 10-fold dilutions on BGA in order to enumerate viable SE.

Quantification of nitrite production

Nitrite production, an indicator of NO synthesis, was measured in the culture supernatant as previously described by Kleinerman *et al.* (1987) with modifications. Cells were seeded at 1 x 10^5 cells/well in 96-well tissue culture plates (Corning Inc.) and cultured in the presence of MNB for 48 hrs at the indicated concentrations. Culture medium alone was used as control. After 48 hrs, 100 µL of Griess reagent (1% sulphanilamide and 0.1% naphthylenediamide in 5% phosphoric acid) (Sigma-Aldrich) was added to 100 µL of culture supernatant and absorbance at 570 nm was measured using a microplate reader. Nitrite concentrations were determined from a standard curve of sodium nitrite (Sigma-Aldrich) in culture medium.

Quantification of H_2O_2 production

H_2O_2 production was measured using an Amplex® Red Hydrogen Peroxide Kit (Molecular probes, Eugene, OR, USA). Cells were seeded at 1 x 10^5 cells/well in 96-well tissue culture plates and cultured in the presence of MNB for 24 hrs at the indicated concentrations. Culture medium alone was used as control. After 24 hrs, the culture medium was aspirated and cells were washed with cold PBS. 100 µL of Amplex Red working reagent (50 µM Amplex Red and 0.1 U/ml HRP) in Krebs-Ringer phosphate buffer (KRPG, 145mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl, 1.22mM $MgSO_4$, 5.5 mM glucose, pH 7.35) was added to the cells and absorbance at 570 nm was measured using a microplate reader. H_2O_2 concentrations were determined from a standard curve of H_2O_2 in KRPG.

RNA extraction and real-time RT-PCR

The effect of MNB on gene expression was examined by real time quantitative PCR. MQ-NCSU cells were seeded at 2.5 x 10^5 cells/well in 24-well plates and incubated with 40 µg/mL MNB in a total volume of 0.5 mL medium for 16 hrs at 41°C. Total RNA was extracted from the cells using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories) according to the manufacturer's

instructions. The quantity and quality of the RNA was verified by spectrophotometry and gel electrophoresis. First-strand cDNA synthesis was carried out using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions, and 100 ng cDNA was used for each real-time PCR reaction. Real-time PCR was carried out using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) on a MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using the following conditions: denaturation 15 s at 95°C, annealing 15 s at 56 °C, and extension 30 s at 72 °C. Chicken primers were designed using Primer3 v.0.4.0 (Rozen and Skaletsky, 2000) and synthesized by the University of Guelph Laboratory Services Molecular Biology Section (Guelph, ON), and were used at a final concentration of 400 nM per reaction. The primers used were as follows: GAPDH, 5'- CAA CAT CAA ATG GGC AGA TG-3' and 5'-AGC TGA GGG AGC TGA GAT GA-3' (NM_204305); iNOS, 5'- GGC TGT GCT TCA TAG CTT CC-3' and 5'- TAT GCT CCC CGA CAT AGG AG'3' (NM_204961); NOX 1, 5'- CAT GGT CAC ATC CTC CAC TG-3' and 5'- CACCTCCTTCATGCTCTCCT-3' (NM_001101830); NRAMP, 5'- CTG CAC TCC TCA TTG GTG AA-3' and 5'- GCC ATG ACG AAG AGG TTG AT-3' (NM_204964); LITAF, 5'- TTC AGA TGA GTT GCC CTT CC-3' and 5'- TCA GAG CAT CAA CGC AAA AG-3' (NM_204267); IFN-γ, 5'-GGC GTG AAG AAG GTG AAA GA-3' and 5'-TCC TTT TGA AAC TCG GAG GA-3' (NM_205149). Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method using GAPDH as internal control, and results were expressed as fold change relative to untreated (control) cells.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Comparisons between treatment groups were performed with one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Results are presented as mean values ± SEM of three independent experiments. Results were considered significant if $P < 0.05$.

Results and discussion

Examination of the phagocytic response of MQ-NCSU cells in the presence of MNB revealed that pre-treatment of the cells with MNB significantly increased phagocytosis ($P < 0.05$) when compared to cells alone, to levels similar to that elicited by LPS (**Figure. 1**). A >2-fold phagocytic increase was observed in cells treated with MNB when compared to untreated cells. The improvement in such non-specific phagocytosis not only has implications for *Salmonella* pathogenesis, but also suggests that *in vivo*, macrophages may be better able to trap foreign antigens and initiate the adaptive phase of the immune response by antigen processing and presentation (Lim *et al.*, 2004).

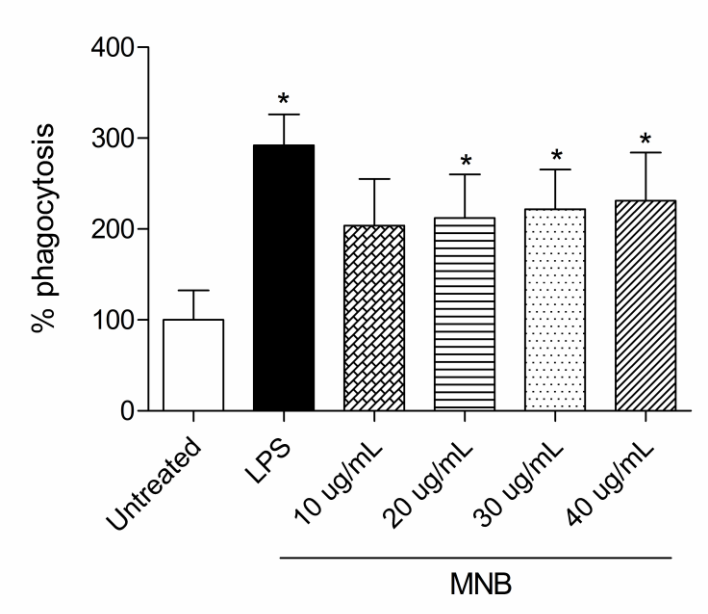


Figure 15 Effect of MNB on phagocytic activity of chicken macrophages. MQ-NCSU cells were treated with increasing concentrations of MNB for 2hrs, followed by incubation with fluorescein-labeled *E. coli* BioParticles. Data shown are mean ± SEM. Results are expressed as % phagocytosis relative to untreated cells. *$P <$ 0.05 compared to untreated cells.

MNB time- and dose-dependently increased the *Salmonella*-killing activity of macrophages, with the most marked reduction in SE viability seen at 40 μg/mL after 48 hrs (**Table 10**), where a >2-fold reduction in log CFU values

was observed when compared to untreated control cells infected with SE, which has previously shown to be highly invasive and capable of survival within MQ-NCSU cells (Withanage *et al.*, 2005).

Table 10 Effect of MNB treatment on *Salmonella*-killing activity of chicken macrophage cells. Values represent changes in mean CFU (log10) of *S. enteritidis* PT4 during phagocytosis with MQ-NCSU cells after pretreatment with MNB (±SEM, n=3).

Time (hr)	Treatment						
			MNB (μg/mL)				
	Control[a]	Control[b]	1	5	10	20	40
1	7.80	7.79	7.88	7.95	7.71	7.89	7.66
4	7.48 ± 0.04	7.65 ± 0.29	7.48 ± 0.14	6.88 ± 0.17	6.10 ± 0.32	6.06 ± 0.22	6.10 ± 0.30
12	7.56 ± 0.06	6.95 ± 0.18	7.48 ± 0.07	6.11 ± 0.07	6.06 ± 0.17	4.78 ± 0.11	4.85 ± 0.29
24	7.61 ± 0.12	6.12 ± 0.19	6.44 ± 0.11	5.78 ± 0.16	5.14 ± 0.09	4.11 ± 0.29	3.69 ± 0.16
48	7.45 ± 0.07	6.05 ± 0.12	6.10 ± 0.15	5.28 ± 0.14	4.35 ± 0.05	3.78 ± 0.33	3.33 ± 0.19

[a]SE alone

[b]MQ-NCSU cells infected with SE, no MNB added

Macrophages exert their potent antimicrobial effects by the release of a number of cytotoxic/cytostatic factors such as reactive oxygen species (ROS), such as H_2O_2 and superoxide, as well as reactive nitrogen species (RNS) and other enzymes and chemical mediators which can result in the destruction of intracellular pathogens (Lim *et al.*, 2004). The importance of both oxidative and nitrosative responses in the clearance of *Salmonella* infection is well known, and has been demonstrated *in vivo*, where mice deficient in both NADPH phagocyte oxidase (NOX-1) and inducible nitric oxide (NO) synthase (iNOS) were unable to clear infection with *Salmonella* (Mastroeni *et al.*, 2000).

Furthermore, the reaction of RNS with ROS generates peroxynitrite, a reactive molecule with potent antimicrobial activity against *S. typhimurium in vitro* (Nathan and Shiloh, 2000). As would be expected, MNB-induced *Salmonella*-killing activity correlated with increases in both nitrite and H_2O_2 production by MQ-NCSU cells. MNB treatment dose-dependently increased both H_2O_2 (**Figure. 16A**) and nitrite (**Figure. 16B**) production. When compared to untreated cells, MNB-induced H_2O_2 production was significantly higher ($P < 0.05$) at doses greater than 10 μg/mL, (resulting in 17-fold to 33-fold increases in H_2O_2 production). MNB-induced nitrite production was also significantly higher when compared to untreated cells, at all doses tested ($P < 0.05$ for 10 and 20 μg/mL; $P < 0.001$ for 30 and 40 μg/mL), resulting in a >4-fold increase in NO production when compared to untreated cells.

In order to further examine the innate immune-modulating effects of MNB on macrophages, MQ-NCSU cells were treated with MNB and the expression of several genes involved in innate immunity and antibacterial activity were measured. MNB-treatment significantly increased expression of iNOS ($P < 0.01$), NOX-1 ($P < 0.05$), LITAF ($P < 0.05$), NRAMP1 ($P < 0.05$), and IFN-γ ($P < 0.05$) when compared to untreated cells (**Figure 17**). MNB-induced gene expression was equivalent to, if not higher than, that induced by LPS, indicating that like LPS, MNB may be capable of stimulating early response of macrophages to bacterial infection.

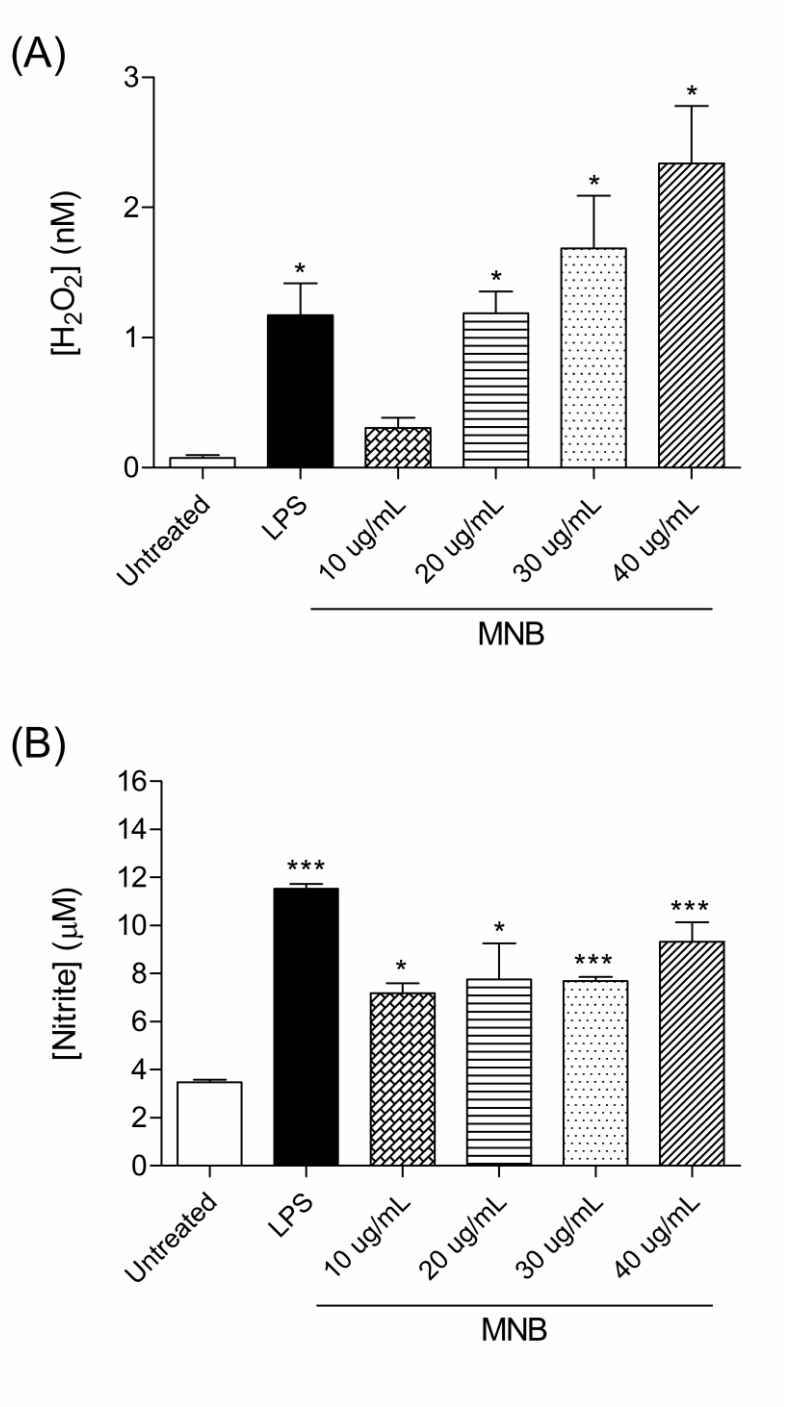


Figure 16 Effect of MNB on H_2O_2 (A) and NO (B) production in chicken macrophages. MQ-NCSU cells were treated with increasing concentrations of MNB for 24 (H_2O_2) or 48 (NO) hrs. Data shown are mean ± SEM of three independent experiments. *$P < 0.05$, ***$P < 0.001$ compared to untreated cells.

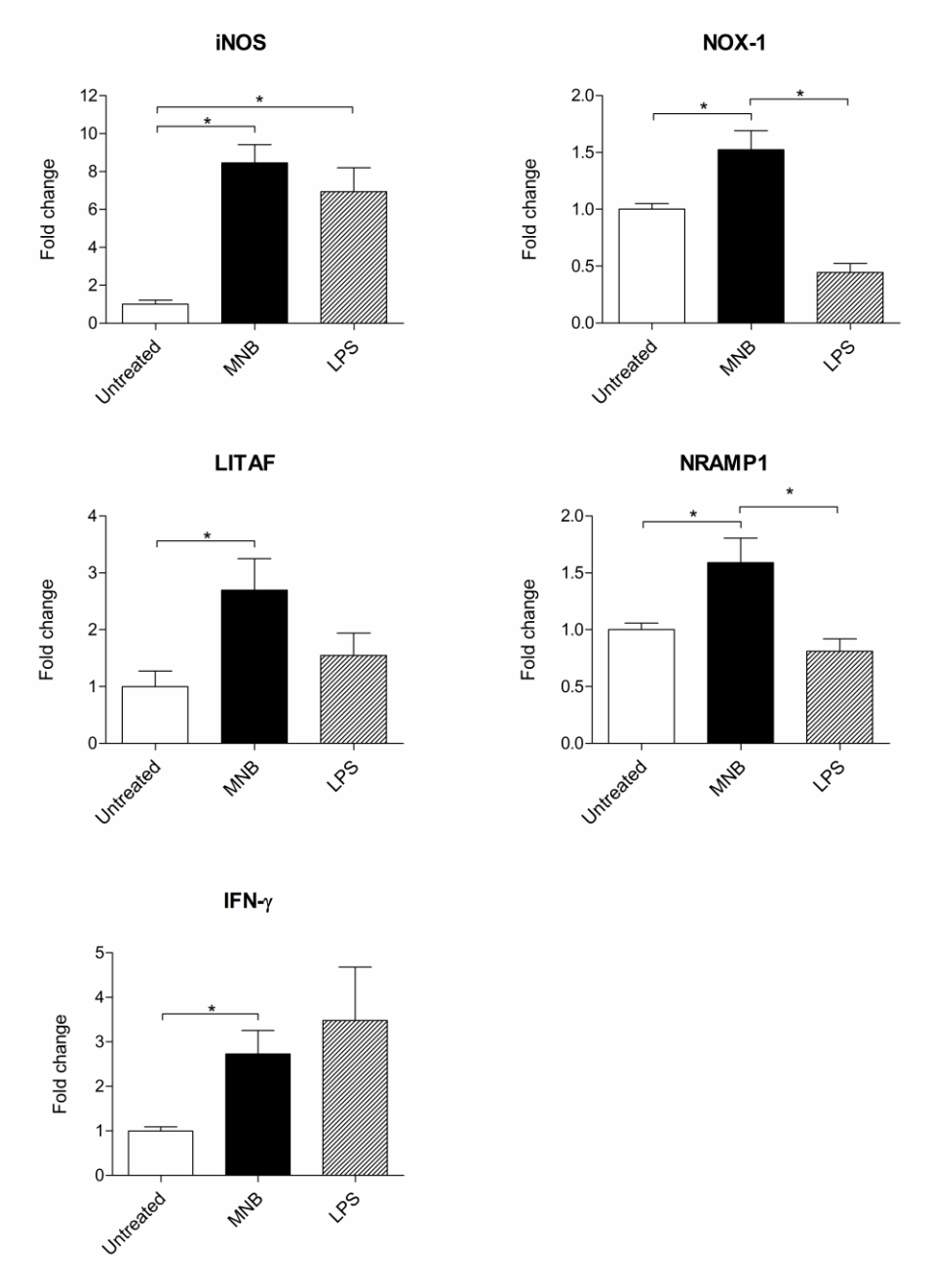


Figure 17 Effect of MNB on gene expression of iNOS, NOX-1, LITAF, NRAMP1, and INF-γ in chicken macrophages. MQ-NCSU cells were treated with 40 μg/mL MNB for 16 hrs, and total RNA was isolated and gene expression analyzed by real-time RT-PCR. Results are expressed as fold change relative to untreated (control) cells. $*P < 0.05$.

Both NOX-1 and iNOS have been found to be required for effective host resistance against *Salmonella in vivo* in mice (Rosenberger and Finlay, 2002)

and mRNA expression of both of these genes was significantly increased following MNB treatment when compared to cells alone. NOX-1 activity produces superoxide that can lead to the generation of other toxic reactive oxygen intermediates, such as H_2O_2, which can directly cause oxidative damage to bacteria (Rosenberger and Finlay, 2002). NADPH oxidase has been shown to mediate iNOS expression in mouse macrophages (Lanone *et al.*, 2005), which leads to the enhanced production of reactive oxygen species (Zhao *et al.*, 2010), and indeed we observed a concomitant increase in the expression of both NOX-1 and iNOS in MNB-treated cells

Interferon (IFN)-γ plays an important role in the early phase of resistance during bacterial infection, and Nauciel and Espinasse-Maes (1992) (Nauciel and Espinasse-Maes, 1992) found that ablation of IFN-γ by administration of anti-IFN-γ antibodies enhanced bacterial proliferation and death in mice infected with a sub lethal dose of *S. typhimurium*. IFN-γ stimulation up-regulates the expression of many antimicrobial effectors and impairs replication of *S. typhimurium* within macrophages (Gulig *et al.*, 1997), and IFN-γ-activated macrophages display enhanced microbicidal activities upon bacterial expression, due to changes in the expression of genes such as iNOS (Held *et al.*, 1999). It has been suggested that priming cells with IFN-γ may enable the host to respond quickly to relatively low doses of LPS, thereby activating antibacterial defenses (Held *et al.*, 1999). Here, MNB treatment increased IFN-γ, and therefore may have provided the necessary first signal to enable enhanced killing of SE.

Resistance to intracellular pathogens such as *Salmonella*, is also strongly influenced by the expression of NRAMP1 (Fritsche *et al.*, 2008), which may act by modulating iron transport, limiting its availability to intracellular microorganisms (Biggs *et al.*, 2001; Mulero *et al.*, 2002; Schaible and Kaufmann, 2004) and effecting macrophage immune function, since iron decreases the activity of IFN-γ-mediated antimicrobial pathways, including the formation of NO (Fritsche *et al.*, 2008). NRAMP-1 functionality increases NO formation (Fritsche *et al.*, 2003), the importance of which has been illustrated by the

observation that mice lacking iNOS are rendered susceptible to *Leishmania* infection (Wei *et al.*, 1995), and Govoni *et al.* (1999) found that expression of recombinant NRAMP1 by mouse macrophage (RAW 264.7) cells abrogated intracellular replication of *S. typhimurium.* In addition, iNOS and NRAMP1 expression have been shown to be associated with responses to SE *in vivo*, in both vaccinated and non-vaccinated chickens (Kramer *et al.*, 2003a; Malek and Lamont, 2003).

Lipopolysaccaride-induced TNF-α factor (LITAF) binds to a critical region of the TNF-α promoter and is reported to be involved in activation of TNF-α expression during LPS induction (Myokai *et al.*, 1999), however, little is known about the function of LITAF in poultry (Malek and Lamont, 2003). LITAF expression was found to be up-regulated following *in vitro* stimulation of macrophages with *S. typhimurium LPS*, as well as after treatment with *Eimeria*, the causative agent of avian coccidiosis (Hong *et al.*, 2006), suggesting that it may play a role in bacterial clearance and inflammatory responses, and may have been involved in the MNB-induced anti-SE activity observed here.

These results indicate that MNB can increase the *Salmonella*-killing activity of macrophages, and may act as a potent immunomodulator, via its ability to up-regulate the expression genes involved in host-defense and stimulate the production of reactive oxygen and nitrogen species. Additional study is required to further elucidate the mechanism of action of MNB as well as explore additional roles of MNB as an antimicrobial and innate immune-enhancing agent.

Chapter V

Effect of dietary mannanase-hydrolyzed copra meal (MCM) on growth performance and intestinal histology in broiler chickens.

Introduction

A number of chemical or immune-based approaches, including the use of disinfectants, insecticides, vaccines, and antibiotics, have been widely used for preventing infections with animal pathogens and for improving meat production. However, the continued use of dietary antibiotics has given rise to the development of drug-resistant bacteria (Sørum *et al.*, 2001). In addition, recent trends in food animal health research has suggested an increased awareness and need for 'natural' dietary additives versus chemicals or antibiotics for the purpose of improving animal health and performance (Al-Batshan *et al.*, 2001).

Food-derived compounds that can stimulate or enhance innate immune responses to pathogens are therefore an attractive alternative to antibiotics. Although the dominant prebiotics are fructooligosaccharide products, additional prebiotics, such as trans-galactooligosaccharides, glucooligosaccharides, lactose, and sucrose, have been investigated (Collins and Gibson, 1999). Moreover, Fritts and Waldroup(2003) and Ferket (2004) have reported that mannan oligosaccharides, which are derived from the yeast cell wall, might be useful as part of an overall feeding strategy to aid in overcoming the potential loss of growth-promoting antibiotics (Fritts and Waldroup, 2003; Ferket, 2004). In particular, MCM and its main component, MNB, have been reported to improve *Salmonella enterica* clearance, to reduce the degree of intestinal pathology in cecal tonsils, and to increase IgA production and the number of intraepithelial mononuclear cells in chicks (Agunos *et al.*, 2007). Moreover, in a study of 200,000 commercial broilers, MCM was reported to promote growth with increasing IgA production (Fukui *et al.*, 2009).

Furthermore, in a study using microarray and real-time transcription polymerase chain reaction analysis, MCM including MNB was suggested to act

as a potent immune-modulator and to exert a combination of effects on the intestinal immune system (Ibuki *et al.*, 2010).

The *in vitro* immuno-modulating activities and antibacterial effects of MNB were also confirmed in chicken macrophages (Ibuki *et al.*, 2011). Based on the above studies, We expected that MCM including MNB (or a component within it) would have an effect on the immunomodulatory system and thereby lead to changes in the intestinal histology. If so, MCM including MNB could improve growth performance in broiler chickens. However, the data on the relationship between growth performance and the effects of MCM administration on the small intestinal tissues in chickens are currently insufficient. Therefore, the purpose of this chapter was to observe the effects of MCM including MNB on growth performance and intestinal histology in broiler chickens.

Materials and Methods

Source of feed additives

MCM including MNB was provided by Fuji Oil, Ltd. (Osaka, Japan) and contained 11.4% MNB (MCM-B1). The different MCM-B1 components are shown in **Table 11.**

Table 11 MCM-B1 components

General composition (%)	Crude protein*	23.0
	Crude fat	8.2
	Ash	6.0
	Moisture	5.0
	Carbohydrate	57.8
Free sugar contents (%)	Glucose	1.6
	Mannose	2.4
	Fructose	1.9
	Sucrose	4.5
	Mannobiose	11.4

* Kjeldahl method: N*6.25

Experimental diet

The conventional mash diets (Nichiwa Sangyo Co. Ltd., Kagawa, Japan) were provided as the basal diet. The experimental diets were supplemented with 0.1% and 0.02% MCM-B1. The starter or grower crumble was without antibiotics or anticoccidial drugs (**Table 12**). The dose of MCM-B1 was based on the previously reported supplementation of chicks with 0.1% MCM-B1 in feed (Agunos *et al.*, 2007; Fukui *et al*, 2009).

Feeding experiments

All experiments were performed according to the humane care guidelines for the use of animals for experimentation as provided by Kagawa University in Japan (Kagawa University, 2006). Forty-eight male Marshall Chunky broilers that were 1 day in age were fed for 7 days. Afterwards, the birds were divided into 3 groups with 4 replicates of 3 birds with an equal mean body weight.

The birds were housed in wire pens under a dairy lighting regimen of 13-h light and an environmental room temperature. The broilers were fed with experimental starter and finisher diets from 7–21 days of age and from 22–49 days of age, respectively. The birds were allowed feed and water *ad libitum* throughout the feeding periods. Feed consumption and body weight were recorded weekly.

Tissue sampling

At the end of the feeding experiment, 4 birds from each group were weighed and killed by decapitation under light anesthesia with diethyl ether. The entire small intestine was quickly excised and placed in a fixative of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). The intestinal segment from the gizzard to the pancreatic and bile ducts was regarded as the duodenum; from the ducts to Meckel's diverticulum, as the jejunum; and from the diverticulum to the ileo-cecal-colonic junction, as the ileum. The same fixative solution was injected into each section of the intestinal lumen, and the middle portion of each section was used for

observation.

Table 12 Feed formulation and nutrient composition of the commercial broiler starter diet and the finisher mash diet

	Starter (days 1–21)	Finisher (days 22–49)
Ingredients (%)		
Ground maize	51.00	58.00
Milo	2.00	9.00
Soybean meal	37.00	22.00
Fish meal	4.00	4.00
Animal fat	4.00	4.60
Rapeseed meal	-	0.60
Calcium carbonate	1.00	0.85
Calcium phosphate tribasic	0.70	0.65
Sodium chloride	0.15	0.15
Vitamin/mineral premix *	0.15	0.15
Calculated component		
Crude protein	23.50	18.00
Metabolisable energy (kcal kg^{-1})	3100.00	3200.00
Crude fibre	4.00	4.00
Crude fat	4.50	6.00
Calcium	0.80	0.70
Phosphorus	0.60	0.55

*Concentrate mixture of the diet (per kg of diet): vitamin A, 10,800 IU; vitamin D3, 2,000 IU; vitamin E, 25 mg; vitamin K3, 2 mg; vitamin B1, 5.4 mg; vitamin B2, 7.2 mg; vitamin B6, 10.2 mg; vitamin B12, 8 mg; biotin, 0.3 mg; folic acid, 1.1 mg; pantothenic acid, 17 mg; nicotinic acid, 70.2 mg; choline, 1,500 g; zinc, 80 mg; copper, 16 mg

Light microscopy

The segments were cut transversely ~2 cm from the duodenum, jejunum, and ileum, fixed in Bouin's fixative solution at room temperature, embedded in paraplast, and sectioned at a thickness of 4 µm. Every tenth section was

collected and stained with hematoxylin-eosin.

Four different parameters (villous area, villous height, epithelial cell area, and mitotic cell number) were examined by light microscopy (Nikon Cosmozone 1S, Nikon Co., Tokyo, Japan) and measured for each intestinal segment using an image analyzer (Nikon Cosmozone 1S, Nikon Co., Tokyo, Japan).

For the measurement of the villi, the villi with lamina propria were chosen for analysis. The mean villus height in each bird was based on a total of 4 villi. The villus areas were calculated from the basal width, apical width, and villus height, with 4 villus areas determined for each bird.

For measuring the epithelial cell area, an area of the epithelial cell layer was randomly sampled at the middle of the villi, and the number of cell nuclei within this layer was counted. The area of the epithelial layer was divided by this number to estimate the area per cell. A total of 4 epithelial areas was counted for each bird. For measuring the number of mitotic cells in the villus crypt, 5 sections were randomly selected per bird; the cells within each section were scored as mitotic if the cells had homogenous, basophilic nuclei that intensely stained with hematoxylin-eosin. The number of mitotic cells was calculated from 5 different sections for each bird, and these 5 values were used to calculate a mean cell mitosis for a single bird. Finally, the mean cell mitoses from 4 birds were expressed as the mean cell mitosis for each individual group.

Scanning electron microscopy (SEM)

Sections (~2 cm in length) of the duodenum, jejunum, and ileum, which were close to those used for the light microscopic sample, were slit longitudinally. The intestinal contents were washed with 0.1 M phosphate buffered saline (pH 7.4). The tissue samples were pinned flat and fixed in this flattened position in a 3% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M cacodylate buffer (pH 7.4) for 1 h at room temperature, cut into 4 mm × 4 mm squares, and then fixed further for 1 h. The pieces were rinsed with 0.1 M sodium cacodylate buffer and were post-fixed with 1% osmium tetroxide in 0.1 M ice-cold sodium cacodylate buffer for 2 h.

The specimens were dried in a critical point drying apparatus. The dried specimens were coated with platinum and observed at 8 kV with SEM (Hitachi S4300SE/N, Hitachi Ltd., Tokyo, Japan).

Statistical analyzes

The results were reported as means ± SE. All data were statistically analyzed by one-way analysis of variance (ANOVA). Subsequently, data statistically significant different at $P < 0.05$ due to treatments were separated by Duncan's multiple range test. Correlations between the dose of MCM-B and each parameter were also calculated using Spearman's correlation test. All statistical analyzes were performed by the Statistical Analysis System (SAS Inst. Cary. NY, 2000) and Dr.SPSS-II (SPSS-Inc. Chicago, IL, 2001).

Results

Growth performance

The body weight gain and the FE were significantly higher in the 0.1% MCM-B1 group relative to that in the control group, while feed intake tended to be higher in the 0.02% and 0.1% MCM-B1 groups. For the correlation analysis, a significant correlation was observed between the dosage of MCM-B1 and the body weight gain and FE (**Table 13**).

Histological analysis of cellular parameters

The height and area of the intestinal villi in the experimental groups were not significantly different from those in the control group. However, the epithelial cellular area of the ileum was significantly higher in the 0.02% and 0.1% groups compared to that in the control group ($P < 0.05$). Furthermore, the cellular area of the duodenum and the jejunum tended to be higher in the 0.02% and 0.1% MCM-B1 groups.

For the correlation analysis, a significant correlation was found between the dosage of MCM-B1 and the cell area of the duodenum, jejunum, and ileum. Cell mitosis in the duodenum was significantly higher in the 0.1% MCM-B1

groups than that in the control group. In terms of the correlation analysis, a significant correlation was seen between the dosage of MCM-B1 and cell mitosis in the duodenum (**Table 13**).

Table 13 Results of Growth Performance and Intestinal Cellular Histological Parameters

	Control	0.02% MCM-B1	0.1% MCM-B1	p-value	Correlation coefficients
Growth performance in broilers fed dietary MCM-B from 7–49 days of age					
Feed Intake (g)	6097 ± 200	6557 ± 299	6707 ± 310	0.308	0.414
Body Weight Gain (g)	2459 ± 110 a	2902 ± 122 ab	3165 ± 205 b	0.027	0.710**
Feed Efficiency	0.403 ± 0.013 a	0.444 ± 0.018 ab	0.471 ± 0.013 b	0.031	0.798**
Intestinal cellular histological parameters measured using light microscopy					
Villus Height (mm)					
Duodenum	1.623 ± 0.109	1.859 ± 0.058	1.743 ± 0.095	0.233	0.237
Jejunum	1.269 ± 0.109	1.283 ± 0.112	1.366 ± 0.120	0.813	0.207
Ileum	0.840 ± 0.061	0.877 ± 0.048	0.889 ± 0.042	0.782	0.118
Villus Area (mm^2)					
Duodenum	0.661 ± 0.046	0.748 ± 0.044	0.752 ± 0.043	0.310	0.384
Jejunum	0.322 ± 0.038	0.362 ± 0.017	0.458 ± 0.083	0.233	0.384
Ileum	0.250 ± 0.026	0.253 ± 0.014	0.240 ± 0.016	0.885	-0.089
Epithelial Cell Area (μm^2)					
Duodenum	431 ± 14	480 ± 21	505 ± 21	0.058	0.650*
Jejunum	406 ± 12	471 ± 24	467 ± 17	0.057	0.591*
Ileum	314 ± 14 a	367 ± 11 b	381 ± 66 b	0.004	0.785**
Cell Mitosis (number)					
Duodenum	1072 ± 41 a	1122 ± 42 ab	1282 ± 66 b	0.041	0.650*
Jejunum	908 ± 54	914 ± 8	1032 ± 92	0.323	0.266
Ileum	639 ± 42	602 ± 49	527 ± 191	0.792	-0.148

a, b, values that do not share common letters within a row differ significantly by Duncan's multiple range rage test ($P < 0.05$).
*, **, statistically significant correlations were found ($P < 0.05$ and $P < 0.01$)

Morphology on the villus tip surface

Compared to the relatively flat cells of the duodenal villus tip in the control (**Figure 18-A**), those of the 0.02% and 0.1% MCM-B1 treatments were

conspicuously protuberant in appearance (**Figure 18-B,C arrow**). In addition, as shown in **Figure 18-C**, some of the cells around the central sulcus were devoid of any microvilli (arrowhead). The same pattern of cellular features (i.e., flat vs. protuberant) in the different treatments was also observed in the jejunal villus tip (**Figure 19**). However, similar to the cells of the ileal villus tip in the control group, that of the 0.02% MCM-B1 treatment group was comparatively flat (**Figure 20-B**). Meanwhile, a clear cell line and more protuberant cells were observed in the ileal villus tip in the 0.1% MCM-B1 treatment

Discussion

In this chapter, we examined the effect of MCM-B1 on growth performance and intestinal histology in broiler chickens. The growth performance was found to improve with MCM-B1 treatment, and histological analysis of the small intestine revealed that dietary supplementation with MCM-B1 resulted in an increase in the cell area. A strong relationship between mucosal histology and body weight has been previously reported (Awad, *et al,* 2006). In particular, an increase in the area of the small intestine results in the increased absorption of nutrients (Johnson and Gee, 1986; Awad, *et al*, 2006; Adibmoradi, *et al*, 2006; Yamauchi, *et al*, 2006; Samanya and Yamauchi, 2002) and thereby contributes to an improved digestion coefficient (Onderci, 2006).

On the other hand, when the size of the small intestine is reduced, the body weight decreases (Batal, *et al*, 2002), and a decrease in lactase, sucrase, and peptidase expression is observed (Hedemann, *et al*, 2006). Prebiotics, sugar cane (Khambualai, *et al*, 2010), chitosan (Khambualai, *et al*, 2009), and sesame meal (Yamauchi, *et al*, 2006) have been observed to promote growth through changes in villus histological parameters. In this chapter, we also observed that the MCM-B1 contribution to growth correlated with increased activation of the villus and confirmed that dietary supplementation with MCM-B can increase the cell area in the duodenum, jejunum, and ileum.

In terms of the relationship between intestinal histology and immunomodulation, Liu *et al.* (2008) reported that rabbit sacculus rotundus

antimicrobial peptide (RSRP) can increase villus height and the area of IgA-secreting cells in each intestinal region, indicating that RSRP can affect both Intestinal histology and mucosal immunity. In addition, Bao *et al.* (2009) reported that broilers treated with pig antibacterial peptide had improved body weight and average daily gain, greater villus height and gut mucosal thickness, increased alkaline phosphatase activity, and a higher ratio of IgA-secreting cells when compared to the control group. Lee *et al.* (2010) reported that Bacillus spp., as direct-fed microbials, can affect villus morphology and systemic-inflammation immunity. These reports reveal potential relationships between immunomodulation and intestinal histology. Further, these findings indicate potential relationships between growth promotion, villus morphology, and the immunomodulatory system.

In this chapter, the increased values in the light microscopy parameters and the hypertrophied epithelial cells indicate that MCM-B1 could stimulate intestinal function, thereby resulting in improved growth performance in chickens. These results suggest that MCM-B1 (or a component within it) may affect the immunomodulatory system and produce changes in the intestinal histology. Thus, MCM-B1 may promote growth performance in broiler chickens. However, because an improvement in the intestinal histology and immuno-modulation by MNB or MCM-B1 were not observed simultaneously, this relationship remains unclear. Research addressing the relationship between these effects, including growth promotion by MCM-B, may be the focus of a future study.

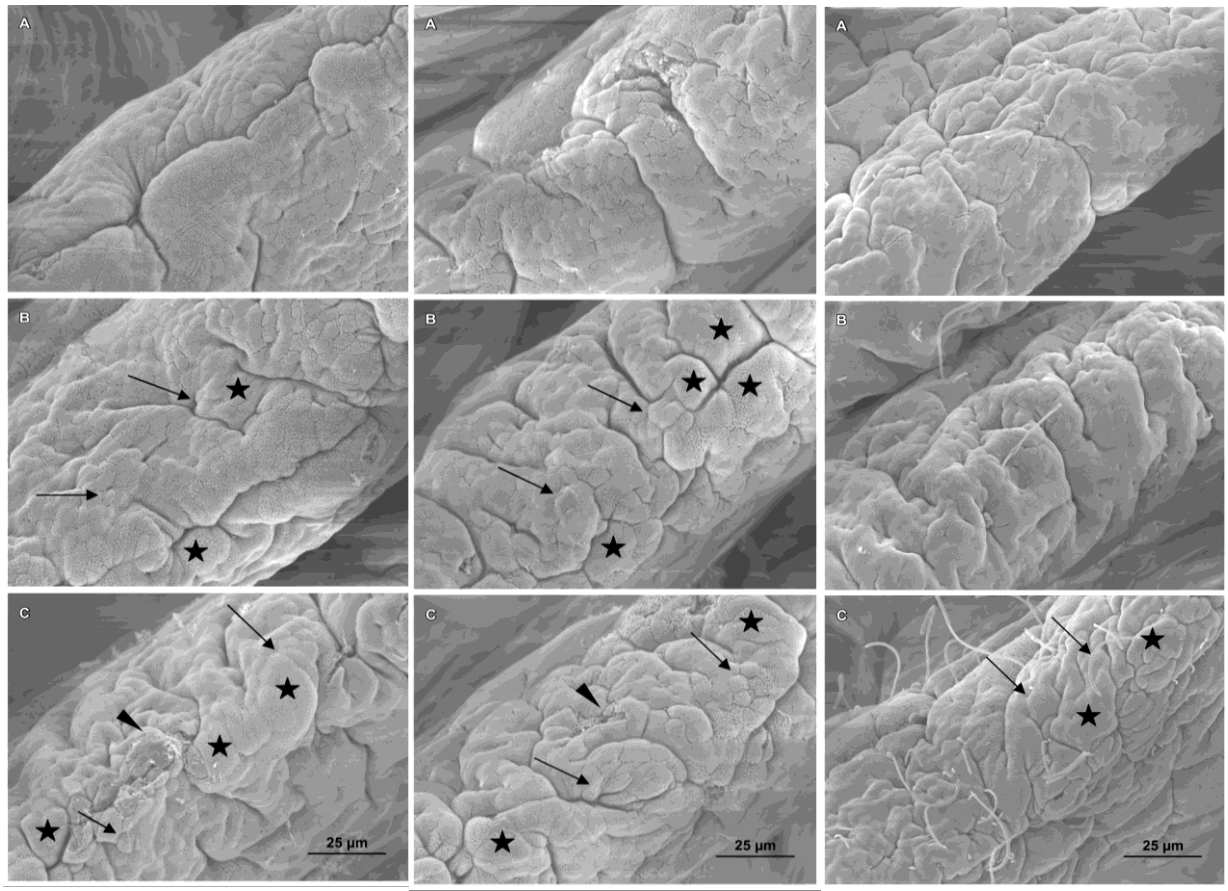


Figure 18 Scanning electron micrographs of epithelial cells on the duodenal(left), jejunal (center), and ileal(right) villus apical surface in chickens fed control (A), 0.02% (B), and 0.1% (C) MCM-B1 diets.

Scale bar = 25 μm (×1000). (Arrows = protuberant cells; arrowhead = cell devoid of any microvilli; stars = cell clusters)

Chapter VI

Effect of Dietary β1,4 mannobiose (MNB) on the Growth of Growing Broiler Chicks

Introduction

In-feed antibiotics have been utilized for over half a century to promote growth in domestic animals (Moore *et al.*, 1946; Leitnterer *et al.*, 2001; Sun *et al.*, 2005). Meanwhile, the extensive use of antibiotics in livestock production has increased the risk of development of resistance in human and animal pathogens (Bates *et al.*, 1994; Witte, 1998). Thus, the use of antibiotics to promote growth in domestic animals has been banned in the European Union (Dibner and Richards, 2005). However, the withdrawal of in-feed antibiotics suppresses growth in domestic animals, which in turn results in an increase in production costs (Casewell *et al.*, 2003; Sun *et al.*, 2005). Therefore, there is a pressing need to develop new feed additives that can be used to improve the growth of domestic animals.

Yang *et al.* (2009), in their review of alternatives to in-feed antibiotics, showed the growth-promoting effects of several oligosaccharides in broiler chickens. Mannanoligosaccharides have been shown to provide various health benefits in broiler chickens. Three major modes of action by which broiler performance is improved by Mannanoligosaccharides have been proposed: 1) control of pathogenic or potentially pathogenic bacteria which possess type-1 fimbriae (mannosesensitive lectin), 2) immune modulation, and 3) modulation of intestinal morphology and expression of mucin and brush border enzymes (Yang *et al.* 2009). We previously demonstrated that MCM including MNB could act as an immune-modulating agent *in vivo*, preventing SE infection in broilers by increasing IgA production and improving SE clearance (Agunos *et al.*, 2007), as well as up-regulating the local expression of genes involved in host defense and innate immunity (Ibuki *et al.*, 2010). Recently, we also found that MNB enhances *Salmonella*-killing activity, activates innate immune responses in chicken macrophages (Ibuki *et al.*, 2011). MCM-B1, which contains 11.4%

MNB, was effective for improving intestinal morphology in broiler chickens (Ibuki *et al,* 2013). For example, on the duodenal villus surface of the 0.1% MCM-B1-fed chickens, some cells devoid of microvilli were observed, suggesting the increased protuberance of these cells represents increased absorption activity. In addition, dietary 0.1% MCM-B1 increased body weight gain in chickens. These findings raise the hypothesis that MNB can improve growth performance in broiler chickens. However, the effects of MNB on growth in broiler chickens have not been examined.

The objective of the present study was to investigate the effects of dietary MNB on the growth of growing broiler chicks.

Materials and Methods

Preparation of MNB

MNB was prepared from Philippine coconut flour as described previously (Ibuki *et al.*, 2011). Coconut flour was defatted with 2 g/g hexane, and then suspended in 10 ml/g distilled water at 80°C for 2 h. The solid was collected by centrifugation at 10,000×g for 20 min, then suspended in 10 ml/g distilled water at room temperature for 10 min, and again collected by centrifugation. These steps were repeated, and the solid was hydrolyzed with -mannanase (Shin-Nihon Chemical Co. Ltd., Aichi, Japan). Following centrifugation at 10,000×g for 20 min, the supernatant was lyophilized and dissolved in deionized water at 10% (w/v). This crude sample was used for further purification. To obtain highly purified MNB, 5ml of 10% (w/v) crude MNB solution was applied to a Bio-Gel P2 Fine gel filtration column (ϕ75mm×1m; Bio-Rad Laboratories, Hercules CA, USA). The sample was eluted with distilled water at 4.5 ml/min, and 25-ml fractions were collected. Saccharide concentrations were measured by the phenol-sulfuric acid method (DuBois *et al.*, 1956) and MNB-containing fractions were combined and lyophilized. According to this method, 99% pure MNB was obtained and its purity and structure were confirmed by HPLC.

Table 14 Composition of experimental diet (g/100g diet)

	Control	MNB*
Soybean meal	45.08	45.08
1% MNB*	0	1.00
Water	0.99	0
Methionine	0.12	0.12
Threonine	0.10	0.10
Corn Starch	39.11	39.10
Soybean oil	6.70	6.70
Vitamin mixture**	1.50	1.50
Mineral mixture***	6.26	6.26
Cellulose	0.14	0.14
Calculated value		
Crude protein (%)	21	21
ME (Kcal/100g)	310	310

Animals and feed

Day-old male broiler chicks (chunky) were purchased from a local hatchery (Ishii Co. Ltd., Tokushima, Japan). They were given free access to water and a commercial chicken starter diet (Nosan Co., Kanagawa, Japan, 20% of crude protein, 310 kcal of ME/100g diet) and acclimated to the facility for 6 days before feeding of experimental diets (**Table 14**). The dose of MNB 0.01% was based on our previous report (Agunos *et al.*, 2007; Ibuki *et al.*, 2010). Methionine, threonine and arginine were added to diets to meet the recommendations of National Research Council for broiler chicks (National Research Council, 1994). Chicks were given free access to water and the experimental diets throughout the experimental period. All experimental diets were provided in powder form.

A total of 24 eight day-old male broiler chicks were weighed, allocated based on the body weight to two cages (1,725 mm x 425 mm x 320 mm, 12 birds in each group) and fed a control diet or a MNB diet (**Table 1**) for 14 days. At the end of the experimental period, body weight was measured, and chicks were sacrificed by decapitation. This chapter was approved by the Institutional

Animal Care and Use Committee (Permission number: 22-05-10) and carried out according to the Kobe University Animal Experimentation Regulation.

Sampling and preparation

Blood was collected from carotid artery. Plasma was separated immediately by centrifugation at 3,000 × *g* for 10 min at 4°C, frozen by liquid nitrogen and stored at -80°C for 3-methylhistidine analysis. The liver, abdominal adipose tissue, breast muscles and thighs were excised and weighed. A portion of the pectoralis major muscle, a major part of breast muscles, was frozen immediately for total RNA, protein, and real-time PCR analyzes.

Real-time PCR analysis.

Total RNA was extracted from the hypothalamus using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from 5 μg of DNase I (Ambion Inc., Austin, Texas, USA)-treated total RNA using a ReverTra Ace® qPCR RT Kit (TOYOBO CO. LTD., Osaka, Japan) with random primers. Complementary DNAs of myostatin, atrogin-1, 20S proteasome subunit C1, 20S proteasome C2 subunit, m-calpain large subunit, caspase 3, and cathepsin B were amplified with the primers described in **Table 15**. As an internal standard, complementary DNA of ribosomal protein S17 (RPS17) was also amplified with the primers described in **Table 15**. All primers were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). THUNDERBIRD™ SYBR® qPCR Mix was purchased from TOYOBO CO. LTD. (Osaka, Japan), and mRNA expression was quantified in duplicate using the Applied Biosystems 7300 Real-Time PCR system according to the supplier's recommendations.

Plasma 3-methylhistidine analysis.

Plasma 3-methylhistidine level was measured by the method of Yamaoka *et al.* (2008).

Statistical analysis

Data were analyzed using Student's *t*-test. All statistical analyzes were performed using the commercial package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

Table 15 Primer sequences for real-time PCR analysis

Gene		Primer sequences	Accession number *
Myostatin	sense	5' -ATG CAG ATC GCG GTT GAT C-3'	NM_001001461
	antisense	5' -GCG TTC TCT GTG GGC TGA CT-3'	
Atrogin-1	sense	5' -CAC CTT GGG AGA AGC CTT CAA-3'	NM_001030956
	antisense	5' -CCG GGA GTC CAG GAT AGC A-3'	
20S proteasome C1 subunit	sense	5'-TGA GGA ACA AGG AGC CCA TCT-3'	AB001935
	antisense	5'-TGC CCT TGT ACT GAT ACA CCA TGT-3'	
20S proteasome C2 subunit	sense	5'-CCA GTA TCT CGT TTG GTG TCA CTA C-3'	AF027978
	antisense	5'-CAT AGC GCT GCG TTG GTA TC-3'	
m-Calpain large subunit	sense	5'-GTG GCT CGG TTT GCT GAT G-3'	D38026
	antisense	5'-AAT CAA GCA CCG GAC ACA ATT-3'	
Caspase 3	sense	5'-GGA ACA CGC CAG GAA ACT TG-3'	AF083029
	antisense	5'-TCT GCC ACT CTG CGA TTT ACA-3'	
Cathepsin B	sense	5'-GCT ACT CGC CTT CCT ACA AGG A-3'	U18083
	antisense	5'-GCG AGG GAC ACC GTA GGA T-3'	
Ribosomal protein S17	sense	5' -GCG GGT GAT CAT CGA GAA GT-3'	NM_204217
	antisense	5' -GCG CTT GTT GGT GTG GAA GT-3'	

*, Refer to GenBank accession number.

Results

Table 16 shows the effects of dietary MNB on body weight and several tissues in growing broiler chicks. Dietary MNB significantly increased the relative weight of breast muscles ($P < 0.01$). The weight of breast muscles tended to increase in the MNB group ($P = 0.119$). Dietary MNB did not affect the weights of the body, thighs, liver, or abdominal adipose tissue. In the present study, we did not measure individual feed intake. Therefore, the feed intake and feed conversion ratio could not be statistically analyzed.

Table 16 Effects of dietary β 1,4 mannobiose (MNB) on body, breast muscles, liver, abdominal adipose tissue weights in growing broiler chicks.

	Control	MNB
Body weight (g)	948.4 ± 21.4	950.0 ± 19.0
Breast muscles weight (g)	139.3 ± 3.6	148.1 ± 4.0
Breast muscles weight (g/100g BW)	14.7 ± 0.2	15.6 ± 0.2 *
Thighs weight (g)	172.6 ± 4.8	176.5 ± 4.3
Thighs weight (g/100g BW)	18.2 ± 0.2	18.6 ± 0.2
Liver weight (g)	22.9 ± 0.8	23.0 ± 0.9
Liver weight (g/100g/BW)	2.4 ± 0.1	2.4 ± 0.1
Abdominal adipose tissue weight (g)	14.9 ± 0.9	14.7 ± 0.9
Abdominal adipose tissue weight (g/100g BW)	1.6 ± 0.1	1.5 ± 0.1

Data represent the mean ± SEM of 12 birds in each group. *, significant with respect to the control group ($P < 0.01$).

Figure 19 shows the effects of dietary MNB on the plasma 3-methylhistidine level and breast muscle atrogin-1 mRNA level. Dietary MNB did not affect the plasma 3-methylhistidine level or breast muscle atrogin-1 mRNA level.

Figure 20 shows the effects of dietary MNB on skeletal muscle protease mRNA levels. Dietary MNB did not affect the mRNA levels of any protease.

Figure 21 shows the effects of dietary MNB on the myostatin mRNA level in the breast muscles. The myostatin mRNA level was significantly ($P < 0.05$) decreased by dietary MNB.

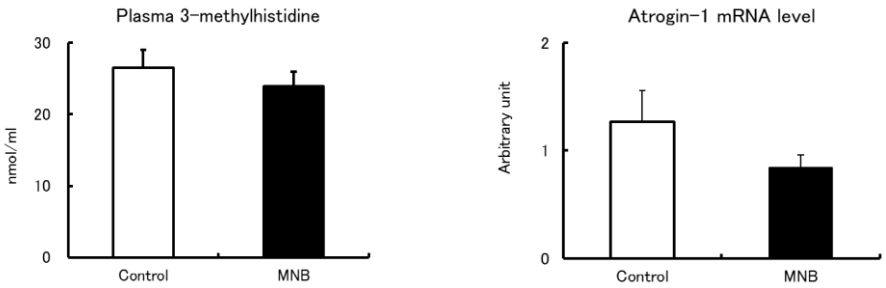


Figure 19 Effects of dietary MNB on the plasma 3-methylhistidine concentration and muscle atrogin-1 mRNA level in broiler chicks. Data represent the mean ± SEM of 12 birds in each group.

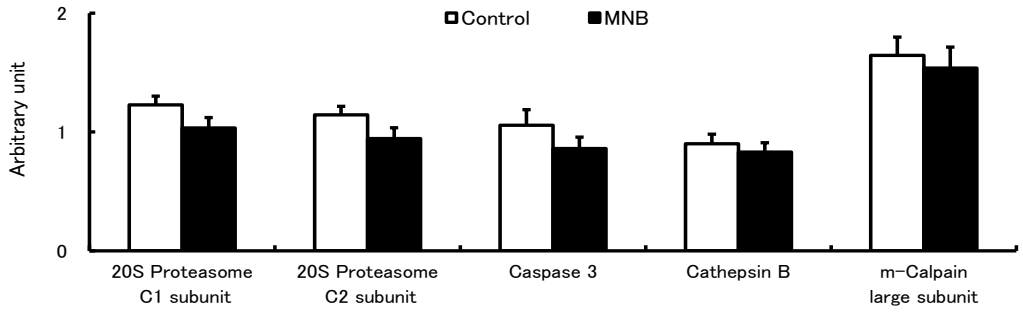


Figure 20 Effects of dietary MNB on the mRNA levels of proteases in the breast muscles of broiler chicks. Data represent the mean ± SEM of 12 birds in each group.

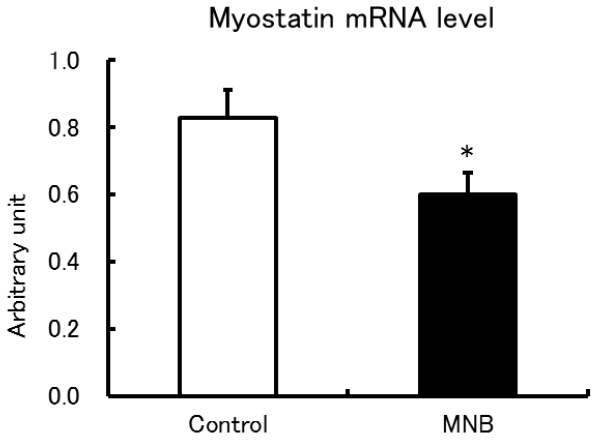


Figure 21 Effects of dietary MNB on myostatin mRNA level in the breast muscles of broiler chicks. Data represent the mean ± SEM of 12 birds in each group. *, Significant with respect to the control group ($P < 0.05$).

Discussion

In the MNB group, the relative weight of breast muscles was significantly increased, but the weight of the thighs was not affected (**Table 16**). The reason for the difference is not clear. One possible explanation is that the effect of dietary MNB on the skeletal muscle weight was different between muscle fiber types in the chicks. There is evidence that more than 99% of muscle fiber in the breast muscle is type II B fiber (Barnard *et al.*, 1982). On the other hand, thigh muscles consist of several types of muscle fiber including type I, II A, II B, III A, and III B. It is therefore possible that dietary MNB

specifically increases type II B fibers in the skeletal muscle in chicks. Further studies are required to address this possibility.

Dietary MNB significantly increased the relative weight of breast muscles, whereas the relative weight of abdominal adipose tissue was not affected (**Table 16**). In chickens, the percentage of carcass fat is quite consistent with that from the abdominal fat adipose tissue (Havenstein, 2003). It is therefore possible that MNB specifically increases the breast muscle ratio without affecting body fat accumulation in broiler chicks. Further studies are required to address this possibility.

The rate of skeletal muscle growth is altered by the rate of protein degradation in skeletal muscle (Goll *et al.*, 2008). Plasma 3-methylhistidine is known to be a nonmetabolizable amino acid marker of myofibrillar protein catabolism (Hayashi *et al.*, 1985; 1994; Fetterer and Allen, 2000; 2001). Therefore, we measured the effects of dietary MNB on plasma 3-methylhistidine level in growing broiler chicks. However, plasma 3-methylhistidine was not affected by MNB (Fig. 1). In mammals, mRNA of atrogin-1 is markedly induced during muscle atrophy (Dehoux *et al.*, 2004; Costelli *et al.*, 2006; Gomes *et al.*, 2001; Bodine *et al.*, 2001). Recently, atrogin-1 expression under normal conditions has been thought to be related to the rate of skeletal muscle proteolysis and muscular size of chickens (Nakashima *et al.*, 2009, Kamizono *et al.*, 2010). Ohtsuka *et al.* (2011) showed that atrogin-1 expression appears to be closely related to the development of skeletal muscle proteolysis and skeletal muscle wasting suggesting its usefulness of as a reliable index of myofibrillar proteolysis in broiler chicks. However, the atrogin-1 mRNA level in the breast muscles was not affected by dietary MNB in the presence study (**Figure 19**). In addition, there was no significant effect on the skeletal muscle protease mRNA levels in the breast muscles of broiler chicks (**Figure 20**). All our findings suggest that the increase in the breast muscle ratio in the MNB group was not due to the inhibition of skeletal muscle proteolysis.

Myostatin is a negative regulator of myoblast proliferation and

differentiation (Otto and Patel, 2010; Elkina *et al.*, 2011). Therefore, we analyzed myostatin mRNA level in the breast muscles and found that the myostatin mRNA level was significantly ($P < 0.05$) decreased by MNB (**Figure 21**). It is therefore possible that the downregulation of myostatin gene expression affected myoblast proliferation or differentiation, which in turn resulted in a high breast muscle ratio. Further studies are required to clarify this point.

Recently, a model whereby myostatin induces skeletal muscle wasting through targeting sarcomeric proteins via Smad3-mediated up-regulation of Atrogin-1 and forkhead box O1 was proposed (Lokireddy *et al.*, 2011). In the present study, we found that the myostatin mRNA level was significantly ($P < 0.05$) decreased by MNB (Fig. 3, $P < 0.05$). Dietary MNB did not affect the mRNA level of breast muscle atrogin-1. However, the means of atrogin-1 mRNA level in the MNB group was about 66.2 % of that in the control levels. It is therefore possible that dietary MNB-decreased myostatin mRNA level is related to the decrease of atrogin-1 mRNA level in the breast muscle.

As mentioned in the introduction section, three major modes of action by which broiler performance is improved by mannanoligosaccharides have been proposed, such as the effects on the undesirable microbiota, immunity, and intestinal mucosa (Yang *et al.* 2009). We recently found that MNB enhances *Salmonella*-killing activity, activates innate immune responses in chicken macrophages (Ibuki *et al.*, 2011), and possibly improves intestinal morphology in broiler chicks (Ibuki *et al.*, 2013). In the present study, we suggest that dietary MNB may induce protein synthesis in breast muscles. Thus, further study will be needed to investigate the involvement between the stimulation of protein synthesis by MNB and the suppression of pathogenic bacteria or the modulation of intestinal morphology or immune responses.

As observed in other studies showing the effect of feed ingredients on growth performance in broiler chicks (Yamamoto *et al.*, 2007; Baurhoo *et al.*, 2009; Khambualai *et al.*, 2009; Saleh *et al.*, 2011), study with a small number of chickens (n = 7 - 12) have been published in several international scientific

journals. Therefore, in the present study, 12 birds were assigned in each group. However, the number of chickens should be increased in future study.

In conclusion, we investigated the effects of dietary MNB on the growth of broiler chicks. Our results showed that MNB increases the relative weight of breast muscles to body weight in growing broiler chicks. These results suggest that MNB could be a promising candidate feed additive to improve meat yield of broiler chickens.

Concluding Remarks

The aim of this study is that the evaluation of the natural feed ingredients MNB and MCM as alternatives of antibiotics or growth promoters in poultry industry.

In Chapter I, The author introduced the background and aim of this study.

In Chapter II, The author showed the effects of MCM-B1-supplemented feeds on the kinetics of SE in broilers and the ensuing histopathological changes. MCM-B1-supplementation reduced SE organ colonisation, cecal carriage and faecal shedding in a time-dependent manner. The high concentrations and persistency of the SE-specific IgA response in those birds given rations supplemented with MCM-B1 or MAN were associated with a decline in SE shedding and cecal carriage in the later stages of infection. MCM-B1 was more effective against SE infection than MAN. Histological examination of the cecal wall and cecal tonsils at 23d post-infection indicated a lesser degree of intestinal pathology. An increased number of intra-epithelial mononuclear cells (mature lymphocytes and macrophages) in the lining epithelium of birds fed on the diet supplemented with MCM-B1 was accompanied by an increased number of lamina propria cells. These results indicate that feeding a diet supplemented with MCM-B1 during the first two weeks after hatching reduced susceptibility to SE infection. Supplementing the diet with MCM-B1 or MAN increased IgA production and improved SE clearance by acting as immunomodulatory agents that prevented intestinal pathology. Feeding a MCM-B1-supplemented diet to broilers could be used as an alternative to antibiotics, because it has no adverse effects on mortality or weight gain.

In Chapter III, The author showed the gut immune-modulating activity of MCM-B2 using microarray and real-time RT-PCR. No significant difference in BW or organ weights was observed between MCM-B2-treated and untreated control birds, and no histological abnormalities were observed in any of the tissues examined. The MCM-B2-treated chickens had significantly higher levels of fecal IgA over all 4 weeks when compared with control birds. Microarray and real-time RT-PCR analysis revealed the upregulation of several genes involved in immune responses, including those involved in antigen recognition, processing and presentation (MHC class I and II),

interferon-related genes, and genes involved in host defense. These results provide insight into the mechanism of action of dietary MNB in the intestine and confirm that MNB acts as a potent immune-modulating agent, exerting combined effects on the intestinal immune system.

In Chapter IV, The author showed the in vitro immune-modulating effects of MNB using chicken macrophage (MQ-MCSU) cells. Treatment of MQ-NCSU cells with MNB dose-dependently increased both phagocytic activity and *Salmonella*-killing activity of macrophages, with the highest reduction in SE viability observed at a concentration of 40 μg/ml at 48 h post-infection. Likewise, both hydrogen peroxide (H_2O_2) and nitric oxide (NO) production were increased in a dose-dependent manner by MNB. Gene expression analysis of MNB-treated macrophages revealed significant increases in the expression of iNOS, NOX-1, IFN-γ, NRAMP1, and LITAF, genes critical for host defense and antimicrobial activity, when compared to untreated cells. This data confirms that MNB possesses potent innate immune-modulating activities and can up-regulate antibacterial defenses in chicken macrophages.

In Chapter V, The author showed MCM-B1 for its capacity to improve growth performance and activate intestinal villus function. Although feed intake was not significantly different among the experimental groups, the body weight gain and FE were significantly higher in the 0.1% MCM-B1 group than in the control group ($P < 0.05$), while feed intake tended to be higher in the 0.02% and 0.1% MCM-B1 groups. The cellular area of the ileum was significantly higher in the 0.02% and 0.1% groups in relation to that in the control group ($P < 0.05$). Furthermore, the cellular area of the duodenum and the jejunum tended to be higher in the 0.02% and 0.1% MCM-B1 groups. For the correlation analysis, a significant correlation was observed between the dosage of MCM-B1 and the cell area of the duodenum, jejunum and ileum. Moreover, the number of mitotic cells was higher in the 0.1% MCM-B1 group. As shown by SEM, the cells at the villi tips were protuberant in appearance in the 0.02% and 0.1% MCM-B1 treatments when compared with the relatively flat cells of the control. On the duodenal villus surface of the 0.1% MCM-B1 group, some cells devoid of microvilli were observed, suggesting that the increased protuberance of these cells represents increased absorption activity. Although intestinal villus height and area did not significantly differ among groups, the levels of these parameters tended to increase in the experimental groups relative to the control. The present morphological findings reveal that

MNB might be effective for activating intestinal absorptive function, and that the functional activation promotes the growth of the chickens.

In Chapter VI, The author showed the effects of dietary MNB (99%MNB) on the weights of body, breast muscles, thighs, liver, and abdominal adipose tissue in growing broiler chicks to evaluate whether MNB can be used as a feed additive for broiler chicks. Dietary MNB significantly increased the relative weight of breast muscles, whereas the weights of the body, thighs, liver and abdominal adipose tissue were not affected. The myostatin mRNA level in the breast muscle was significantly reduced by MNB. Since myostatin is a negative regulator of myoblast proliferation and differentiation, it is possible that the downregulation of myostatin gene expression is involved in the increased breast muscle growth with MNB. The plasma 3-methyl histidine level, which is known to be a nonmetabolizable amino acid marker of myofibrillar protein catabolism, and the breast muscle atrogin-1 mRNA level, which is involved in protein catabolism, were not affected by dietary MNB. In addition, MNB did not affect protease mRNA levels in the breast muscles. These results suggest that MNB does not affect proteolysis in the breast muscles.

The author infer that the natural ingredient MCM including MNB, which is exported from Japan to worldwide as a feed ingredient , is a promising candidate for use as a feed additive to promote growth and prevent in *Salmonella* incident in chickens. The author hopes that MCM including MNB will be used as an alternative of antibiotics not only for chickens but also for other domestic animals.

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List of Publications

Chapter Ⅱ : Agunos, A., Ibuki, M., Yokomizo, F., Mine, Y.
Effect of dietary ß 1,4 mannobiose in the prevention of *Salmonella enteritidis* infection in broilers.
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Chapter Ⅲ : Ibuki, M., Kovacs-Nolan, J., Fukui, K., Kanatani, H., Mine, Y.
Analysis of gut immune-modulating activity of ß 1,4 mannobiose using microarray and real-time reverse transcription polymerase chain reaction.
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Chapter Ⅳ : Ibuki, M., Kovacs-Nolan, J., Fukui, K., Kanatani, H., Mine, Y.
ß1,4 mannobiose enhances *Salmonella*-killing activity and activates innate immune responses in chicken macrophages.
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Chapter Ⅴ : Ibuki, M., Fukui, K., Yamauchi, K.
Effect of dietary mannanase-hydrolyzed copra meal on growth performance and intestinal histology in broiler chickens
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Chapter Ⅵ : Ibuki, M., Yamamoto, Y., Yamasaki, H., Honda, K., Fukui, K ., Yonemoto, H., Hasegawa, S., Kamisoyama, H.
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