ABSTRACT

Singboottra, Panthong. Reduction of Inflammatory Responses by Mannan Rich Fraction. (Under the direction of Dr. F. W. Edens)

The aims of this research include: 1) comparison of the agglutination-inducing activity of yeast cell wall products; 2) determination whether mannan rich fraction (MRF), a yeast cell wall product, reduces inflammatory responses of chicken macrophage cell lines exposed to lipopolysaccharide (LPS); 3) identification of mechanisms involved in the reduction of inflammatory responses of chicken macrophage cell lines exposed to LPS by MRF; and 4) to investigate whether MRF can reduce inflammatory responses *in vivo*.

Six yeast cell wall products were examined for their ability to agglutinate *Escherichia coli* (*E. coli*) or *Salmonella typhimurium* (*S. typhimurium*) cells. The results of the investigation indicated that Product B, Product E, and Product A agglutinated more than 70% of *E. coli* cells. Product F and Product C agglutinated approximately 69% and 60% of *E. coli* cells, respectively, while Product D agglutinated only approximately 46% of the *E. coli* cells. When *S. typhimurium* was used to test the ability of these yeast products to agglutinate bacterial cells, Product E and Product A agglutinated nearly 70% of *S. typhimurium* cells. Product F agglutinated approximately 62% cells, and Product B and Product C agglutinated close to 60% of *S. typhimurium* cells. Product D agglutinated only 46% of *S. typhimurium* cells. Mannose residues mediated the agglutination of *E. coli* and *S. typhimurium* by these yeast products.

Infections within the gastrointestinal tract (GIT) with pathogenic strains of *E. coli* or *Salmonella* cause fever, diarrhea, anorexia, somnolence, droopy wings, and ruffled feathers in infected chickens and turkeys. These clinical signs are a result of an endotoxin called lipopolysaccharide (LPS), that resides within the outer membrane of these bacteria and stimulates target cells to produce pro-inflammatory cytokines and nitric oxide that mediate clinical signs described above.

An *in vitro* study of the effect of mannan rich fraction (MRF) of yeast *Saccharomyces cerevisiae* in BioMOS® on the inflammatory responses of chicken macrophage cell lines (MQ-NCSU and HTC) to LPS was conducted. MRF at 2.5 mg/ml stimulated MQ-NCSU cell proliferation, whereas MRF at 10 mg/ml or higher was toxic to the cells. At the concentration of 2.5 mg/ml, MRF by itself did not induce either MQ-NCSU or HTC chicken macrophage cell lines to produce nitrite or interleukin-6 (IL-6). The nitrite and IL-6 production of MQ-NCSUs and HTCs were significantly increased when cells were stimulated with LPS, but MRF significantly reduced nitrite and IL-6 production of these LPS-stimulated cells. MRF down-regulated inducible nitric oxide synthase (*iNOS*) and *IL-6* gene expression of cell lines stimulated with LPS. An attempt to identify the possible mechanisms involved in the reduction of the inflammatory response by MRF indicated that MRF reduced the expression of LPS receptor toll-like receptor-4 (TLR-4), and possibly activated the transcription factor NF-κB that represses gene expression. It was further investigated whether MRF could also reduce the inflammatory responses *in vivo* as an *in vitro* finding often times does not translate to *in vivo* situation. The results showed that abdominal exudate macrophages from three-week

old broiler chickens fed with a basal diet and challenged with enteropathogenic *E. coli* had a significantly higher nitrite production than similar cells from non-challenged broiler chickens fed with a basal diet. On the other hand, abdominal exudate macrophages of birds challenged with *E. coli* and fed with a diet supplemented with MRF showed a significantly lower level of nitrite production than cells from challenged birds that were fed with a basal diet. Unexpectedly, macrophages isolated from three weeks old non-challenged broiler chickens fed with MRF produced a slight, but significantly higher level of IL-6 than macrophages from non-challenged birds fed with a basal diet or challenged birds fed with either a basal diet or MRF. MRF was also found to significantly increase body weight gain of *E. coli*-challenged or non-challenged broiler chickens compared to the body weight gain of challenged birds fed with a basal diet.

REDUCTION OF INFLAMMATORY RESPONSES BY MANNAN RICH FRACTION

by

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TO

MY LATE FATHER AND MY MOTHER

BIOGRAPHY

Panthong Singboottra was born on November 9, 1972 in a small province in Thailand called Roi-Ed. Though she grew up in a poor family, she managed to get her high school degree from the best high school in Roi-Ed, Sathree-surksa Roi-Ed High School, in 1991 on scholarship. She went to Chiang Mai University and received her Bachelor's degree in Medical Technology from the School of Associated Medical Sciences in 1995 also through a prestigious scholarship.

One year later, she won another prestigious scholarship from the Royal Thai Government to study abroad. She was accepted and started her Master's program in Immunology in August of 1997 under the guidance of Dr. Teresa Nadder at the Department of Clinical Laboratory Sciences, School of Allied Health of Medical College of Virginia/Virginia Commonwealth University in Richmond, Virginia**.** Ms. Panthong received her Master's degree in December of 1999.

Panthong then moved from Virginia to North Carolina and started her Ph.D. at North Carolina State University in January 2000. She began in the Immunology Program but later transferred to the Physiology Program in the Poultry Science Department, College of Agriculture and Life Sciences, North Carolina State University in March 2003.

Upon completion of her Ph.D. degree, Panthong will return to Thailand to teach as well as perform research in the Immunology Program of Chiang Mai University.

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Finally, but most importantly, I give thanks to my Lord Jesus for wisdom, strength, joy, and all the things You have done in my life. You are the source of everything and the center of my life. ***In God, All Things are Possible***.

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LIST OF ABBREVIATIONS

AEC	abdominal exudate cell
AGP	antibiotic growth promoter
ALV	avian leukosis virus
AMM	avian macrophage-specific complementary deoxyribonucleic acid micro array
ANOVA	analysis of variance
AP	alkaline phosphatase
APP	acute phase protein
APR	acute phase response
B. bifidum	*Bifidobacterium bifidum*
B. infantis	*Bifidobacterium infantis*
B. longum	*Bifidobacterium longum*
BPI	bactericidal/permeability-increasing protein
C. jejuni	*Campylobacter jejuni*
C. perfringens	*Clostridium perfringens*
cAMP	cyclic-adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CE	competitive exclusion
CL	cellulose
CO_2	carbon dioxide
CM	complete medium

C-terminal	carboxy-terminal
DD	death domain
DIG	digoxigenin
E. coli	*Escherichia coli*
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
FOS	fructooligosaccharide
GIT	gastrointestinal tract
GM	glucomannan
GPI	glycosylphosphatidylinositol
GRAS	generally recognized as safe
HTC	heterophil culture
IκB	inhibitor of kappa B
IDS	indigestible saccharide
Ig	immunoglobulin
IKK	inhibitor of kappa B kinase
IL	interleukin
IL-1R	interleukin-1 receptor
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
KDO	2-keto-3-deoxy-D-mannooctonate

L. casei	*Lactobacillus casei*
L. delbruekii	*Lactobacillus delbruekii*
LAB	lactic acid bacteria
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
Mal	myeloid differentiation factor 88 adaptor-like protein
MAPK	mitogen activated protein kinase
MBW	mean body weight
mCD14	membrane-bound form CD14
MDV	Marek's disease virus
MOS	mannanoligosaccharide
mOsm	milliosmol
MQ-NCSU	macrophage-North Carolina State University
MR	mannose receptor
MRF	mannan rich fraction
MTT	3-(5,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWG	mean body weight gain
MyD88	myeloid differentiation factor 88
NDO	non-digestible oligosaccharide
NF-κB	nuclear factor-kappa B

NLS	nuclear localization signal
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
N-terminal	amino-terminal
O_2	oxygen
OD	optical density
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffer saline
PGE_2	prostaglandin- E_2
POD	peroxydase
PRR	pattern-recognition receptor
RHD	rel-homology domain
rhIL-6	recombinant human interleukin-6
RNA	ribonucleic acid
RT-PCR	reverse trancriptase-polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
S. choleraesuis	*Salmonella choleraesuis*
S. dublin	*Salmonella dublin*
S. enteritidis	*Salmonella enteritidis*
S. give	*Salmonella give*
S. kedougou	*Salmonella kedougou*

S. montevideo	*Salmonella Montevideo*
S. pullorum	*Salmonella pullorum*
S. typhimurium	*Salmonella typhimurium*
sCD14	soluble form CD14
SCFA	short-chain fatty acids
SDS	sodium dodecyl sulfate
SIRS	systemic inflammatory response syndrome
ST	*Salmonella typhimurium*
TBS	tris buffer saline
T_c	core temperature
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
VFA	volatile fatty acid
WB	western blot
WBC	white blood cell

INTRODUCTION

Gastrointestinal tract (GIT) infections, induced by pathogenic Gram-negative bacteria, often cause fever, diarrhea, anorexia and somnolence in infected chickens and turkeys. These clinical signs are a result of an endotoxin called lipopolysaccharide (LPS), which resides within the outer membrane of these bacteria. LPS binds its receptor complex, toll-like receptor-4 (TLR-4)/MD-2, expressed on several cell types including macrophages (MQ) through association with LPS-binding protein (LBP) and CD14. Binding of LPS to its receptor complex triggers the signaling cascade that leads to the translocation of the transcription factor NF-κB from the cytoplasm to the nucleus. Once it reaches the nucleus, NF-κB binds to and activates the transcription of genes encoding proinflammatory cytokines (Interleukin-1 (IL-1), IL-6 and TNF-α) and inducible nitric oxide synthase (*iNOS*), which function to mediate the clinical signs described above.

BioMOS® is a glucomannoprotein complex isolated from the outer cell wall of a strain of the yeast *Saccharomyces cerevisiae* (Alltech, Inc., Nicholasville, KY). BioMOS® has long been used as a feed supplement to improve animal health and performance. Empirical data have demonstrated several mechanisms by which BioMOS® enhances health and growth of the animals. These mechanisms include: binding of BioMOS® to bacterial lectin via its mannose residues followed by the excretion of these bacteria through the peristaltic process; modification of the population of bacterial microflora within the GIT as well as modulation of their metabolic products; and enhancing an adaptive immune response of animals.

The goal of this project was to determine whether BioMOS® promoted growth and performance of animals by reducing an inflammatory response in addition to the mechanisms mentioned above. However, BioMOS® itself is an insoluble product. Therefore, a mannan rich fraction (MRF), a soluble product of BioMOS®, was used in this study. Chicken macrophage cell lines were used in an *in vitro* system as macrophages express an LPS receptor (TLR-4) and produce an ample amount of pro-inflammatory cytokines and nitric oxide (NO) upon activation. The MRF was tested initially to determine if it reduced IL-6 and NO production as well as down-regulated *IL-6* and *iNOS* gene expression of LPS exposed macrophage cell lines. If so, would MRF reduce that production by down-regulating TLR-4 expression and reduce NF-κB activation? Finally, the results from the *in vitro* system investigations were tested in an *in vivo* study to determine if there would be the possiblity of direct translation of *in vitro* to *in vivo* conditions.

LITERATURE REVIEW

Bacterial Infections within the Gastrointestinal Tract of Poultry

Most of the bacteria that cause enteric diseases can be placed within the Enterobacteriaceae family. Although there are more than 32 genera of bacteria in this family, primarily, *Escherichia* and *Salmonella* are involved in poultry diseases. The major causes of economic loss in the poultry industry worldwide are bacterial diseases that include Salmonellosis, Pullorum Disease, Fowl Typhoid, Paratyphoid Infection, Arizonosis, and Colibacillosis. Birds that are infected and symptomatic with these diseases show very similar clinical symptoms which include anorexia, somnolence, diarrhea, droopy wings, and ruffled feathers (Calnek et al., 1997; Jordan and Pattison, 1996). These causative bacterial agents are Gram-negative, rod-shaped, nonspore-forming, aerobic or facultative anaerobic organisms. Most are motile with flagelli.

Within the outer membrane of these bacteria, there is an endotoxin known as lipopolysaccharide (LPS). LPS is composed of three main components; an O-antigen, a core region, and a lipid A. O-antigen is a repeating unit of polysaccharides. The structures of this component among the same species have been found to be remarkably diverse compared to the core and lipid A due to variability in the number of sugar polymers, the position of the monosaccharide within the structure, and the possibility of formation of either branched or linear polymers, which can be either homo- or heteropolysaccharides. O-antigen is the region within LPS that is targeted by the host and against which an immune response is raised.

The core region of LPS is a non-repeating unit of polysaccharide. In contrast to the O-antigen, the structure of the core is more conserved. Bacteria within the same genus and occasionally within a family frequently share a common core sequence.

The last component of LPS, lipid A, is also highly conserved. Lipid A is a hydrophobic glycolipid composed of two residues of phosphorylated glucosamines. Each glucosamine is attached to fatty acids (Raetz, 1990; Raetz and Whitfield, 2002; Schnaitman and Klena, 1993; Ulevitch and Tobias, 1995). Lipid A is the component that contains the full endotoxin activity of LPS (Takada and Kotani, 1989). To exert this biological activity, lipid A, either as a free molecule or attached to the membrane of intact bacteria, is recognized by an acute phase protein known as LPS-binding protein (LBP). LBP is a 60-kDa glycoprotein that is synthesized by liver cells and secreted into the blood (Ramadori et al., 1990). It is expressed at less than 0.5 μg/mL in normal individuals and increases to approximately 50 μg/mL in an acute phase response (Tobias et al., 1986).

The amino acid sequence at the N-terminal of the LBP is 69% identical to the amino acid sequence of another protein that binds LPS found in granulocytes known as the bactericidal/permeability-increasing protein (BPI) (Schumann et al., 1990). LBP binds specifically to the lipid A of LPS and shows the strongest binding when the unique sugar, 2-keto-3-deoxy-D-mannooctonate (KDO) is present (Tobias et al., 1986). The binding of LBP to LPS results in the formation of a LBP-LPS complex that enhances the binding of LPS with another glycoprotein slightly smaller than LBP and is named CD14 (Juan et al., 1995b).

There are two forms of CD14. The first is a membrane-bound form (mCD14), which is a 53-kDa glycoprotein that is expressed on the surface of monocytes, macrophages, and neutrophils (in mammals; heterophils in avian species). The mCD14 does not possess transmembrane and cytoplasmic domains, but it is expressed on the cell surface by inserting its anchor, glycosylphosphatidylinositol (GPI) into the cell membrane (Haziot et al., 1988). The second is a soluble form (sCD14), which is a 48-kDa protein that lacks the GPI anchor (Haziot et al., 1988). The sCD14 assists cells such as endothelial cells and epithelial cells that do not express mCD14 in binding to LPS (Arditi et al., 1993; Bazil et al., 1989; Frey et al., 1992; Haziot et al., 1993; Pugin et al., 1993). By converting the conventional CD14 to a transmembrane protein with the cytoplasmic domain, Lee et al. (Lee et al., 1993) demonstrated that both conventional and transmembrane forms of CD14 respond to LPS identically in terms of IgM expression, NF-κB activation, and protein tyrosine phosphorylation. However, the addition of an antibody that did not block LPS binding to a genetically expressed CD14 did not inhibit NF-κB activation. Thus, Lee and co-workers (Lee et al., 1993) concluded that CD14 is only involved in LPS binding but not directly involved in the signaling process. However, other signaling molecules likely are involved.

The binding site of LPS on CD14 is confined within the 152-amino acids of the N-terminus (Juan et al., 1995b), and the amino acids 57-65 are particularly important (Juan et al., 1995a). After binding to LPS, CD14, via an unknown mechanism, transfers LPS to its receptor composed of a Toll-like receptor-4 (TLR-4) and MD-2. MD-2 is a novel molecule that is requisite for TLR-4 recognition of LPS (Shimazu et al., 1999).

TLR-4 was the first mammalian Toll-like receptor to be discovered and characterized (Medzhitov et al., 1997). It is a type-I transmembrane protein which is characterized by the presence of multiple leucine-rich repeats (LRRs) on its extracellular domain and the Toll/Interleukin-1 Receptor (TIR) domain within the cytoplasmic portion (Hashimoto et al., 1988; Kobe and Deisenhofer, 1995; Medzhitov et al., 1997).

The LRRs have been found in several proteins that are involved in protein-protein interactions (Kobe and Deisenhofer, 1995). The TIR domain is a conserved protein found mostly within the cytoplasmic portion of the receptors which functions as a mediator of protein-protein interactions (Aravind et al., 2001). A high-level of the amino acid sequence homology between the TIR domains of TLRs and IL-1R has been demonstrated (Medzhitov et al., 1997), and it has been postulated that TLRs and IL-1 share the signaling cascade.

MD-2 is a small protein that does not possess any transmembrane or cytoplasmic portion and is expressed on the surface of several cell types, including monocytes/macrophages in association with TLR-4. (Shimazu et al., 1999) identified MD-2 and demonstrated that the IL-3 dependent Ba/F3 cell line transfected with TLR-4 and MD-2 responded to LPS activation, whereas cells transfected with TLR-4 alone did not respond to LPS activation indicating that MD-2 is essential for LPS signal transduction.

The exact role of MD-2 on LPS signaling is not clear, but it may provide the binding site for a nearby TLR-4 molecule thereby pulling the TLR-4 receptors closer together (Shimazu et al., 1999). The binding of LPS to the receptor complex has been

suggested to cause induction of receptor aggregation (Martin and Wesche, 2002), which brings in to close proximity the TIR domains, allowing protein-protein interaction between these domains.

The interaction of the TIRs causes domain conformational changes that allow Myeloid Differentiation Factor 88 (MyD88) and MyD88 Adaptor-like protein (Mal) to bind to the cytoplasmic portion of the receptors via their TIR domains. MyD88 is an adaptor protein that is made up of a C-terminal TIR domain and an N-terminal death domain (DD; (Bonnert et al., 1997). Results from a study using MyD88 knockout mice indicated that MyD88 is required for LPS signaling (Kawai et al., 1999). Mal is an adaptor protein containing a C-terminal TIR domain. However, Mal does not contain the DD. Using a yeast two-hybrid system and co-immunoprecipitation, it was shown that Mal forms homodimers and/or heterodimer with MyD88 and is essential for TLR-4 signaling (Fitzgerald et al., 2001). Activated MyD88 then recruits a downstream protein called IL-1R-Associated Kinase (IRAK) to the receptor complex (Wesche et al., 1997).

There are four protein members within the IRAK family: IRAK-1, IRAK-2, IRAK-M and IRAK-4 (Janssens and Beyaert, 2003). Similar to MyD88, all IRAKs consist of an N-terminal DD that may assist with the binding of IRAK to MyD88. All IRAK members contain a central kinase domain (Martin and Kollewe, 2001). However, only the kinase domains of IRAK-1 and IRAK-4 are active (Wesche et al., 1999). Furthermore, IRAK-1, IRAK-2, and IRAK-M, contain a C-terminal stretch that is important to the interaction of IRAK with another downstream signaling molecule called

the Tumor necrosis factor receptor-associated factor 6 (TRAF-6) (Darnay et al., 1999; Ye et al., 2002).

IRAK-1 and IRAK-4 have been shown to be involved in LPS signaling (Hu et al., 2002; Picard et al., 2003; Suzuki et al., 2003; Swantek et al., 2000). IRAK-4 has been demonstrated to work upstream from IRAK-1, possibly functioning to phosphorylate IRAK-1 (Li et al., 2002). Binding of IRAK-1 and IRAK-4 to MyD88 triggers the phosphorylation of these two kinase proteins causing the dissociation of IRAK-1 from the receptor complex. IRAK-1 then moves to the cytosol where it binds to and further phosphorylates TRAF-6.

TRAF-6 is composed of a conserved carboxy-terminal domain (TRAF-C) and an α-helical N-terminal domain (TRAF-N) that contains a RING finger and five zinc-finger structures (Cao et al., 1996). Unlike the other members of the TRAF family, which utilize the RING finger to mediate Nuclear Factor-κB (NF-κB) activation, the NF-κB activation mediated by TRAF-6 also requires zinc-fingers (Cao et al., 1996). Activated TRAF-6, in turn, interacts with some downstream MAP kinase kinase kinase (MAPKKK) proteins in cytosol (Qian et al., 2001) to activate an enzyme complex known as Inhibitor of κB kinase complex (IKK).

The IKK complex is composed of three subunits; two are the homologous catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ) (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Rothwarf et al., 1998). The activated IKK complex is able to phosphorylate Inhibitory κB (IκB), particularly at $Serine^{32}$ and $Serine^{36}$ of IκB-α and $Serine^{19}$ and $Serine^{23}$ of Iκ-β (Chen et al., 1995). IκB is a family

of related proteins that contain multiple copies of a 30-33 amino acid sequence called ankyrin repeats.

There have been seven IκB proteins identified to date. These include IκB-α, IκB-β, IκB-γ, IκB-ε, Bcl-3, p100 and p105. IκB binds to the transcription factor (NF-κB) via its ankyrin repeats (Huxford et al., 1998; Jacobs and Harrison, 1998). NF-κB is also a family of related proteins. Proteins that belong to this family are RelA (p65), RelB, c-Rel, p50/p105, and p52/p100.

Each member of the Rel family contains a conserved N-terminal region of about 300 amino acids called the Rel-homology domain (RHD). This region contains the DNA binding site, the dimerization domain, and the nuclear localization signal (NLS) (Ghosh et al., 1998). IκB binds to the RHD domain of NF-κB and masks these domains within the RHD, thereby preventing NF-κB from translocating to the nucleus. When IκB is phosphorylated by IKK, IκB is dissociated from NF-κB revealing NF-κB NLS domain and allows NF-κB to translocate to the nucleus. Inside the nucleus, NF-κB binds to specific genes and up-regulates the transcription of those genes. Most of these genes encode proteins of the pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and are involved in immune responses such as an inducible nitric oxide synthase (iNOS) and some adhesion molecules.

Host Responses to Infection

In a healthy individual, homeostasis regulates the normal functions of cells, tissues, and organs within each body system. When homeostasis is disturbed by, for

example, microbial infections, the body will attempt to reestablish homeostasis back to normal and eliminate the disruptive factor(s). The body employs the mechanism known as an 'acute phase response' (APR) to create this new balance. The phenomenon of APR has been well summarized by Kushner (Kushner, 1982). The general characteristics of APR include the onset of fever, an increase in the granulocyte number in blood (neutrophilia/heterophilia in avians) facilitation of blood clotting ability, increased muscle protein catabolism, increased gluconeogenesis, induced changes in lipid metabolism and hormone syntheses, increased the level of copper, reduced plasma iron and zinc, enlarged liver to increase acute phase protein synthesis, induce anorexia and somnolence, and increased activation of complement components.

Fever is an elevation of the body core temperature (T_c) caused by a change in a 'set point' of the T_c of the thermoregulatory center within the anterior hypothalamus (Dinarello et al., 1999; Saper and Breder, 1994). Pro-inflammatory cytokines, mainly IL-1β, IL-6 and TNF-α, secreted during infections, are among several factors important in mediating fever (Dinarello, 1996; Dinarello et al., 1986; Kozak et al., 1998; Rothwell et al., 1991). The exact mechanism of how these cytokines communicate with the thermoregulartory center in the brain is still under debate. Three hypotheses have been proposed to explain how these cytokines work with the brain to trigger fever. 1) These cytokines, after being secreted into the circulatory system, travel to the brain and cross the blood brain barrier (BBB) by an active transport process. 2) These cytokines also cross the BBB where it is leaky. 3) Instead of traveling to the brain, these cytokines stimulate the peripheral nerves to transmit a signal to the brain (Blatteis and Sehic, 1997).

Once these cytokines or their signals reach the brain, they stimulate cells within the hypothalamus to produce and release prostaglandin E_2 (PGE_2), which mediates the onset of fever (Milton and Wendlandt, 1970) by stimulating the release of cAMP (Woolf et al., 1976). The increase in body temperature during infection yields several benefits to the host. Small et al. (1986) reported that fever suppresses bacterial growth. In addition, an elevation of body temperature results in the reduction of a serum level of an iron that restricts the growth of some microorganisms that require iron for their growth (Weinberg, 1971). Green and Vermeulen (1994) found that elevated temperature inhibited the synthesis of LPS of *Klebsiella, Enterobacter, Serratia, Salmonella, Proteus*, and some serotypes of *E. coli***.** In addition, investigators have demonstrated that fever enhances the function of neutrophils (Rosenspire et al., 2002).

Acute phase proteins (APP) are synthesized and secreted by hepatocytes in response to various infections and are induced by pro-inflammatory cytokines, particularly IL-6 (Castell et al., 1989). These APPs act as chemoattractants to recruit immune cells to clear up pathogens (Badolato et al., 1994; Olsson et al., 1999; Xu et al., 1995) or as inducers of the antagonists of pro-inflammatory cytokines (Tilg et al., 1993).

Pro-inflammatory cytokines also induce anorexia and somnolence (Plata-Salaman, 1998) that result in the decrease of food and water intake. As a consequence, anorexia and somnolence may indirectly inhibit microbial growth by limiting their nutrient sources. Meanwhile, the body will rely on stored energy to maintain the proper functions of cells, tissues, and organs during the period of acute phase response. A study of the effect of endotoxin on carbohydrate metabolism in rats showed that blood glucose

level was increased while the percentage of liver glycogen was decreased 30 minutes after endotoxin injection and that plasma glucose level was decreased in five hours (Filkins and Cornell, 1974). These results indicate the mobilization of glucose stored in liver in the form of glycogen. Once the liver glycogen was depleted, muscle protein catabolism will occur to mobilize amino acids to the liver for gluconeogenesis (Holecek et al., 1995; Jepson et al., 1986). Lipid metabolism is also altered during the infection. Several investigators demonstrated that animals receiving LPS showed an increase blood level of triglyceride or total lipid (Feingold et al., 1992; Lequire et al., 1959).

Endotoxin and an acute phase response in poultry

Birds injected with LPS also exhibited acute phase responses including increased body temperature, anorexia, somnolence, increased plasma concentration of iron and zinc, reduced plasma concentration of copper, and increased expression of APP and corticosterone (Johnson et al., 1993; Nakamura et al., 1998). Ferket et al. (2002) reported that turkeys injected with LPS exhibited mild fever (0.25°C increase in body temperature) at 8 hours post injection. In addition, Xie and co-workers (Xie et al., 2000) demonstrated that 21-day-old broiler chickens injected intravenously with *Salmonella typhimurium* LPS showed ruffled feathers, reduced body weight gain, increased liver weight, which suggested increased APP synthesis by hepatocytes (Mireles et al., 2005), increased total white blood cell count, elevated heterophil concentration, and reduced plasma glucose concentration (Xie et al., 2000). Furthermore, the increase of protein catabolism in the wing muscles incubated with crude-IL-1 prepared from the LPS-treated

peritoneal monocytes was also reported (Klasing et al., 1987). The effect of LPS on lipid metabolism in chickens has been studied. In contrast to what has been found in mammals, lipogenesis by chicken adipocytes treated with supernatants collected from endotoxin-treated chicken macrophages was increased (Butterwith and Griffin, 1989). This unexpected result conflicts with the belief that stored energy is mobilized to compensate for low food intake during infection. As in mammals, these acute phase responses presumed to be mediated by pro-inflammatory cytokines. A study of the effect of LPS on gene expression using an avian macrophage-specific cDNA microarray (AMM) revealed that genes encoding IL-1β and IL-6 were up-regulated (Bliss et al., 2005). In addition, an increase in IL-1- and IL-6-like activities in blood drawn from chickens injected with an LPS was reported and incubation of the wing muscles with this IL-1 resulted in increased degradation of muscle protein (Klasing et al., 1987; Nakamura et al., 1998; Xie et al., 2000). IL-1 prepared from peritoneal monocytes treated with LPS also caused the blood level of iron and zinc to decrease (Klasing, 1984).

Antibiotic Growth Promoters

Antibiotics have been used at a subtherapeutic level to promote growth in food animals for almost fifty years. However, the concern about bacterial drug resistance in humans caused by the utilization of these antibiotics as growth promoters (AGP) is also rising and is being debated intensely. In fact, European countries, particularly Denmark, have already banned and voluntarily withdrawn the use of most AGPs and plan to ban all AGPs by 2006. In the United States, several AGPs are still being used in animal feed, but

the practice is under surveillance. Increased pressure from the consumers may lead to the removal of AGPs from animal feed in the US in the future. Due to this bacterial drug resistance problem, researchers, together with the food industry, are now actively seeking alternatives to AGPs.

Growth Promoting Mechanism of Antibiotics

Mechanisms by which antibiotics promote growth are still not completely understood. Antibiotics in germ-free animals do not promote growth. Thus, it was suggested that antibiotics have no direct effect on host gut cells or tissues in promoting growth and that these effects are mainly due to the actions of antibiotics on gut microflora (Coates et al., 1955; Jukes and Williams, 1953). In a review by Francois (Francois, 1961), the mode of action of antibiotics on metabolism included: a reduction in the feed conversion ratio (the ratio between the amount of food consumed and the weight gain); lowered energy expenditure, indicated by the reduction of O_2 consumption and heat production; and increased absorption of calcium. In addition, Francois summarized the effect of antibiotics on the digestion, as antibiotics cause the pH of the ceacal contents to decrease. Antibiotics also increase the absorption of amino acids within the gastrointestinal tract by inhibiting the destruction of these amino acids by bacteria. Antibiotics also increase glucose retention in blood as well as glucose absorption. Furthermore, the activity of some digestive enzymes was found to increase in animals receiving antibiotics. Francois (1961) reported that antibiotics caused a thinning of the intestinal wall that may allow more nutrients to be absorbed. Antibiotics have been

shown to inhibit the formation of ammonia, amines, phenols, and indoles that cause growth-depression. Franscois (1961) noted that antibiotics alter the number of bacteria within the intestine, as well as their metabolism, in a way that facilitates animal growth. Gaskins et al. (2002) have proposed that there are at least four mechanisms by which antibiotics promote growth: 1) Antibiotics inhibit sub-clinical infections; 2) Antibiotics reduce growth-depression caused by microbial metabolites; 3) Antibiotics reduce microbial use of nutrients; and 4) Antibiotics enhance the uptake and use of nutrients through the thinner intestinal wall**.** Understanding how antibiotics work as growth promoters provides a guideline for investigators to search for the alternatives to AGPs.

Alternatives to Antibiotics

The following are some of the potential alternatives to antibiotics that have been proposed as growth promoters:

1) Bacteriocins

Definition and Classification

Bacteriocins are small peptides synthesized by bacteria to kill other closely related bacteria (Tagg et al., 1976). Though first recognized in Gram-negative bacteria (Lazdunski, 1988) due to their benefit on food production, bacteriocins from Gram-positive bacteria are the main focus of this review. According to Klaenhammer, bacteriocins of Gram-positive bacteria, particularly lactic acid bacteria (LAB), are divided into four classes, I-III (Klaenhammer, 1993).

Class I, also known as lantibiotics, are bacteriocins that contain about 19-50 amino acids and usually contain unusual amino acids such as lanthionine, methyl-lanthionine, dehydrobutyrine, and dehydroalanine. An example of lantibiotics is nisin produced by *Lactococcus lactis* (Hurst, 1967). Nisin has received GRAS status (generally recognized as safe, 21 CFR 184.1538) (Joerger, 2003) and has been approved to used as a food preservative for more than fifty years (Cleveland et al., 2001).

Class II bacteriocins are small heat-stable peptides that are not modified with any unusual amino acids as in class I. Bacteriocins in class II are further subdivided into two subgroups: 1) IIa that contain a conserved amino acid sequence (Tyr-Gly-Asn-Gly-Val) at the N-terminus and a disulfide-bridge that is formed by two cysteins at the N-terminal half of the peptides. The target of bacteriocins class-IIa is the foodborne pathogen *Listeria monocytogenes* (Kaiser and Montville, 1996); 2) IIb are the bacteriocins composed of two different peptides.

Class III bacteriocins are large heat-labile proteins. There is still limited information on this class of bacteriocins.

Mode of Action

Reviews by several groups have concluded that bacteriocins kill target bacteria primarily by forming a pore in the cytoplasmic membrane that causes the dissipation of the transmembrane potential, the pH gradient and, ultimately, leads to the release of cellular materials (Cleveland et al., 2001; Hechard and Sahl, 2002; Venema et al., 1995).

Roles of Bacteriocins as Alternatives to AGP

Though bacteriocins have been effectively utilized as food preservatives for several years, there have been few studies investigating the role of these compounds on animal growth. Studies of the effects of bacteriocins in animals have shown impressive results. Wooley et al (1999) generated a non-virulent *Escherichia coli* that produced the bacteriocin mincrocin 24 and orally administered these bacteria to chickens continually via their water supply. They found that the number of *Salmonella typhimurium* within the intestinal tracts of broiler chickens was significantly decreased (Wooley et al., 1999). Another study investigated the inhibitory effect of bacteriocin enterocin CCM 4231 produced by an ovine ruminal strain *Enterococcus faecium* CCM 4231 on other sheep ruminal bacteria has also shown positive results (Laukova and Czikkova, 1998). Entorocin CCM 4231 was found to dramatically reduce the total bacterial cell count of the closely related bacterium *Enterococcus faecium* EF 26/42, slightly decrease the number of *Streptococcus bovis* AO 24/85, and strongly inhibit the growth of *Escherichia coli* from ruminal fluid obtained from the sheep. Recently, Stern et al. (2001) investigated whether bacteriocin with a size of about 4 kDa secreted by *Paenibacillus polymyxa* NRRLB-30509 could reduce the number of *Campylobacter jejuni*, the foodborne pathogen that colonize chicken intestine. Stern reported that *C. jejuni* were not detected in the feces from chickens fed with this bacteriocin and the colonization of *C. jejuni* within the intestine was also reduced.

2) Bacteriophages

Bacteriophages are viruses that replicate in bacteria. The fact that bacteriophages cannot infect animal cells but only its bacterial host has drawn attention from several investigators to explore their role as alternatives to antibiotics. Toro and colleagues (Toro et al., 2005) demonstrated that chicks exposed orally with *Salmonella*-specific bacteriophage and followed with *Salmonella typhimurium* challenge tended to ($P>0.05$) have higher body weight than *S. typhimurium*-challenged chicks that did not received any bacteriophage. Inhibition of *S.typhimurium* colonization within the gastrointestinal tract by exposure to this bacteriophage was also tested. This *Salmonella*-specific bacteriophage was found to significantly reduce the number of *S. typhimurium* within the ceca four days post challenged. However, when the bacteriophage was combined with a defined competitive exclusion agent (CE), a synergistic inhibitory effect of these two products on *S. typhimurium* colonization was not observed.

Bacteriophages have been reported to protect animals from infections by other bacteria as well. Huff et al. (2003) showed that injecting active *E. coli*-bacteriophages intramusculary in *E. coli*-challenged birds reduced the mortality rate by almost 25%. When the birds received multiple injections of the bacteriophages, mortality rate fell to zero. Additionally, the body weight of birds injected with bacteriophage and challenged with *E. coli* was not changed from non-challenged birds. On the other hand, the body weight of birds receiving heat-killed bacteriophages and challenged with *E. coli* dramatically decreased (Huff et al., 2003). Recently, a protective effect against

Enterococcus faecium (Biswas et al., 2002) and *Vibrio vulnificus* (Cerveny et al., 2002) by host-specific bacteriophages has been demonstrated.

3) Competitive Exclusion, Probiotics and Prebiotics

The concept of competitive exclusion is credited to Nurmi and Rantala (Nurmi and Rantala, 1973), who looked for an alternative approach to counteract a severe antibiotic resistant outbreak of *Salmonella infantis*. The Nurmi or competitive exclusion concept was to establish an adult-type resistance to salmonellas in newly hatched chicks by administering adult intestinal microorganisms to the chicks. The term *competitive exclusion* (CE) was proposed by Lloyd et al. (1974) and implied 'the use of undefined cultures of the cecal contents from healthy chickens in the newly hatched chicks' (Lloyd et al., 1974). This cecal content is presumed to contain beneficial bacteria that will colonize within the gastrointestinal tract of the chicks and protect them from harmful bacteria by competing for limited nutrients and attachment sites and by secreting some substances (e.g. volatile fatty acids and bacteriocins) that are toxic to pathogenic microorganisms (Nurmi et al., 1992). However, with the risk of the undefined cecal content being contaminated with some pathogenic bacteria, plus the medium used for differentiating different species of bacteria and several studies of the functions of microflora becoming more widely available, the concept of probiotics has emerged.

Probiotic is defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989)**.** According to Fuller (1989), for any microorganism to be used as a probiotic, it must meet

the following criteria: 1) It must be capable of being prepared in a viable manner and on a large scale (e.g., for industrial purposes); 2) During use and under storage the probiotic should remain viable and stable; 3) It should be able to survive in the intestinal ecosystem; 4) The host animal should gain beneficially from harboring the probiotics. Examples of some bacteria that have been used as probiotics in humans are lactobacilli (e.g. *Lactobacillus acidophilus, L. casei, L. delbruekii*), and bifidobacteria (e.g., *Bifidobacterium adolescentis, B. bifidum, B. longum, B. infantis*). Numerous studies have been conducted to demonstrate the benefits of lactobacilli and bifidobacteria (Asahara et al., 2004; Branca and Rossi, 2002; Cui et al., 2004; Orrhage and Nord, 2000; Reid, 2001). The mode of action of probiotic bacteria is suggested to be multifaceted and is unique in different bacterium. For example, bifidobacteria was found to require iron for growth (Bezkorovainy and Topouzian, 1981) and was suggested to exert its beneficial effect to the host by limiting the iron resources available to pathogenic bacteria (Bezkorovainy and Kot, 1998). In addition, *Lactobacillus reuteri* produces an antimicrobial substance known as reuterin that may be toxic to some pathogens (Talarico et al., 1988). Probiotic bacteria have been incorporated into fermented dairy food such as yogurt, milk, and fruit juices, and are also available to the public as freeze-dried bacteria in capsule, tablet, or powder form (Hamilton-Miller, 2004). However, since probiotic bacteria have to pass through the stomach, an organ providing a rich source of proteases and strong acids, colonization within the intestine may be reduced. Moreover, these bacteria have to compete for nutrients and sites for colonization with indigenous bacteria within the gastrointestinal tract. To overcome some of these limitations, the prebiotic concept was established.

A prebiotic is defined as 'the nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). The criteria for any food ingredient to be classified as a prebiotic are as follows: 1) It must be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract; 2) It must be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated; 3) It must be able to alter the colonic flora in favor of a healthier composition; 4) It must induce luminal or systemic effects that are beneficial to host health. Examples of some prebiotics are non-digestible carbohydrates (oligo- and polysaccharides), some peptides and proteins, and certain lipids. Though many non-digestible oligosaccharides (NDO) have been investigated, the most extensively studied NDO are fructooligosaccharides (FOS) and inulin that are commercially available as RAFTILOSE® and FAFTILINE®, respectively.

4) Mannanoligosaccharide

Mannanoligosaccharide (MOS) is a glucomannoprotein complex isolated from the outer cell wall of yeast (Newman 1994). It is one of the leading products that are potential candidates for antibiotic alternatives. Several studies have been conducted to characterize the effects of MOS on different aspects of animal performance.

MOS agglutinates type-1 fimbriae bacteria

Spring et al. (Spring et al., 2000) used an *in vitro* study to demonstrate that MOS (Alltech, Inc., Nicholasvile, KY) was able to agglutinate *Escherichia coli*, *Salmonella typhimurium, S. enteritidis, S. montevideo, S. give, S. kedougou, and S. dublin*, but not *S. choleraesuis* or *S. pullorum*. This agglutination could be blocked by mannose or fructose sugars. However, a much higher amount of fructose was required to obtain the same inhibitory effect as the lowered amount of mannose. It has been known for a number of years that enteric bacteria have to attach to the epithelial cells within the gastrointestinal tract in order to establish an infection.

Ofek et al. (1977) incubated epithelial cells with *E. coli* K_{12} in the presence of D-mannose or methyl α-D-mannopyranoside and found that these sugars inhibited the adherence of this bacterium to oral epithelial cells. They proposed that the binding of *E. coli* to epithelial cells was mediated by a mannose-specific, lectin-like substance present on the surface of this bacterium which binds to a mannose-like receptor site on epithelial cells (Ofek et al., 1977). Oyofo et al. (1989b) showed that D-mannose and mythyl α-D-mannoside significantly reduced the attachment of *Salmonella typhimurium strain ST-10*, which represented the type-1 fimbriae, mannose-sensitive bacteria, to the small intestine of the one-day-old chicks (Oyofo et al., 1989b). Moreover, the binding of *E. coli* to mouse peritoneal macrophage cells was also blocked by yeast mannan (Bar-Shavit et al., 1980). Firon et al. (1983) concluded that some components within the fimbriae of bacteria might be responsible for the mediation of the attachment of bacteria to epithelial cells because the inhibition of the agglutination assay by mannose, which is carried out

using the fimbriae with yeast mannan, exhibited a similar inhibitory pattern to the agglutination assay with when the intact bacteria were used. Later it was shown that FimH, a mannose-binding lectin, and a shaft comprised mainly of FimA are involved with the attachment of type-1 fimbriae enterobacteria to target cells (Duncan et al., 2005). Thus, mannose or mannan might be used as a therapeutic compound to inhibit infection by enterobacteria. The outer cell wall of yeast *S. cerevisiae* is rich with mannan (Lipke and Ovalle, 1998) and the isolation of mannose from the yeast cell wall is a more practical source of mannose than synthetically produced mannose.

Since MOS is derived from the outer yeast-cell wall and has been shown to be able to agglutinate several strains of *E. coli* and *Salmonella*, it could be used to reduce the number of *S. typhimurium* 29E within the cecum of birds (Spring et al., 2000). Spring et al. (2000) suggested that MOS may acts as a decoy of enterobacteria, employing its mannose residues to bind to lectin-like molecules on the surface of those bacteria. Moreover, MOS is an oligosaccharide that cannot be digested within the gut due to the absence of digestive enzymes. As a consequence, MOS-bound bacteria are discarded from the body through the peristaltic process.

MOS modifies the population of microorganisms within the gut

MOS has been shown to alter the number of commensal bacteria within the gastrointestinal tract. The number of enterobacteria in cecal content prepared from hens fed a MOS-supplemented diet was found to be significantly reduced, whereas the number of *Enterococcus spp.* was significantly increased (Fernandez et al., 2002). Although the authors did not mention the benefit of these changes in the number of the aforementioned

bacteria, it is possible that the increased number of *Enterococci* may contribute some benefit to the host via bacteriocin enterocins (Abriouel et al., 2001; Herranz et al., 2001; Leroy et al., 2003; Oyofo et al., 1989a) produced by *Enterococcus spp.* One study in dogs showed that MOS tended (P=0.09) to decrease the concentration of *C. perfringens* (Strickling et al., 2000), the gram-positive anaerobic spore-forming bacterium found in its natural habitat within the intestine of animals and humans. *C. perfringens* is also capable of causing foodborne diseases (Hatheway, 1990; Rood and Cole, 1991). Additionally, the concentration of *C. perfringens* was also significantly decreased in MOS-fed turkeys, whereas, the concentration of bifidobacteria was found to be significantly increased (Sims et al., 2004). Bifidobacteria are the desirable bacteria that have been reported to enhance human health (Abe et al., 1995; Ouwehand et al., 2002a; Ouwehand et al., 2002b; Saavedra et al., 1994).

MOS modulates the metabolic products of gut microflora

Besides changing the number of normal flora within the gastrointestinal tract, MOS alters the level of metabolite products of these bacteria, as well. The concentration of ammonia in the feces from dogs fed MOS was significantly lower than from dogs fed a basal diet (Zentek et al., 2002). Similar results were obtained from a study in young turkeys (Juskiewicz et al., 2003). Ammonia is a toxic metabolic product resulting from protein fermentation of bacteria within the large intestine. It has strong odor, causes growth depression of animals, and may contribute to colon carcinogenesis (Lin and Visek, 1991; Salminen et al., 1998; Visek, 1978). However, studies of the effects of MOS on other bacterial metabolic products, such as volatile fatty acid (VFA), lactate, and

short-chain fatty acids (SCFA), in various animals showed no effect (Spring et al., 2000; Strickling et al., 2000; Swanson et al., 2002; White et al., 2002).

Effect of MOS on animal growth and performance

Zdunczyk et al. (2005) demonstrated that turkeys receiving diets supplemented with a medium or high-level of MOS gained significantly more weight than those receiving a basal diet or a diet supplemented with a low level of MOS 16 weeks later. However, there was no difference in the body weight of these turkeys at the ages of 4 and 8 weeks (Zdunczyk et al., 2005). Additionally, turkeys fed MOS were significantly heavier than the ones that did not receive MOS at the age of 18 weeks (Sims et al., 2004). (Parks et al., 2001) found the BW of turkeys fed with MOS was significantly higher than the BW of turkeys fed with a typical US corn-soybean meal diet. The hens that were challenged with *E. coli* and fed with MOS showed a significantly greater body weight gain compared to a basal diet (Fairchild et al., 2001). However, MOS had no effect on body weight gain in dogs, pigs, and calves (Heinrichs et al., 2003; Strickling et al., 2000; White et al., 2002).

MOS modulates immune responses in animals

A study of the effect of glucomannan (GM) on the immune response revealed that the proportion of IgA-presenting lymphocytes, analyzed by flow cytometry, within the cecum of rats were significantly higher than when cellulose (CL) was used (Kudoh et al., 1999). In this study, GM was used as a highly fermentable indigestable saccharide (IDS), while CL was used as non-fermentable IDS. The amount of IgA within the cecal contents was also markedly increased. Swanson et al. (2002) reported that the percentage of

lymphocytes in the total white blood cell (WBC) count in blood collected from dogs fed MOS was significantly increased (Swanson et al., 2002). Nevertheless, MOS did not affect the level of serum IgG and IgM in dogs.

Given the fact that cecal IgA can be elevated with MOS supplementation while neither IgG nor IgM are elevated, but yet lymphocytes numbers in blood are increased, it is important, therefore, to determine if there is a possibility that early responses of a cell mediated dependent development in the immune response might be affected by MOS. Specifically, it is important to determine if macrophages, which are the first cells to respond to a potential bacterial pathogen, react by inducing production of cytokines that will drive the inflammatory reaction that is the first defined immunological response to bacterial invasion and to determine the influence MOS exerts on that response.

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CHAPTER 1

Agglutination of Type-1 Fimbrial Bacteria by Different Yeast Cell Wall Products

Abstract

The agglutination-inducing activity of six commercial products of mannan oligosaccharide contained in yeast cell wall was examined using an agglutination assay previously described (Peng et al., 2001). When the assay was performed using *Escherichia coli*, the agglutination-inducing activities of Product B, Product E, and Product A were the highest with percentages of aggregated cells being in excess of 70%. Product F and Product C moderately agglutinated *E. coli* with percentages of aggregated cells of 68.5% and 60.27%, respectively. Product D had only 45.69% aggregated cells. When *Salmonella typhimurium* was used in the agglutination assay, Product E and Product A agglutinated the most *S. typhimurium* cells with 69.60% and 68.93% aggregated cells, respectively. Product F, Product B and Product C exhibited lower agglutination-inducing activity than Product E and Product A with percentage of aggregated cells of 61.67%, 57.69% and 58.91%, recpectively. As seen in the agglutination assay with *E. coli*, Product D was the product with the lowest agglutination-inducing activity that only 45.90% of *S. typhimumrium* cells. The agglutination of *E. coli* and *S. typhimurium* by these yeast products was mediated apparently by the mannose residue on the yeast cell wall since mannose significantly reduced the percentage of aggregated cells by these products.

Introduction

Enterobacteria, particularly *Escherichia* and *Salmonella,* are the major causative agents of infections within the gastrointestinal tract (GIT) of both humans and animals. Infections of animals with these bacteria, especially livestock and poultry, not only cause an economic loss for the producers, but also serve as the reservoir of contaminated meats that are the sources of foodborne diseases in humans. In order to establish the infection within the GIT, these bacteria have to attach to target cells such as enterocytes, M cells, or macrophages. Many members of the family *Enterobacteriaceae* possess type-1 fimbriae or pili (Fader et al., 1982; Kohno et al., 1984; Korhonen et al., 1980; Salit and Gotschlich, 1977), the rigid, hair-like structures that express peritrichously on the surface of bacteria (Brinton, 1965; Duguid et al., 1966). Type-1 fimbriae are different from the other types of fimbriae in their ability to agglutinate erythrocytes, but this agglutination can be blocked with α-D-mannose (Clegg and Gerlach, 1987). Although the organization of the DNA segment containing genes encoding the protein products that are involved in the synthesis of type-1 fimbriae in different bacterial genera are not identical (Clegg et al., 1987; Orndorff and Falkow, 1984), the basic structure of all type-1 fimbriae is composed of FimA and FimH.

FimA is the major component of type-1 fimbriae. Thousands of copies of FimA, also called pilli, polymerize helically to form a pilus (Orndorff and Falkow, 1985) whose nature is extremely stable as indicated by its resistance to 6M urea, solubilization by sodium dodecyl sulfate (SDS), and hydrolysis by trypsin (McMichael and Ou, 1979; Salit

and Gotschlich, 1977). The transcription of the *fimA* gene, that encodes for protein pilin, is regulated by the products of the genes *fimB* and *fimE* (Blomfield et al., 1991).

FimH is a minor component of type-1 fimbriae. It is located at the tip of the pilus and is the component that mediates the binding of bacteria to host cells (Abraham et al., 1987; Hanson and Brinton, 1988; Jones et al., 1995). The binding of bacteria to host cells via FimH has been shown to be mannose-specific (Abraham et al., 1988; Krogfelt et al., 1990; Ofek et al., 1977).

Several studies have shown reduced adherence of type-1 fimbrial bacteria to epithelia-derived enterocytes in the presence of mannose sugar or its derivatives (Ofek et al., 1977; Oyofo et al., 1989a). Thus, it appears that mannose might be used as a therapeutic agent to prevent or treat the infections by the enterobacteria.

D-Mannose is an aldohexose in mannans, found in polysaccharides, in plant materials that has poor digestibility and absorption from the intestinal tract of poultry (Bogner, 1961). Supplementation of synthesized or purified mannose can be a costly approach in attempts to reduce pathogen colonization of the GIT. Mannan can inhibit the binding of *E. coli* to target cells (Bar-Shavit et al., 1980). The outer cell wall of yeasts such as *Saccharomyces* (Lipke and Ovalle, 1998) or *Candida* (Kapteyn et al., 1995) provide a rich source of mannan. Yeast cells have been shown to be able to agglutinate several bacteria, and this agglutination was blocked by D-mannose or its derivatives (Eshdat et al., 1981; Mirelman et al., 1980). This suggested that yeast cell mannan was sufficient to bind type-1 fimbrial bacteria and that competitively, D-mannose could block

the yeast-induced agglutination because the D-mannose would have bound to the type-1 fimbriae on the bacteria.

In this study, we compared the ability of different yeast cell wall products to agglutinate *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*S. typhimurium*) in *in vitro* tests.

Materials and Methods

Yeast Cell Wall Products and Bacterial Strains

Six yeast cell wall products, designated as A-F, were tested for their agglutination-inducing activity on *E. coli* and *S. typhimurium*. Product A is composed of 14% mannan and 20% β-glucan. Product B contains 11% mannan and 37% β-glucan. Product C has 24-26% mannan and 22-24% β-glucan. Product D consists of 6.2% mannan and 32% β-glucan. Product E is composed of 45% mannan and 30% β-glucan. The last product, Product F contains 11% mannan and 33% β-glucan. These products were dispersed with distilled water to obtain the concentration of 100 mg/ml.

Bacteria used in this experiment were the VPI isolate of an enteropathogenic *E. coli* and a Nal^R/Nov^R *S. typhimurium*. Bacteria were grown in the LB broth at 37°C with agitation at 130 rpm in the shaker overnight. The overnight cultures were harvested by spinning at 1500 rpm in the centrifuge and washed two times with PBS, pH 7.2. Bacterial pellets were re-suspended with PBS and the concentrations of bacteria were determined by measuring the absorbency at 600 nm and adjusted to 10^{10} cells/mL. Bacteria were kept on ice until ready to use which was in the same day.

Agglutination Assay

The agglutination of bacteria by various yeast products was examined as described previously (Peng et al., 2001) with some small modifications. Briefly, in a 15-mL tube, 200 μL of bacterial suspension was added to 2 mL of phosphate-buffer saline (PBS, Sigma, # P4417), pH 7.2, followed by the addition of 1.8 mL of yeast- cell wall suspension or PBS. The agglutination of each yeast product was performed in triplicate. The tubes were then vortex-mixed for 10 minutes. After mixing, the suspension was allowed to settle for 10 minutes prior to removing of 3.5 mL of the upper solution and put into a new tube. Water was added to the upper solution that was removed to bring the volume up to 4 mL. This suspension was designated as A. Volume of the remaining precipitate was also increased with 3.5 mL of water to bring the volume up to 4 mL. This suspension was designated as B. The volume of the precipitate in the tube to which PBS was added instead of the yeast product was also brought up to 4 mL, and this tube was designated as C. Tubes were mixed to homogenize the suspension. The density of the suspension was measured by reading the absorbency at 600 nm using the spectrophotometer. The agglutination-inducing activity of the yeast products was calculated as the percentage of bacterial cells that agglutinated in the yeast product suspension, expressed as $(OD_B-OD_C)/(OD_A+OD_B)$ x 100%. The agglutination assay was repeated two times when *E. coli* was used and three times with *S. typhimurium*. The assay was done in duplicate for each product at each trial.

Agglutination Inhibition Assay

The agglutination inhibition assay was performed to test whether the binding of these yeast products to bacteria is mediated by mannose residue. This assay was basically similar to the agglutination assay described above except 100 µl of 1 mg/mL mannose was added into the tube containing 2 mL PBS along with 200 µl of bacterial suspension, and 1.7 mL yeast suspension was used.

Statistical Analysis

Data were subjected to analysis of variance using the General Linear Models Procedure of SAS (SAS Institute, 1996). When there were significant main effects, means were separated using LSD (SAS, 1996). The correlation between percentage aggregated cells and percentage of mannan or glucan was also analyzed using the Correlation Procedure of SAS. Statements of significance are based on $P \leq 0.05$ or as stated in the text.

Results

Figures 1 and 2 show the agglutination-inducing activity of different yeast cell wall products when the agglutination assay was performed using *E. coli* and *S. typhimurium*, respectively. All yeast cell wall products were able to agglutinate both *E. coli* and *S. typhimurium*. Overall, the ability of these products to agglutinate *S. typhimurium* was slightly lower than their ability to agglutinate *E. coli*.

Product B along with Product E and Product A were among the products that exhibited the strongest agglutination-inducing activity (over 70%) on *E. coli*. Percentage aggregated cells with these three products was significantly higher than those of Product

F, Product D, and Product C. Product F and Product C showed moderate agglutination-inducing activity on *E. coli* with percentage aggregated cells of 68.53% and 60.27%, respectively. It should be noted that, even though we grouped Product F and Product C into the same moderate agglutination-inducing group, percentage aggregated cells of Product F was significantly greater than that of Product C. Product D was found to have the lowest percentage aggregation of *E. coli* cells with 45.69%.

Product E and Product A exhibited the highest agglutination-inducing activity on *S. typhimurium* (69.60% and 68.93% aggregated cells, respectively) compared with all other products. Product F, Product B, and Product C showed moderate agglutination-inducing activity on *S. typhimurium* with percentage aggregated cells of 61.67%, 57.69%, and 58.91%, respectively. Product D was the weakest agglutination inducer on *S. typhimurium* with percent aggregated cells of only 45.90%.

In the presence of mannose, percentages of aggregated cells of all yeast cell wall products with both bacteria were significantly reduced (Figures 3 and 4).

Discussion

Attachment of bacteria to target cells, such as enterocytes, M cells and macrophages, is an essential step for bacterial pathogenesis within the gastrointestinal tract. Enterobacteria that express type-1 fimbriae on their cell surface employ FimH, a minor component of type-1 fimbriae, to bind to mannose residues that are expressed on the surface of target cells (Abraham et al., 1988; Krogfelt et al., 1990). Mannose or its derivatives such as α-D-mannopyranoside has been shown to be able to block or reduce the adherence of enterobacteria to the epithelial cells in the intestine (Ofek et al., 1977;

Oyofo et al., 1989b). The importance of the aforementioned work is that mannan in yeast cell walls, a rich and inexpensive source of mannan, can be used to bind and agglutinate several strains of *E. coli* and *Salmonella* (Spring et al., 2000).

In this report, we compared the ability of six yeast cell wall products, which contained varying amounts of mannan and β-glucan, to agglutinate *E. coli* and *S. typhimurium*. However, the detailed structures and the methods to quantify the percentage mannan and β-glucan in these products were not identified and percentages of each are those provided by the individual companies manufacturing or distributing the products for commercial purposes.

We found that all six products agglutinated both bacteria tested. The agglutination-inducing activity of all yeast products on *S. typhimurium* was slightly lower than on *E. coli*. This result may reflect the variation in the composition of type-1 fimbriae in different genera of bacteria of the family *Enterobacteriaceae* as the organization of genes encoding the components involved in type-1 fimbrial synthesis of these bacteria are not identical (Clegg et al., 1987).

Product B, Product E, and Product A were the products that induced the strongest agglutination of *E. coli* cells with percentages of aggregated cells of over 70%. Product E and Product A exhibited the highest agglutination-inducing activity on *S. typhimurium*. The activity of Product B was more than 10% lower than Product E and Product A in the case of *S. typhimurium*. Product B, Product E, and Product A contain 11%, 45% and 14% mannan, respectively. Product F and Product C showed lower percentages of aggregated cells, and Product D had the lowest percentage of aggregated cells of all yeast products

tested. Percentages of mannan in Product F, Product C, and Product D are 13%, 24-26% and 6.2%, respectively. The correlation coefficients between percentage aggregated *E. coli* cells or *S. typhimurium* and percentage of mannan are 0.326 (P=0.08) and 0.421 (P=0.004), respectively (Table 1). These data indicated that the amount of mannan in the yeast products is, in part, responsible for the ability of yeast products to agglutinate type-1 fimbrial bacteria. As has been previously reported by several investigators, the binding to these yeast products to *E. coli* and *S. typhimurium* was mediated by mannose since percentages of aggregated cells of all products were significantly decreased when mannose was added. However, percentage mannan content is not the complete reason for improved aggregation of bacterial cells. Some product contained a lower percentage of mannan, but still was able to agglutinate more bacterial cells than the ones with higher percentages of mannan. Thus, other factors such as the number of mannose residues on the oligosaccharide or the types of glycosydic linkage appear to influence the ability of yeast products to bind to and agglutinate bacteria. Percentages of β-glucan did not show any correlation with the agglutination-inducing activity of yeast products examined since the correlation coefficient between percentage aggregated *E. coli* and percentage glucan of -0.029 (P=0.88) (Table 1). Interestingly, percentage of β-glucan showed negative correlation with the percentage aggregated *S. typhimurium* with the correlation coefficient of -0.293 (P=0.05) which indicates the inhibitory effect of β-glucan on the agglutination-inducing activity of yeast cell wall products on this bacterium.

The results from this study showed that all mannan containing yeast cell wall products employed in this investigation were capable of inducing agglutination of two

different enterobacteria, *E. coli* and *S. typhimurium*. However, differences of percentage aggregations of cells among various products should not be the sole reason for one to use as a tool to select the best product to use as a feed additive since pilli is not the only factors that determine the virulence of pathogenic bacteria. Factors such as, whether bacteria produce toxins, types of toxins they produce, or the structure and function of polysaccharide in bacterial cell wall are also contributed to the virulence of bacter (Gyles, 1994). In addition, one yeast cell wall product does not agglutinate all enteropathogenic bacteria (Spring et al., 2000). Therefore, as a screen, *in vitro* agglutination test is important in providing only some basis for the action of the MOS product *in vivo*. Given these results, it is imperative that field testing of the products be done to provide full evaluation of the effectiveness of each product. Use of the native product in an *in vitro* environment is not a definitive evaluation because *in vivo* those products will be influenced by gut pH, gut enzymes and even gut bacteria and their metabolic products.

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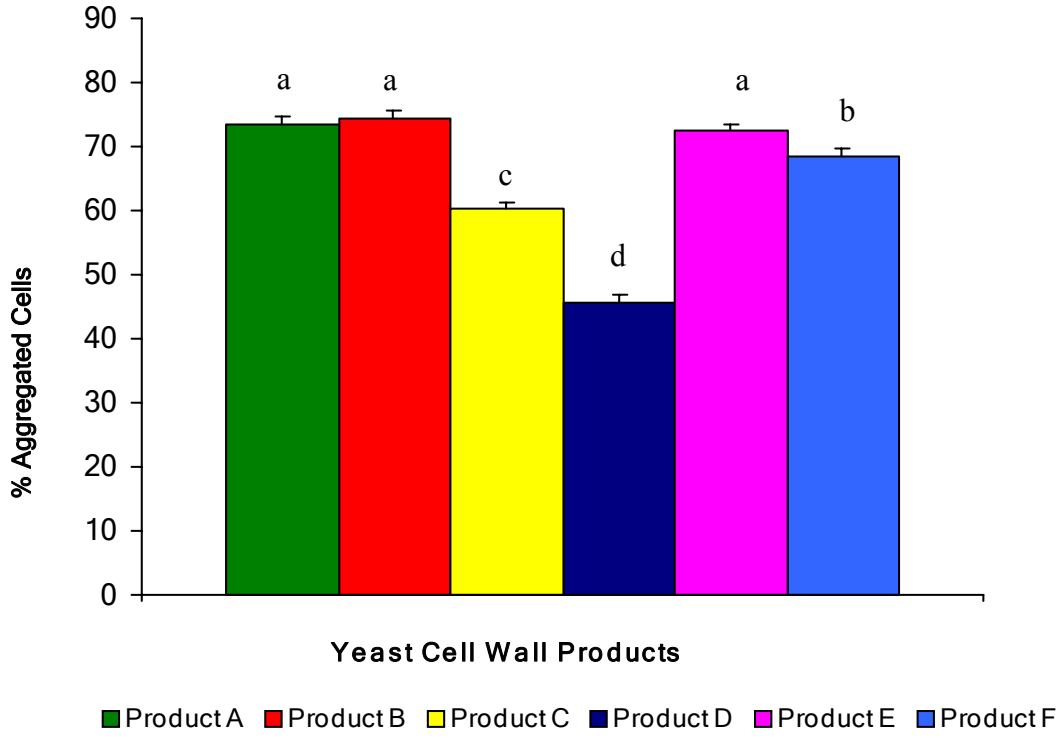
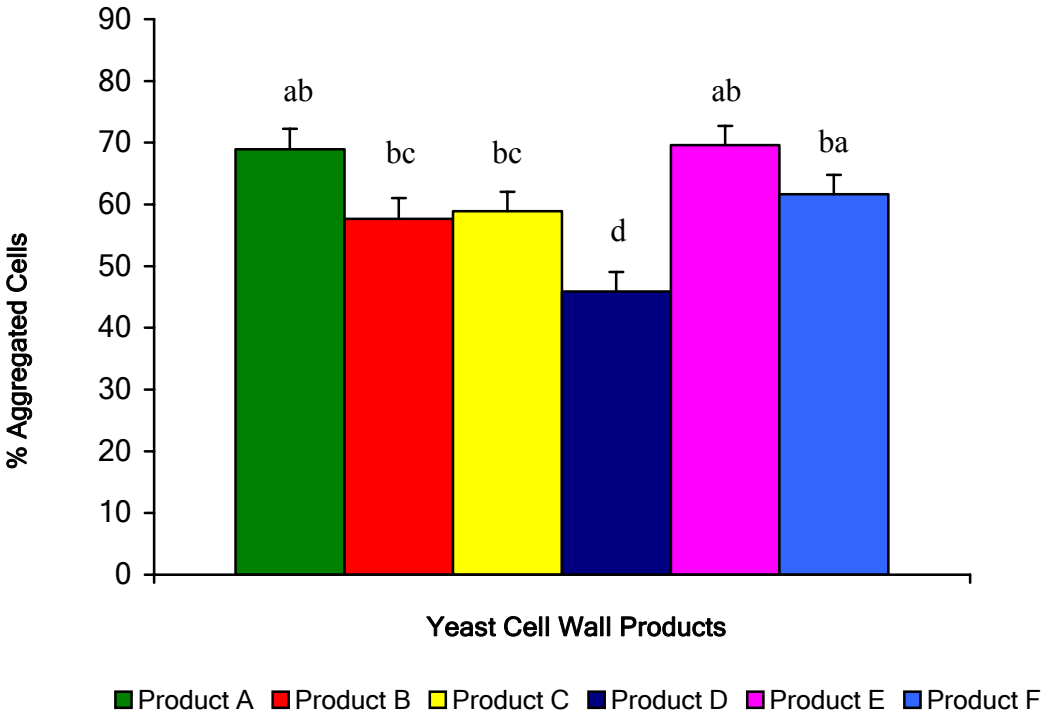
Figure 1. The agglutination-inducing activity of different yeast products on *E. coli* cells.



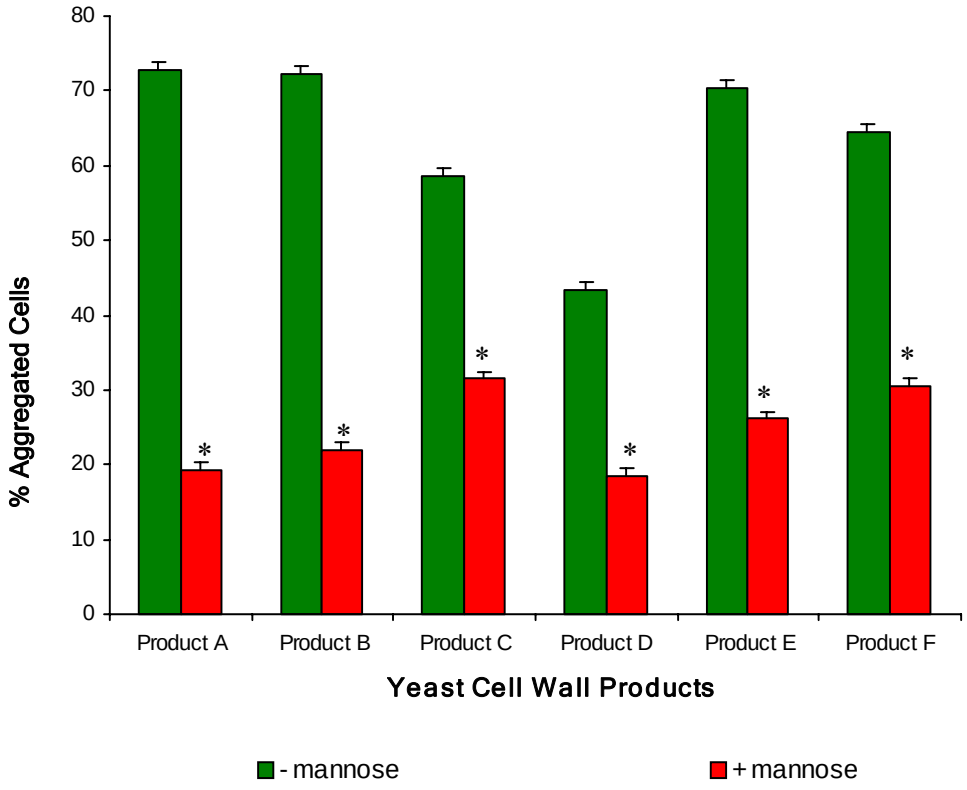
Note: Products with different superscripts are statistically significant different from each other.

Figure 2. The agglutination-inducing activity of different yeast products on *S. typhimurium* cells.



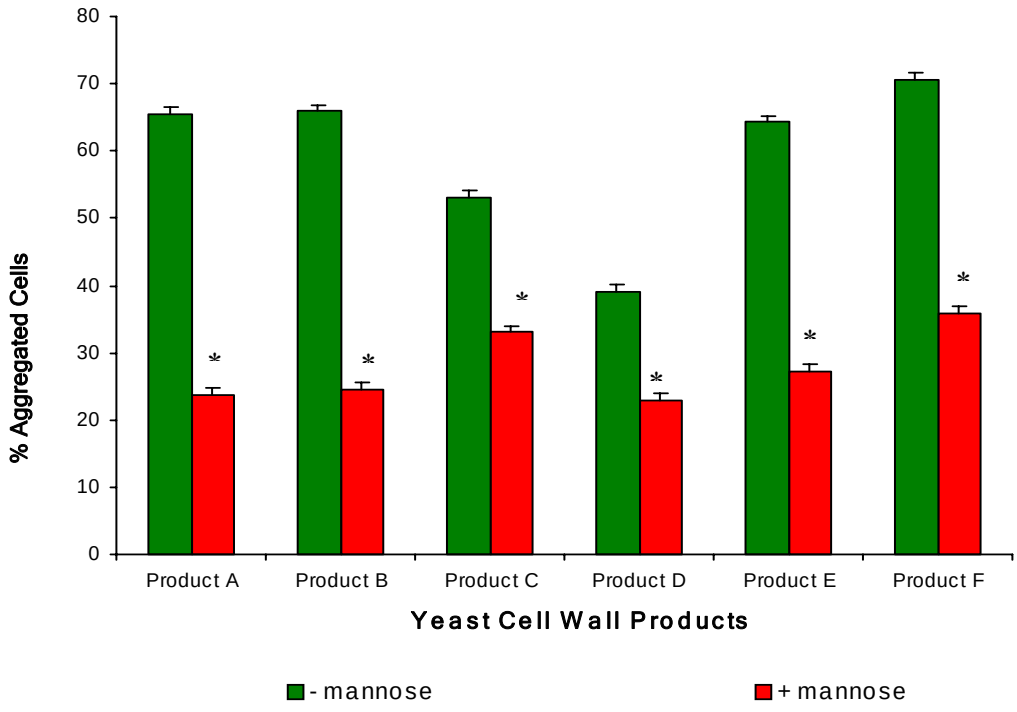
Note: Products with different superscripts are statistically significant different from each other.

Figure 3. The agglutination-inducing activity of different yeast products with or without mannose on *E. coli* cells.



* Represents a significant (P≤0.05) mannose-related inhibition of agglutination by different products containing mannanoligosacharide.

Figure 4. The agglutination-inducing activity of different yeast products with or without mannose on *S. typhimurium* cells.



* Represents a significant (P≤0.05) mannose-related inhibition of agglutination by different products containing mannanoligosacharide.

Table 1. The correlation between the agglutination-inducing activity and percentage of mannan or glucan of yeast cell wall products.

Assay	Correlation Coefficient with Percentage of Mannan	Correlation Coefficient with Percentage of Glucan
Agglutination with E. coli	0.326	- 0.029
Agglutination with S. typhimurium	0.421	- 0.293

CHAPTER 2

The Mannan Rich Fraction of *Saccharomyces cerevisiae* in Bio-Mos® Reduces Lipopolysaccharide-Induced Pro-inflammatory Cytokine and Nitric Oxide Production by Chicken-Macrophage Cell Lines

Abstract

Gastrointestinal tract (GIT) infections induced by pathogenic Gram-negative bacteria, cause revenue losses for livestock producers. Lipopolysaccharide (LPS) located within the outer membrane of Gram-negative bacteria causes the release of pro-inflammatory cytokines and nitric oxide that mediate the signs of gastrointestinal distress in infected animals. In this study, the soluble mannan rich fraction (MRF) from the glucomannoprotein found in *Saccharomyces cerevisiae* yeast cell wall and commercially marketed as BioMOS® (Alltech Inc., Nicholasville, KY) was capable of reducing the inflammatory responses of LPS-stimulated chicken macrophage cell lines (MQ-NCSU and HTC). The MRF (10 mg/mL or higher) was found to be toxic to the cell lines, but MRF at 2.5 mg/mL caused cell line proliferation. The effect of the MRF on nitric oxide and Interleukin-6 (IL-6) production by the chicken macrophage cell lines was determined. By itself, MRF had no effect on nitrite or IL-6 secretion of either the MQ-NCSU or HTC cell lines, but MRF significantly reduced the nitrite and IL-6 production of these LPS-stimulated two cell lines. MRF was found by RT-PCR to down-regulate *iNOS* and *IL-6* gene expression in LPS-stimulated macrophage cell lines. These observations suggest that MRF acts to facilitate an anti-inflammatory response by

reducing the synthesis and release of inflammatory cytokines by LPS-stimulated macrophages. Thus, the benefits of BioMOS® supplementation in livestock and poultry feed could be related to less energy being directed to massive inflammatory reactions and more directed into growth.

Introduction

Lipopolysaccharide (LPS) is an endotoxin embedded within the outer membrane of Gram-negative bacteria. Generally, LPS consists of three main components: (1) an O-antigen, (2) a core region, and (3) lipid A. The O-antigen is a repeating unit of the polysaccharide to which the immune response is directed against. This component of the LPS structure is highly variable among bacterial strains even within the same species, and is used as a tool to characterize of the serotype of each strain of bacteria. The core region is a conserved oligosaccharide and is common among bacteria of a single genus. Lipid A is a glycolipid consisting of a phosphorylated β (1,6)-linked D-glucosamine disaccharide backbone to which long-chain fatty acids are attached (Raetz, 1990; Raetz and Whitfield, 2002; Schnaitman and Klena, 1993; Ulevitch and Tobias, 1995). Lipid A is the toxic part of the LPS molecule (Takada and Kotani, 1989).

The LPS exerts its biological activities when Lipid A is specifically bound by a LPS-binding protein (LBP), a glycoprotein secreted by hepatocytes in response to an infection (Ramadori et al., 1990), to form an LBP-LPS complex. The formation of a LBP-LPS complex facilitates the binding of LPS to CD14, a glycoprotein that exists as both a soluble and a membrane-bound protein (Haziot et al., 1988). Binding of LPS to CD14 brings the latter molecule closer to its potential receptor complex that consists of a

Toll-like Receptor-4 (TLR-4) and a MD-2 molecule. TLR-4 is a type-1 transmembrane protein that contains multiple leucine-rich repeats (LRRs) on the extracellular domain and the Toll/Interleukin-1 Receptor (TIR) domain on its cytoplasmic portion (Hashimoto et al., 1988; Kobe and Deisenhofer, 1995; Medzhitov et al., 1997). MD-2 is a small protein expressed on the surface of several cell types in association with TLR-4 (Shimazu et al., 1999). Binding of LPS to its receptor complex causes the induction of receptor aggregation (Martin and Wesche, 2002) and subsequently triggers the signaling cascade that results in the translocation of the transcription factor nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus. The NF-κB binds to and activates transcription of certain genes. These genes include those that encode pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) and an inducible nitric oxide synthase (iNOS), an enzyme involved in the synthesis nitric oxide (NO) (Muller et al., 1993; Takada et al., 1991; Tamura et al., 1992; Xie et al., 1992).

Pro-inflammatory cytokines have several biological functions. These include increasing body temperature (Dinarello, 1996; Dinarello et al., 1986; Kozak et al., 1998; Rothwell et al., 1991), inducing the synthesis of acute-phase proteins by hepatocytes (Castell et al., 1989), induction of anorexia and somnolence (Plata-Salaman, 1998), and altering nutrient metabolism (Feingold et al., 1992; Filkins and Cornell, 1974). Collectively, the purpose of these responses is to assist the body in its attempts to re-establish homeostasis that was disrupted by infection and to eliminate the invasive pathogen(s). However, excessive production of these pro-inflammatory cytokines brings about sepsis, a systemic inflammatory response syndrome (SIRS) characterized by the

occurrence of the cardiovascular dysfunction, dissemination of intravascular coagulation and hypotension that lead to inadequate organ perfusion and tissue hypoxia (Parrillo, 1993; Van Amersfoort et al., 2003). The latter can cause multiple organ failure (e.g. kidney and liver), which is usually associated with increased death rates in infected individuals (Brun-Buisson et al., 1995).

Nitric Oxide (NO) is a small molecule of free radical gas that is involved in several cell and tissue biological functions in humans and animals. One molecule of NO can be generated from an amino acid L-arginine catalyzed by NO Synthase (NOS). There have been several studies indicating the association of NO with the protective immune responses (James, 1995; Karupiah et al., 1993; Laubach et al., 1995; Lorsbach et al., 1993; MacMicking et al., 1997). However, NO was also suggested to be a key mediator of septic shock (Groeneveld et al., 1994; Parrillo, 1993) due to its ability to act as a potent endogenous vasodilator (Kilbourn et al., 1990).

BioMOS® (Alltech, Inc., Nicholasville, KY 40356) is a glucomannoprotein complex isolated from the outer cell wall of the yeast *Saccharomyces cerevisiae*. BioMOS® is one of the potential alternatives to antibiotics and has been used as a feed supplement to improve animal health and growth performance. It has been shown to be able to agglutinate enterobacteria with type-1 fimbriae and the agglutination was inhibited by the addition of mannose sugar (Spring et al., 2000). Spring et al. (2000) demonstrated that BioMOS® could reduce the concentration of *S. typhimurium* 29E within the cecum of the chickens and suggested that BioMOS® bound mannose receptor ligands on enterobacteria with fimbrae. Moreover, BioMOS® was reported to be able to

increase the number of beneficial bacterial microflora such as *Enterococcus spp.* (Fernandez et al., 2002) and reduce the concentration of pathogenic bacteria such as *C. perfringens* (Strickling et al., 2000). BioMOS® also enhanced the immune responses in animals (Kudoh et al., 1999; Swanson et al., 2002). Interestingly, animals receiving BioMOS® in the feed gained significantly more weight than the ones receiving only the basal diet (Sims et al., 2004; Zdunczyk et al., 2005). Furthermore, the mortality rate in animals fed BioMOS® and challenged with some pathogenic bacteria was lower than in those without BioMOS® as illustrated by significantly reduced mortality in chickens challenged with *Salmonella* (Spring et al., 2000).

In this study, BioMOS® was examined to determine whether it might reduce inflammatory signaling related to LPS-induced stimulation of chicken immortal macrophage lines fed MRF, the soluble fraction of the BioMOS®, in an *in vitro* system.

Materials and Methods

Cell Lines, Media, and Culture Conditions

Three cell lines were used in this present study: 1) The MQ-NCSU cell line (maintained at North Carolina State University Department of Poultry Science) is the chicken macrophage tumor cell line generated from cells isolated from the spleen of the Marek's disease virus (MDV)-infected Dekalb XL chicks (Qureshi et al., 1990). The MQ-NCSU was maintained in Leibovitz-McCoy's complete medium (CM) consisting of 38.5% Leibovitz L-15 medium (Sigma, St. Louis, MO), 38.5% McCoy's 5a medium (Sigma), 5% tryptose phosphate broth solution (Sigma), 8% fetal bovine serum (FBS)

(HyClone Laboratories, Inc., Logan, UT), 10% chicken serum (Sigma), 1% of 200mM L-glutamine (Sigma), 1% of 100x sodium pyruvate (Sigma), 1% of 100x antibiotic antimycotic solution (Sigma), and 1% of 0.001M 2-mercaptoethanol (2-ME), at 41°C, 5% CO_2 humidified incubator. 2) The HTC cell is also a chicken macrophage cell line that was discovered in a broiler chicken-heterophil/granulocyte culture that was inadvertently left in the incubator for a long period and was found to be transformed by avian leukosis virus (ALV) and MDV (Rath et al., 2003). The HTC cell line was maintained in the RPMI medium (Sigma) supplemented with 5% FBS (HyClone Laboratories, Inc.) and 1% antibiotic antimycotic solution (Sigma). The HTC cell line was a gift from Dr. N. C. Rath (US Department of Agriculture and the Department of Poultry Science, University of Arkansas, Fayetteville, AR). 3) The B-9 cell is a murine hybridoma/plasmacytoma B-cell line (DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). It was maintained in RPMI medium supplemented with 10% FBS, 1% antibiotic antimycotic solution, and 25 μL of 20μg/mL recombinant human IL-6 (rhIL-6, Affinity BioReagents™, Golden, CO).

Stimulation of Chicken Macrophage Cell Lines for Nitrite and IL-6 Assays

In 96-well tissue culture plates, the chicken macrophage cell lines at $1x10^4$ cells/well were cultured with medium only, 2.5 mg/mL MRF, 2.5 mg/mL MRF + 5 μg/mL LPS (*E. coli* strain O127: B8, Sigma), or 5 μg/mL LPS at 41°C (for MQ-NCSU) or 37°C (for HTC), in a 5% CO_2 humidified incubator. The supernatants were collected at 1, 2, 3, 4, 5, and 24 hours for kinetic studies and at 0, 2, 5, and 24 hours after incubation for nitrite or IL-6 assays.

Stimulation of Chicken Macrophage Cell Lines for RT-PCR

Approximately $30x10^6$ chicken macrophage cells/flask/treatment/10mL were cultured in T-25 tissue culture flasks (Fisher) with medium only, 2.5 mg/mL MRF, 2.5 mg/mL MRF + 5 μg LPS, or 5 μg LPS alone at 41°C (for MQ-NCSU) or 37°C (for HTC), with 5% CO_2. Cells were collected at 0, 6, and 18 hours by centrifugation at 1200 rpm (400 x g) for 10 minutes, followed by RNA extraction using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The integrity of the RNA was checked using the agarose-formaldehyde gel electrophoresis prior to converting the RNA to cDNA using the ImProm-II™ Reverse Transcription System (Promega). First, the RNA sample and the primers in nuclease-free water (2μL nuclease-free water, 2μL RNA sample, 1μL of 0.5μg/μL oligo $(dT)_{15}$ primer) were preheated at 70°C for 5 minutes and immediately chilled on ice for 5 minutes. Then 15μL of the master mix was added to the denatured sample. Each 15μL of the master mix included: 4μL ImProm-II™ 5x reaction buffer, 5μL of 25mM $MgCl_2$, 1μL of 10mM dNTP mix, 1μL (20U) of recombinant RNasin™ ribonuclease inhibitor, 1μL ImProm-II™ reverse transcriptase, and 3μL nuclease-free water). The reverse transcription reaction was run with the Eppendorf® Mastercycler Gradient (Brinkmann an Eppendorf company, Westbury, NY). The RT-PCR cycle used was: Annealing at 25°C for 5 minutes, Extension at 42°C for 1 hour, and Deactivation of the reverse transcriptase enzyme at 70°C for 15 minutes. The cDNA yield was quantified by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,

Wilmington, DE), the samples were adjusted to the concentration of 1 μg/μL and stored in a -70°C freezer until used.

MTT Assay

The MTT assay was performed to determine the cytotoxic effect of the Mannan Rich Fraction (MRF) on the chicken macrophage cell line. A chicken macrophage cell line, MQ-NCSU, was plated on flat-bottom, 96-well tissue culture plates (Fisher Scientific, Pittsburgh, PA) at $1x10^4$ and $5x10^3$ cells/well. Different concentrations of MRF (Alltech, Inc., Nicholasvile, KY), ranging from 1.25 mg/mL to 80 mg/mL, in CM medium or medium only were added (four wells/ each treatment/each cell number). Plates were incubated at 41°C, 5% CO_2, for 18-24 hours followed by the addition of 50 μL of 1mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) and incubated for an additional 3-4 hours. The viable MQ-NCSUs absorbed the MTT, which was then metabolized by mitochondria yielding a blue crystalline compound. When the plates were removed, 150μL of the supernatant was discarded followed by the addition of 150μL of 0.85% acid alcohol (200mL 2-propanol + 1.32mL HCl + 100mL PBS; all chemicals were purchased from Sigma) with thorough mixing to dissolve the crystalline compound. The plates were centrifuged at 1200 rpm (400 x g) for 10 minutes, the supernatants were transferred to new plates, and the absorbency was read at 540 nm. The averages of the absorbance readings were calculated, and the results, reported as the percentage cell survival, were expressed as a negative for cell death or a positive for viable cells as compared with the negative control (medium only) that had its cell survival equated to 0 or baseline.

Nitrite Assay

It is difficult to directly measure nitric oxide (NO) production due to its short half-life. Thus, NO measurement is often carried out using the conventional Griess assay that detects nitrite (NO_2^-), the more stable metabolite of NO (Nathan, 1992; Stuehr and Marletta, 1987a; Stuehr and Marletta, 1987b). The Griess assay uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions to detect NO_2^- by converting it to an azo compound with an absorbency that can be detected by visible spectrophotometry at 540 nm.

The Griess reagent system (Promega, Madison, WI) was used in the nitrite assay in our study. The standard nitrite solution was prepared according to the manufacturer's instruction to generate a standard reference curve. Volumes of 50μL of the standard solutions or the supernatants were added to the flat-bottom 96-well plate (Immulon® 1B, Thermo Labsystems, Franklin, MA) in triplicate, followed by the addition of 50μL of the sulfanilamide solution. The plate was incubated at room temperature (RT) for 5-10 minutes in the dark. The NED solution was then added at 50μL/well and the plate was further incubated for an additional of 5-10 minutes. The absorbency was measured within 30 minutes using the MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA) at 540 nm.

IL-6 Bioassay

The bioactivity of IL-6 was measured by using murine hybridoma/plasmacytoma cell line B9 (DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) whose growth depends on the presence of IL-6 (Helle, et al.,

1988). The B-9 cells were collected by centrifugation at 1200 rpm (400 x g) for 10 minutes and washed two times with B-9 cell medium without rhIL-6. Cells were re-suspended with a known volume of the B-9 cell medium without rhIL-6. Cells were counted using a trypan blue exclusion method and cell number was adjusted to $1x10^5$ cells/mL concentration. The supernatants were diluted to 1:2 and 1:10 with the B-9 cell medium without IL-6. B-9 cells were added at 100 μL/well in the 96-well tissue culture plates. B-9 cell medium without rhIL-6, B-9 cell medium with rhIL-6, or diluted supernatants were added at 100μL/well in triplicate into the plates. Plates were incubated at 37°C, 5% CO_2 for 68 hours. The proliferation of B-9 cells was then determined using the MTT assay as described above.

Detection of the *iNOS* and *IL-6* Gene Expression by PCR

The samples that were reverse transcribed as above were used as the templates for detection of the *iNOS* and *IL-6* gene expression by the polymerase chain reaction using the PCR Core System (Promega). Each 50μL PCR reaction in a 0.2-mL thin-wall PCR tube (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN) consisted of 3μL (1.5mM) of the 25mM $MgCl_2$, 5μL of the 10x reaction buffer, 1μL (200μM) of the 10mM PCR nucleotide mix, 20pmol of the upstream primer, 20pmol of the downstream primer, 1μL of the 5ug/μL of the *Taq* DNA polymerase, 1μg of cDNA template, and nuclease-free water to a final volume of 50μL. For *iNOS*, the PCR cycle was: Initial denaturation at 95°C for 2 minutes, Denaturation at 95°C for 30 seconds, Annealing at 65°C for 1 minute, Extension at 72°C for 2 minutes, Running the cycle from

denaturation to extension for 30 cycles, and Final extension at 72°C for an additional 4 minutes. The sequences of the chicken iNOS primers were: 5'-AAT GCT GTG CCC ATG GCA GTT TGCA-3', and 5'-CAC CTC AAG GAG CAT GTT GG CAACA-3'. These primers were synthesized by Sigma-Genosys (Cambridge, UK). For *IL-6*, the PCR cycle was: Initial denaturation at 95°C for 2 minutes, Denaturation at 95°C for 30 seconds, Annealing at 58°C for 1 minute, Extension at 72°C for 1 minute, Repeating the cycle from denaturation to extension for 30 cycles, and Final extension at 72°C for 2 minutes. The chicken IL-6 primers were: 5'-AGA AAT GCC TGA CGA AGC TCT CCA-3', and 5'-TAC AAC AGT CTG GCT GCT GGA CAT-3'. These primers were synthesized by the IDT Integrated DNA Technologies, Inc. (Coralville, IA). The PCR products were examined by running on a 1% agarose-gel electrophoresis and the gel was stained with ethidium bromide and visualized with the Kodak High Performance Gel Imaging System (Kodak, New Haven, CT).

Results

The cytotoxic effect of MRF on the chicken macrophage cell line, MQ-NCSU

At $5x10^3$ cells/well (Figure 1), the concentrations of MRF at 10 mg/mL and higher were associated with MQ-NCSU cell death as indicated by the reduction of the percentage of viable cells of at least 25% compared with the negative control (medium only). At this cell number, the treatment of MQ-NCSU with MRF alone at 5 mg/mL resulted in the highest percentage of viable cells (approximately 10% greater than the negative control). The percent viable cells decreased when concentrations of MRF were

reduced below 5 mg/mL. When the number of cells was increased to $1x10^4$ cells/well, a similar response pattern was obtained (Figure 2), with the highest percentage of viable cells shown at the MRF concentration of 2.5 mg/mL.

MRF reduced nitrite production of chicken macrophage cell lines, MQ-NCSU and HTC, in response to LPS

Figures 3 and 4 illustrate the time course response of nitrite concentrations of the MQ-NCSU and HTC cells, respectively, in response to medium only, MRF, or LPS. Both cell lines exhibited similar response patterns. There was zero or small amounts of nitrite production detected at all time points in response to medium or MRF. In contrast, nitrite production of both chicken cell lines was detected as early as 5 hours, and this level was increased significantly at 24 hours after treatment with LPS.

When MQ-NCSU or HTC cells were cultured with LPS in the presence of MRF, the reduction of nitrite production of these cells was observed at 5 and 24 hours (Figure 5 and 6). This reduction was statistically decreased ($P<0.0001$ for both cell types) at 24 hours after treatment but not at 5 hours.

MRF decreased IL-6 production of MQ-NCSU and HTC cells induced by LPS

IL-6-like activity produced by the MQ-NCSU and HTC cell lines, respectively, after exposure to different treatments were shown in Figures 7 to 10. Neither MQ-NCSU nor HTC had a significantly elevated level of IL-6-like activity at any time point when treated with the MRF by itself (green bar, Figures 7 and 9) compared with the negative control (yellow bar). In contrast, the IL-6-like activity was detected as early as 2 hours

after treatment with LPS (red bar, Figures 7 and 9). This activity was significantly elevated and was still evident at 24 hours.

When the MRF was present along with the LPS in the cell culture (blue bar), the IL-6-like activity was decreased. For MQ-NCSU (Figure 8), this IL-6-like activity was found significantly reduced at 2 hours post treatment, and moderately decreased (P=0.056) at 5 hours. However, the IL-6-like activity of MQ-NCSU treated with the LPS and MRF was slightly increased at 24 hours post treatment. For HTC cell (Figure 10), the IL-6-like activity of cells treated with LPS + MRF was significantly reduced at 2, 5 and 24 hours post treatment.

MRF down-regulated nitric oxide and IL-6 production at the transcriptional level

The *iNOS* gene expressions of the RNA samples extracted from the MQ-NCSU and HTC cells, respectively, treated with different treatments collected at 0, 6 and 18 hours are illustrated in Figures 11A and 11B. MRF exerted an effect on *iNOS* gene expressions that was parallel to the nitrite production of the chicken macrophage cell lines that had been exposed to LPS. The expected LPS induction of *iNOS* gene expression by these two cell lines was reduced by MRF. This was indicated by the lower intensity of the bands of the transcriptional products of the RNA extracted from cells treated with LPS + MRF compared to the ones from cells treated with the LPS alone. MRF reduced the *iNOS* gene expression at 6 and 18 hours post treatment in cells treated with LPS, but also at 0 hour or immediately after MRF addition. The intensity of the gene product bands was also found to be lower in the samples from cells treated with MRF alone as compared compared to cells treated with medium only.

Figure 12 show the effect of MRF with and without LPS stimulation on IL-6 gene expression of the HTC cells. The intensity of the banding for the transcriptional products from cells treated with LPS along with MRF was lower than that from cells treated with the LPS alone at 6 and 18 hours post treatment. By 18 hours, the IL-6 expression was found significantly reduced in all treatments compared to 6 hours.

Discussion

Bacterial infections in the GIT of chickens are among several leading causes of revenue loss in the poultry industry. The addition of antibiotics in feed at a sub-therapeutic dose has been shown to reduce the incidence of these infections as well as to promote animal growth and performance (Abdel Hakim et al., 1989; Feighner and Dashkevicz, 1987; Jukes and Williams, 1953). However, there are growing lines of evidence suggesting an association between the use of antibiotic growth promoters and bacterial resistance leading to risks for human health and well-being (Aarestrup et al., 1996; Holmberg et al., 1984; Kaukas et al., 1988; Molbak et al., 1999). Reports of such problems associated with usage of growth promoting antibiotics has raised public awareness and concern, and that concern has led to the ban of nearly all of the growth promoting antibiotics in Europe. Consequently, scientists are involved in intensive searches looking for the alternatives to growth promoting antibiotics.

BioMOS®, a mannan-based carbohydrate, derived from *Saccharomyces cerevisiae* yeast cell wall, is one of several alternatives that have been studied and has been shown to possess beneficial properties similar to those attributed to the prophylactic

use of growth promoting antibiotics in animal feeds. Among the beneficial properties of BioMOS® is its ability to agglutinate type-I fimbrial bacteria via the interaction between its mannose residues and bacterial lectins and reduction in the number of pathogenic bacteria that colonize to the GIT (Spring et al., 2000), altered numbers of intestinal microflora (Fernandez et al., 2002; Strickling et al., 2000), and increased growth rate and improved animal performance (Sims et al., 2004; Swanson et al., 2002). Furthermore, BioMOS® was found to be able to modulate the immune responses in animals (Kudoh et al., 1999; Swanson et al., 2002).

In the present study, the inhibitory effect of mannan rich fraction (MRF), the soluble fraction of the BioMOS®, on the LPS-induced inflammatory response of a chicken macrophage cell, MQ-NCSU, was studied. At concentrations of 20 mg/mL or higher, MRF was found to be toxic to MQ-NCSU cell line as indicated by the reduction of percent viable cell. This toxic effect possibly resulted from the increased osmotic pressure at high concentration of MRF (Table 1), since the osmotic pressure of the MRF at 20 mg/ml MRF or higher was greater than the physiological limit of osmotic pressure (300-310 mOsM). However, at 2.5 mg/mL MRF exerted no toxic effect on the MQ-NCSU macrophages but stimulated the proliferation of this cell line.

Regardless of species, a cell proliferative response to MRF has not been reported previously, and more studies are needed to identify which MRF component is responsible and to determine how the effect was induced. There is evidence showing that a high molecular weight (~ 200 kDa), heat-stable, soluble glycoprotein purified from a crude saline extract of yeast *Saccharomyces cerevisiae* caused proliferation of peripheral blood

mononuclear cells isolated from healthy human subjects (Darroch et al., 1994). Similar results were obtained when the mannan was isolated from *Candida albicans*, a yeast that shares similar but not identical structure to *S. cerevisiae*, was used (Podzorski et al., 1990). Since MRF at 2.5 mg/mL yielded the highest proliferative effect on the MQ-NCSU cells, it was decided to use MRF at this concentration for other investigations.

MRF reduced the nitric oxide production of LPS-stimulated MQ-NCSU and HTC chicken macrophage cell lines. Nitric oxide production was not observed in these two macrophage cell lines when MRF was at 2.5 mg/mL. As expected, nitrite production of the cell lines was increased in response to LPS stimulation being detected as early as 5 hours and significantly increased at 24 hours post LPS-treatment. When the MQ-NCSU and HTC cell lines were stimulated with the LPS in the presence of the MRF, nitrite production was significantly decreased at 24 hours post treatment. Additionally, the expression of an *iNOS*, a gene encoding an enzyme involved in the synthesis of nitric oxide, was also reduced. These results indicated that MRF down-regulated NO production of the chicken macrophage cell lines that we speculated that either at the transcriptional level or by affecting TLR-4 events upstream to the LPS-signaling cascade.

Nitric oxide (NO) is generated from L-arginine metabolism (Bredt and Snyder, 1994; Iyengar et al., 1987; Palmer et al., 1988b) catalyzed by nitric oxide synthase (NOS). Three forms of NOS enzymes, the endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) nitric oxide synthases (Charles et al., 1993; Hope et al., 1991; Lamas et al., 1992) have been described. Both eNOS and nNOS are constitutively expressed (Hope et al., 1991; Palmer et al., 1988a) by endothelial cells and neurons, respectively, but

iNOS is expressed only when cells are induced by stimuli such as inflammatory cytokines and LPS (Stuehr and Marletta, 1985; Xie et al., 1992). Normally, NO is associated with protective immune responses such as those associated with either antibacterial (MacMicking et al., 1997) or antiviral (Karupiah et al., 1993) activity. Nitric oxide is also capable of enhancing tumor cell killing activity by macrophages (Lorsbach et al., 1993) as well as preventing the growth of parasites (James, 1995). On the other hand, excessive NO has been found to be associated with pathologic conditions such as influenza virus-induced pneumonia (Akaike et al., 1996), stroke (Beckman, 1991), atherosclerosis (Cooke and Dzau, 1997), and particularly sepsis (Kirkeboen and Strand, 1999). Thus, the fact that MRF significantly reduced NO production by LPS-stimulated macrophages may also decrease the risk of sepsis in infected animals by maintaining the NO production at an optimal level. MRF did not completely block NO synthesis by the LPS-stimulated macrophage, but regulated NO to a lower level of production and maintained its function as an effective antimicrobial agent (Hope et al., 1991).

The influence of MRF on pro-inflammatory cytokine production by macrophage cell lines with different treatments was also investigated. In this experiment, IL-6, one of pro-inflammatory cytokines secreted by macrophages as a result of the LPS-TLR4 signaling cascade stimulation was studied. The IL-6 assay used in this experiment employed the ability and requirement of the IL-6 to maintain the growth of the B-9 cells. As seen with NO production, the IL-6-like activity of the macrophage cells treated with the MRF alone was not different from the activity found in cells treated with the medium only. The IL-6-like activity of the cells treated with LPS was significantly elevated at 2, 5

and 24 hours. In the presence of MRF, significant reductions in the IL-6-like activity was observed in cells treated with the LPS at 2 hours for the MQ-NCSUs and at 2, 5, and 24 hours for the HTC cells. The nature of this differentiation of the IL-6-like activity between these two cell lines is probably due to several factors such as the differences in the genetic strains of chickens, or tissue sources from which these two macrophage cell lines were isolated (Qureshi et al., 1990; Rath et al., 2003). These differences may contribute to the distribution of the cytokine receptors on the surface of cells, therefore to the strength of their responses. Unfortunately, the information regarding the tissue distribution of IL-6 receptors on the surface of these two cell lines is currently not available. Similar to *iNOS* gene expression, the *IL-6* gene expression of cells treated with LPS + MRF was decreased. MRF, by itself, did not up-regulate the expression of the *IL-6* gene in macrophages.

IL-6 is a multifunctional cytokine that can be produced by several cell types including both myeloid and non-myeloid lineages. IL-6 is an important cytokine for the proliferation of several cell types (Hirano et al., 1986; Houssiau et al., 1988; Ikebuchi et al., 1987; Satoh et al., 1988). Its function as a mediator of an acute phase response is well established (Gauldie et al., 1987; Peters et al., 1997). IL-6 is one of the pro-inflammatory cytokines secreted during the infection that mediates fever (Dinarello et al., 1999; Kozak et al., 1998). It also induces anorexia and somnolence (Plata-Salaman, 1998) in response to infection that results in decreased food and water intake in animals. Generally, the acute phase response, fever, anorexia and somnolence are induced during infection to help the infected individual to regain homeostasis while facilitating pathogen clearance.

However, if these conditions are chronic, infected animals will be cachectic and their mortality rate may be increased. IL-6 was reported to be able to stimulate NO synthesis in association with sepsis (Nathan and Xie, 1994). Increased IL-6 production will amplify NO production and the severity of the infection (Nathan and Xie, 1994). Thus, the reduction of *IL-6* gene expression and IL-6 production by macrophage cell lines exposed to MRF may help control the severity and mortality caused by various enteric infections.

The observations made in this investigation provided evidence that MRF from the yeast cell wall in BioMOS® may in fact function to modulate the immune responses in host animals. Improved performance would appear to be the result of less energy being directed toward the immune responses and more energy being directed toward growth. However, the production of inflammatory cytokines was not suppressed totally but simply lowered by regulatory activity by MRF, and under these conditions, normal, well-controlled inflammatory reactions could be initiated with potentially minimal negative influence on performance in pathogen-challenged host animals. The influence on the control of inflammatory cytokine production by macrophages may in fact be regulated at the transcriptional level or by affecting TLR-4 events upstream to the LPS-signaling cascade as shown by less *iNOS* expression. Obviously, these conclusions are based on the fact that the macrophage plays a major role in the initiation and maintenance of the inflammatory reaction in pathogen-challenged hosts. Thus, if one accepts the possibility that macrophages function *in vitro* similar to their function *in vivo*, then these conclusions could be validated by testing MRF in pathogen-challenged growing broiler chickens, which was addressed in Chapter 4.

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Figure 1. Effect of MRF on the Chicken Immortal Cell Line MQ-NCSU Viability ($5x10^3$ cells/well).

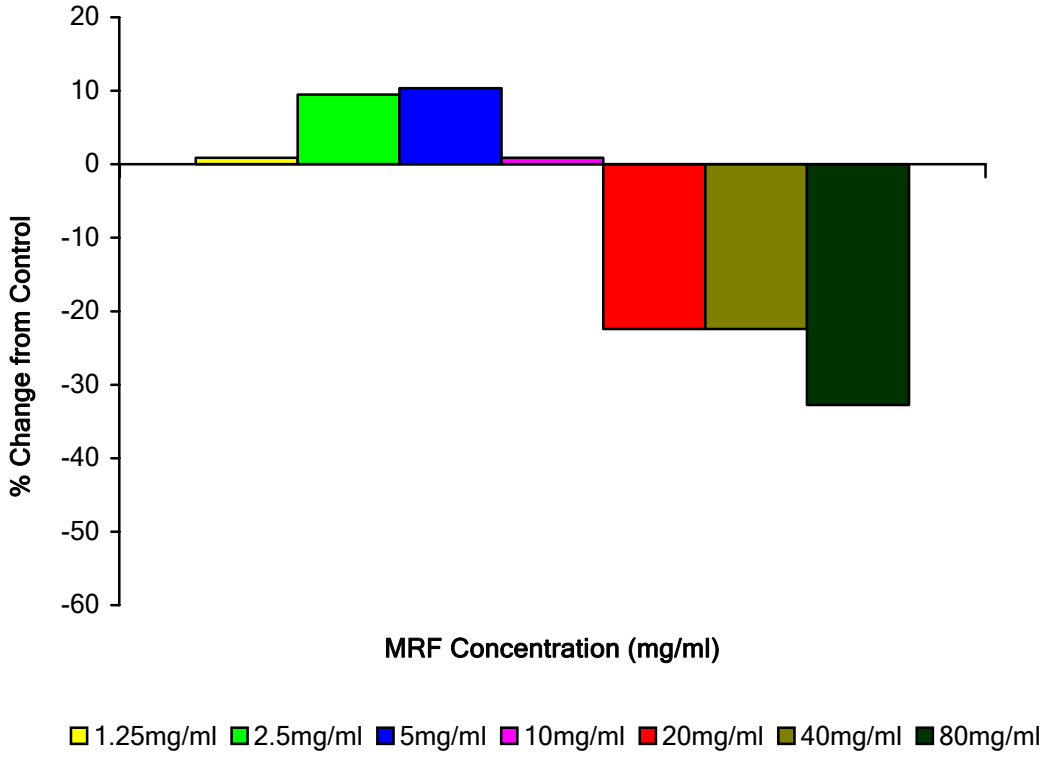


Figure 2. Effect of MRF on the Chicken Immortal Cell Line MQ-NCSU Viability ($1x10^4$ cells/well).

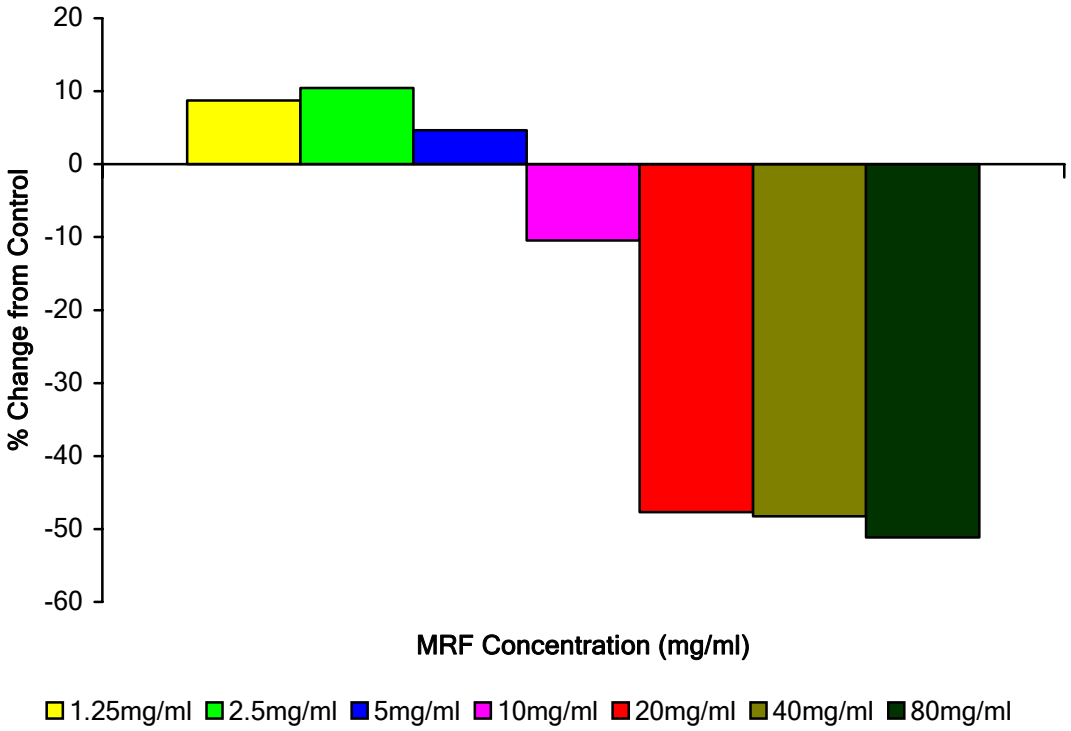


Table 1. Osmotic pressure of different concentrations of MRF.

MRF Concentration (mg/ml)	1.25	2.5	5	10	20	40	80
MRF Osmotic Pressure (mOsM)	296	295	300	306	320	349	411

Figure 3. Time course response of nitrite production of MQ-NCSU cell line. The * represents a significant difference of nitrite production of LPS-treated cells from other treatments, at each time point.

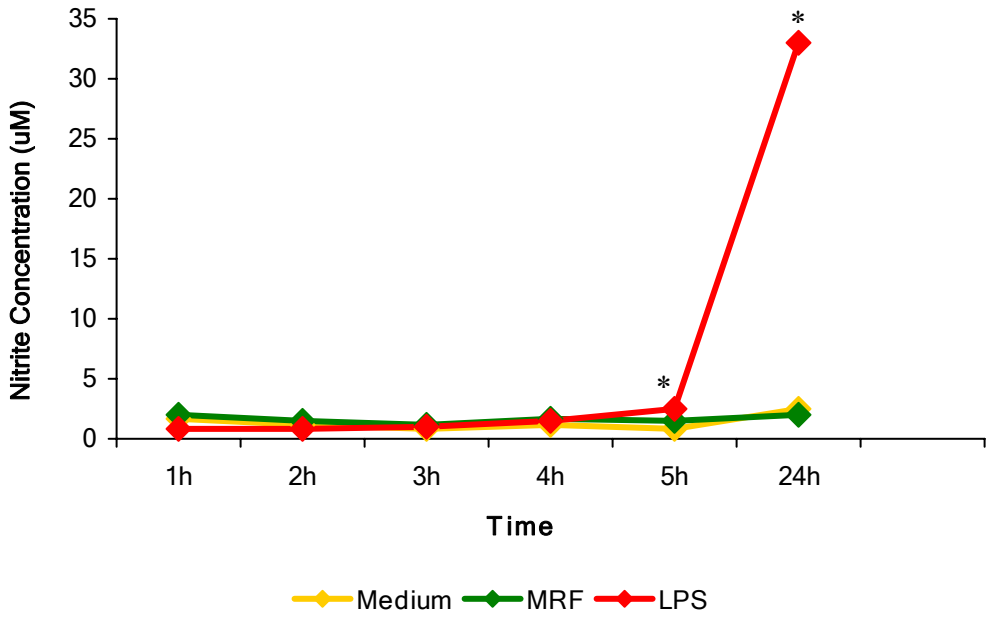


Figure 4. Time course response of nitrite production of HTC cell line. The * represents a significant difference of nitrite production of LPS-treated cells from other treatments, at each time point.

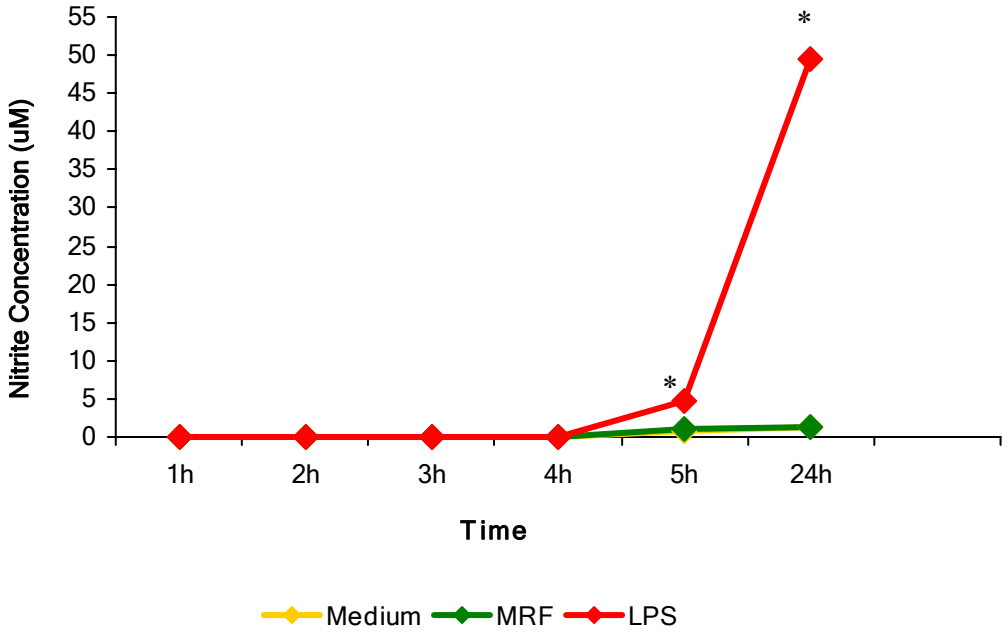


Figure 5. Effect of MRF on nitrite production of the LPS-stimulated MQ-NCSU cell line. Different lower case letters on the histogram bars represents a significant difference among treatments. ($P \leq 0.05$), at each time point.

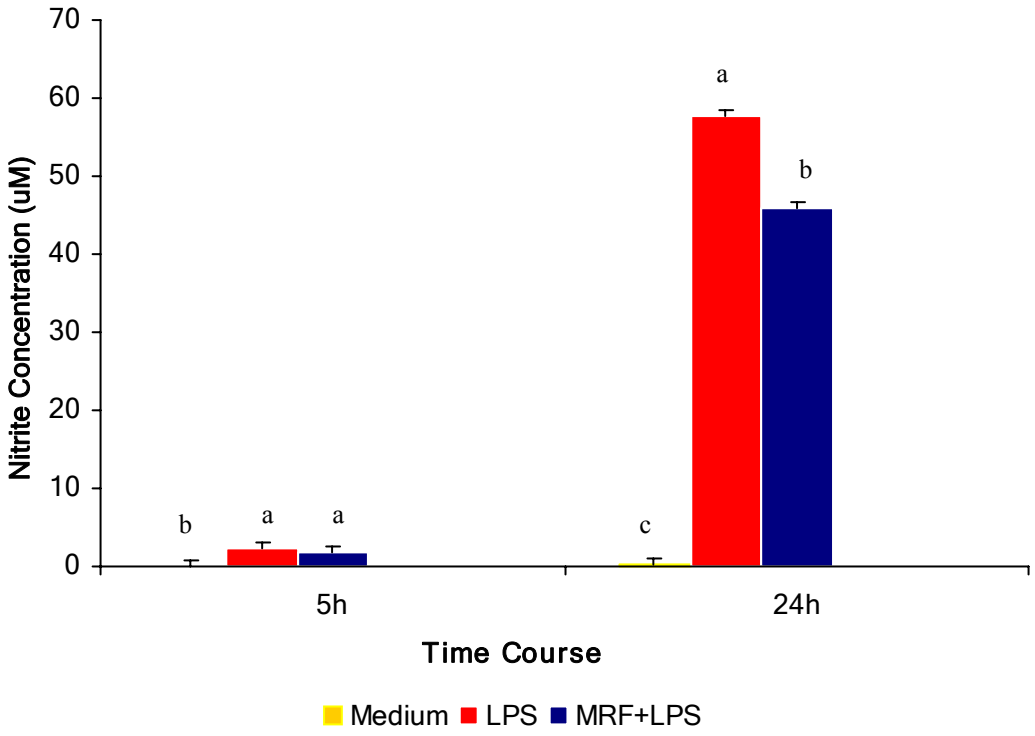


Figure 6. Effect of MRF on nitrite production of the LPS-stimulated HTC cell line. Different lower case letters on the histogram bars represents a significant difference among treatments. ($P \leq 0.05$), at each time point.

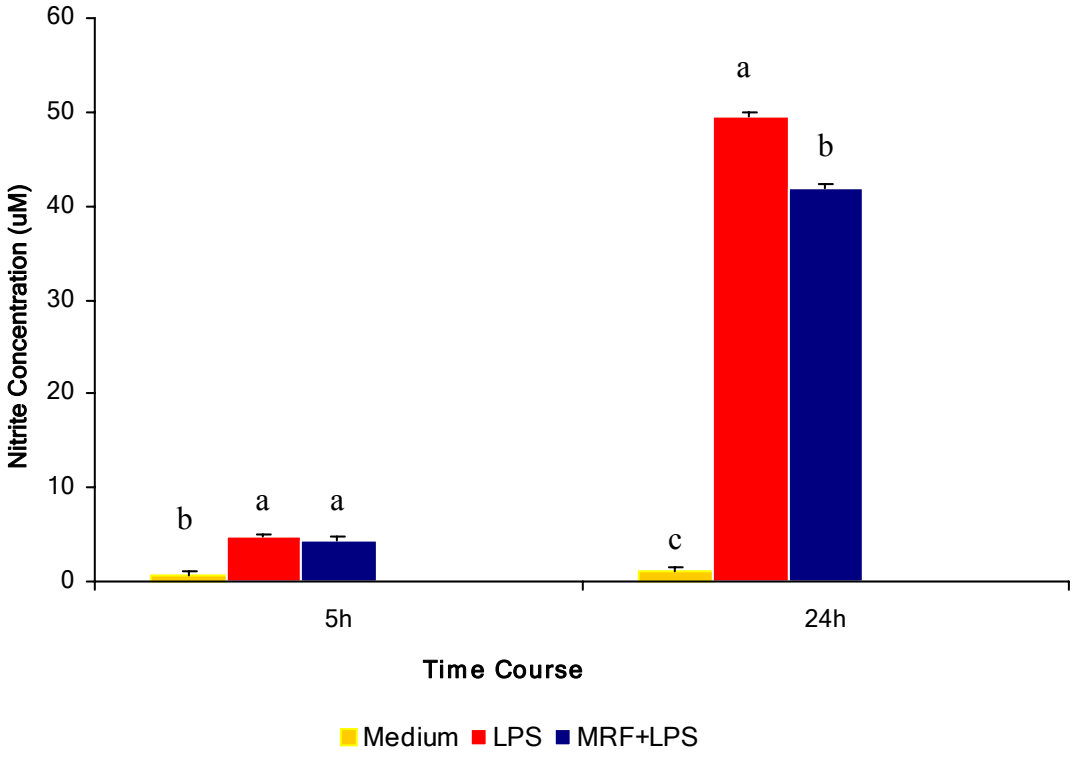


Figure 7. IL-6 production of the MQ-NCSU cells treated with medium, MRF, or LPS. Different lower case letters on the histogram bars represents a significant difference among treatments. ($P \leq 0.05$), at each time point.

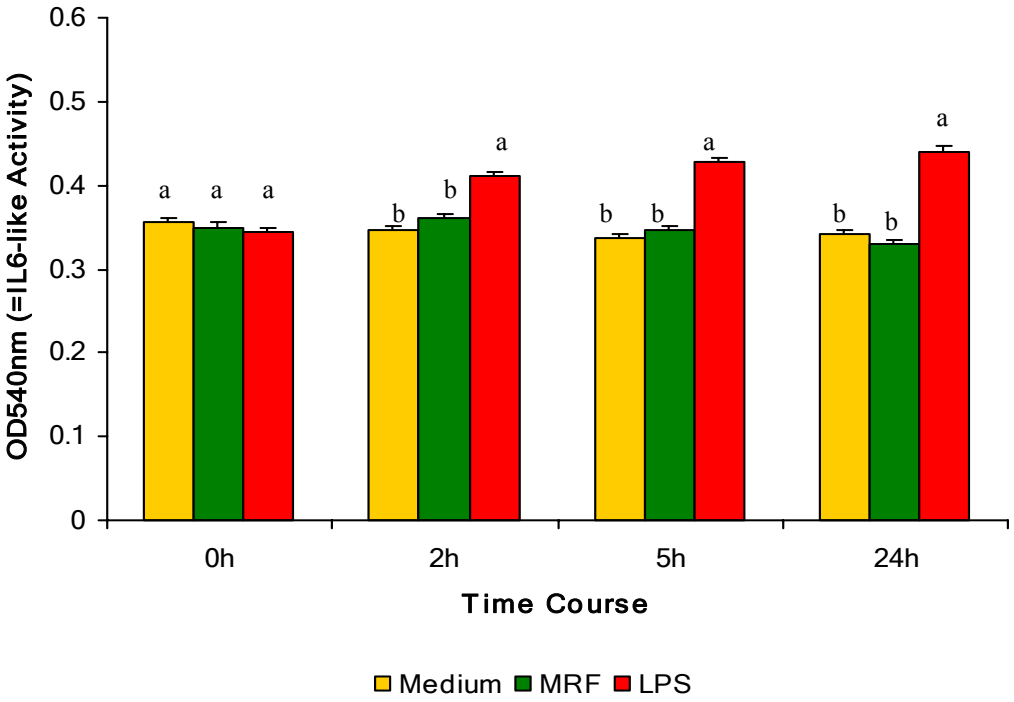


Figure 8. Effect of MRF on IL-6 production of the MQ-NCSU cells induced by LPS. Different lower case letters on the histogram bars represents a significant difference among treatments. ($P \leq 0.05$), at each time point.

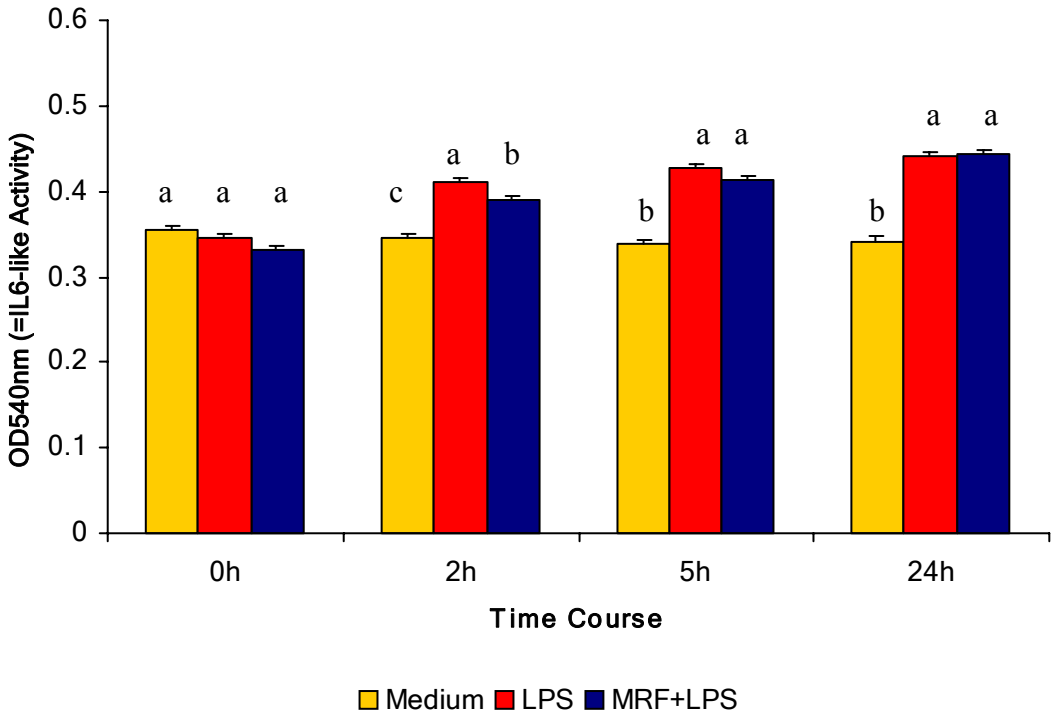


Figure 9. IL-6 production of the HTC cells treated with medium, MRF or LPS. Different lower case letters on the histogram bars represents a significant difference among treatments. ($P \leq 0.05$), at each time point.

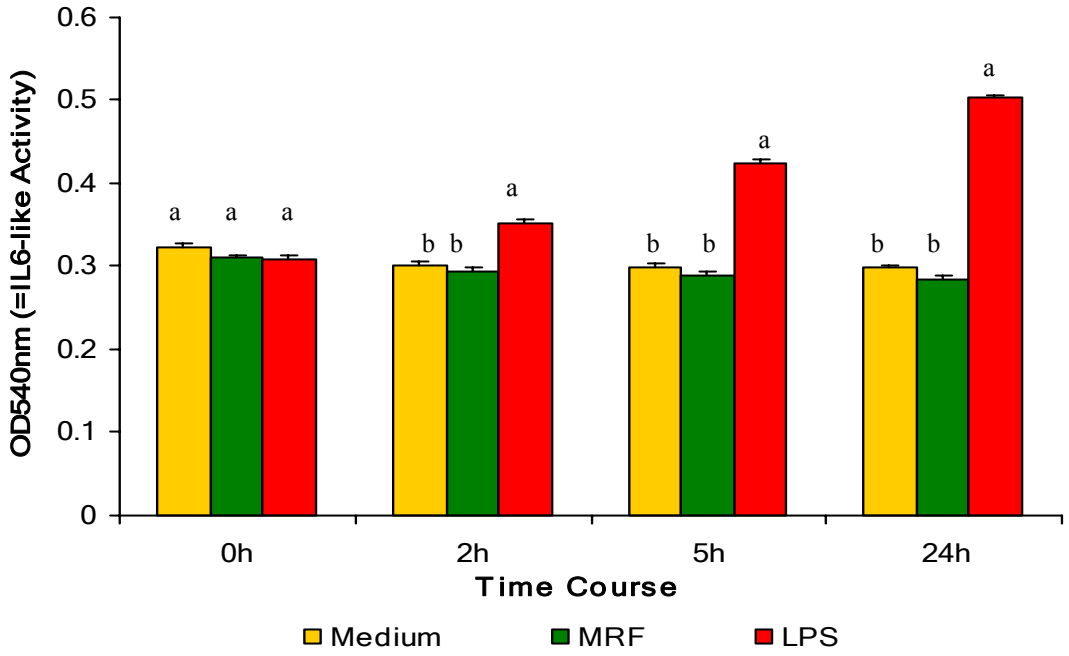


Figure 10. Effect of MRF on IL-6 production of the HTC cells induced by LPS. Different lower case letters on the histogram bars represents a significant difference among treatments. ($P \leq 0.05$), at each time point.

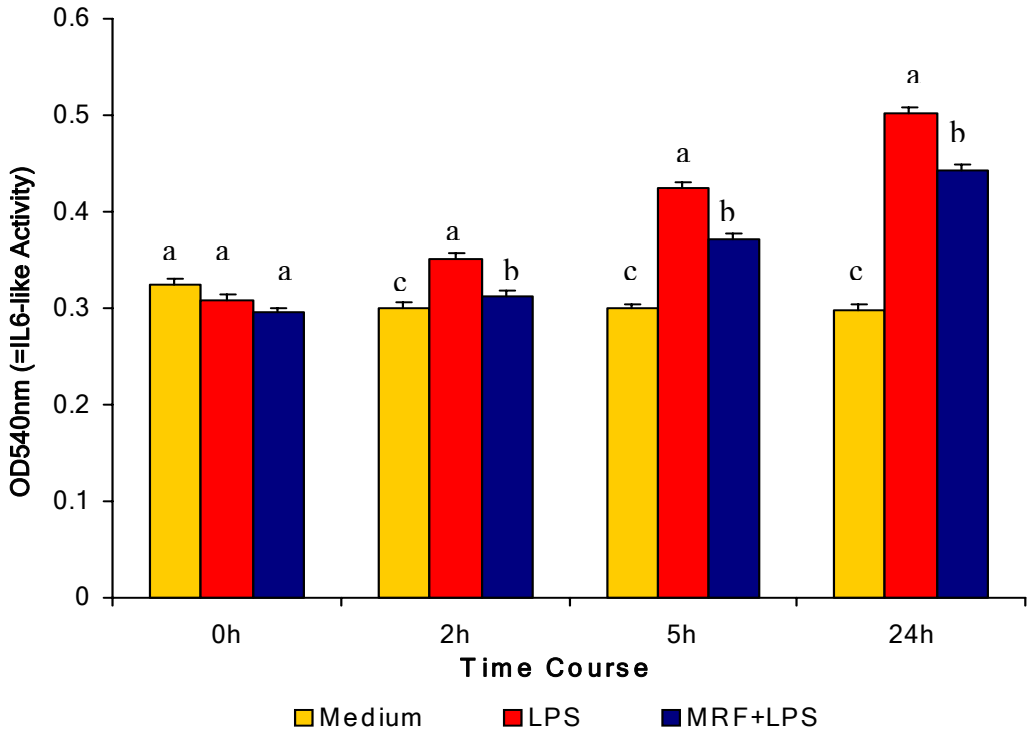
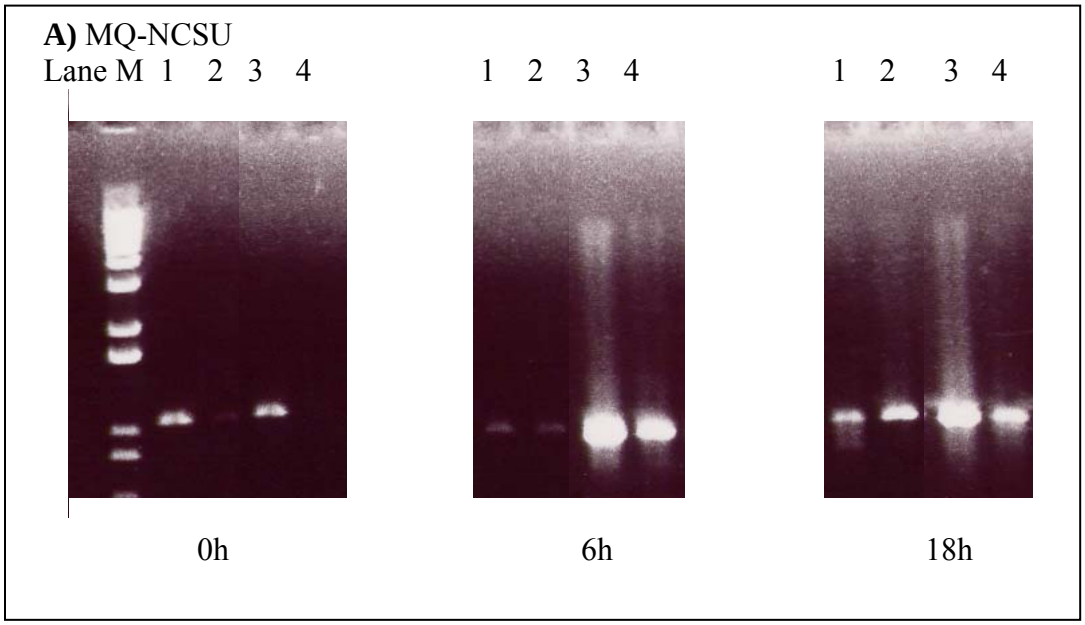
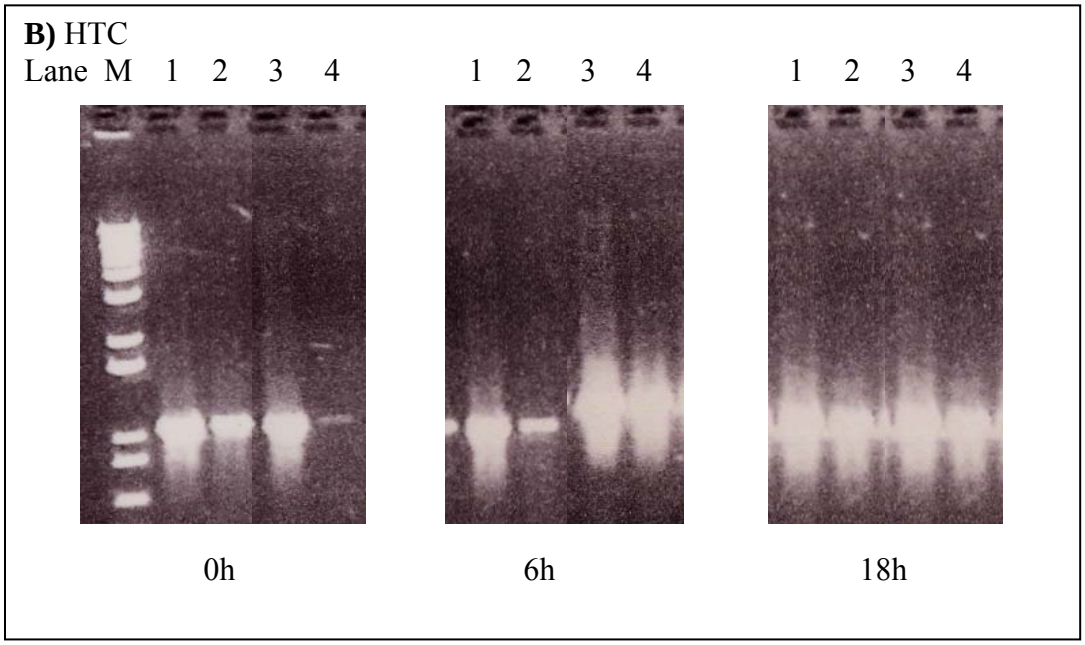


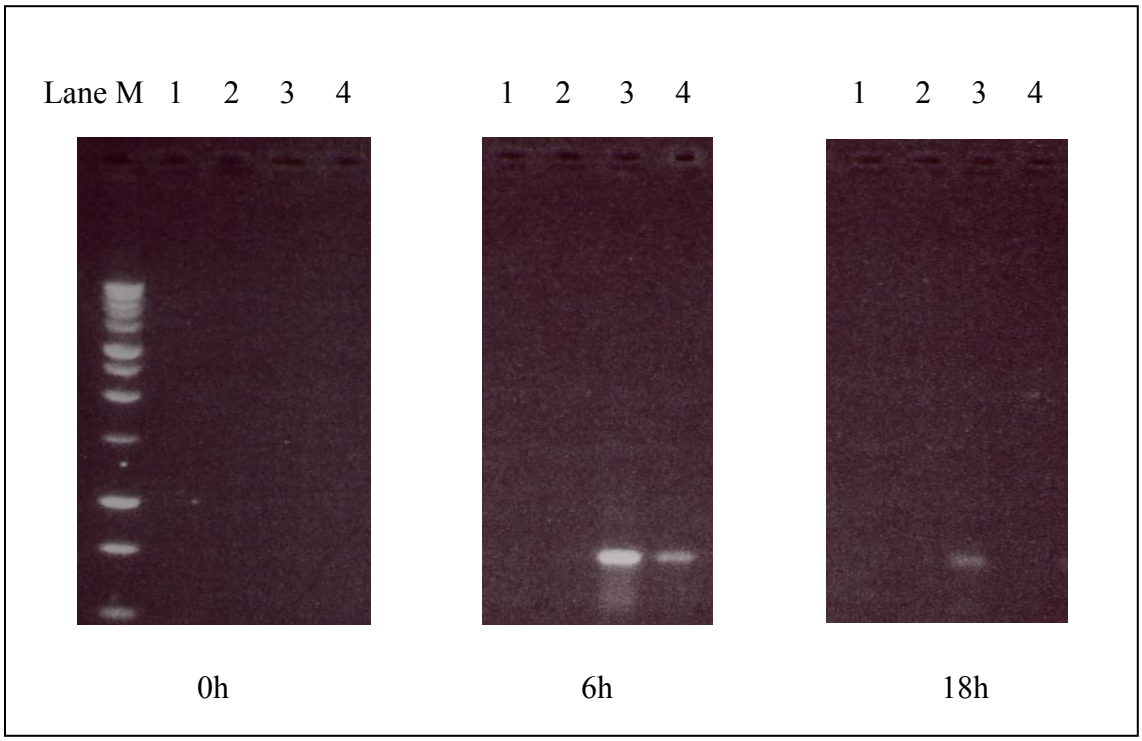
Figure 11. Effect of MRF on *iNOS* gene expression of the chicken macrophage cell line induced by LPS.





Lane M = Marker, 1 = Medium, 2 = MRF, 3 = LPS, 4 = MRF + LPS

Figure 12. Effect of MRF on *IL-6* gene expression of the HTC macrophage cell line induced by LPS.



M = Marker, 1 = Medium, 2 = MRF, 3 = LPS, 4 = MRF + LPS

Table 2. Percentage intensity of RT-PCR bands of *iNOS* and *IL-6* gene expression of the RNA samples from MQ-NCSU or HTC treated with medium, MRF, LPS, or MRF and LPS.

A) *iNOS* gene expression

Treatment	% Intensity of *iNOS* gene expression of MQ-NCSU			% Intensity of *iNOS* gene expression of HTC		
	0h	6h	18h	0h	6h	18h
Medium	4.9	1.0	6.3	8.0	8.0	9.6
MRF	0.9	1.4	8.9	7.6	5.0	9.1
LPS	4.5	22.0	21.2	8.3	11.5	9.6
LPS+MRF	0.5	15.6	12.8	4.2	10.6	8.5

B) *IL-6* gene expression

Treatment	% Intensity of *IL-6* gene expression of HTC		
	0h	6h	18h
Medium	0	0	0
MRF	0	0	0
LPS	0	64.7	71.9
MRF+LPS	0	35.3	28.1

CHAPTER 3

Mechanisms Involved in the Reduction of LPS-Induced Inflammatory Responses of Chicken Macrophage Cell Lines by Mannan Rich Fraction of *Saccharomyces cerevisiae* in BioMos®

Abstract

The mannan rich fraction (MRF) in BioMOS® reduces inflammatory responses of chicken macrophage cell lines that were stimulated by LPS. The inflammation suppressing mechanisms facilitated by MRF were believed to have been associated with down-regulating toll-like receptor-4 (TLR-4) expression. MQ-NCSU cells were treated with medium only, MRF, LPS, or MRF + LPS and harvested at 0, 5, and 24 hours for protein extraction. The TLR-4 expression was analyzed by western blot analysis. The TLR-4 expression was decreased at 0 and 5 hours in cells treated with MRF or MRF + LPS, but MRF had no effect on TLR-4 expression at 24 hours. This suggested that MRF possibly binds competitively to TLR-4 and prevents TLR-4 from binding to its putative ligand, LPS. The effect of MRF on NF-κB activation was also investigated. MQ-NCSU cells were treated with medium only, MRF, or LPS for 30 minutes. Nuclear content was extracted and subjected to NF-κB analysis using an electrophoretic mobility shift assay technique. LPS caused NF-κB activation while MRF repressed it. It is possible that MRF might activate an unconventional NF-κB transcription factor that could compete with a conventional one by binding to the DNA and blocking the transcription of that gene.

Introduction

Gram-negative bacteria contain an endotoxin within their outer membrane known as lipopolysaccharide (LPS). An LPS exerts its endotoxic activity by triggering a Toll-like receptor-4 (TLR-4) signaling cascade. TLR-4 is a type-1 transmembrane protein which is characterized by the presence of multiple leucine-rich repeats (LRRs) on its extracellular domain and the Toll/Interleukin-1 Receptor (TIR) domain within the cytoplasmic portion (Hashimoto et al., 1988; Kobe and Deisenhofer, 1995; Medzhitov et al., 1997). In order to initiate TLR-4 signal transduction, the LPS is first bound by an LPS-binding protein (LBP) to form an LPS-LBP complex (Ramadori et al., 1990). The binding of LBP to LPS facilitates the recognition of LPS to another glycoprotein known as CD14 (Haziot et al., 1988). CD14 then transfers LPS to TLR-4. The binding of LPS to TLR-4 in association with a small protein called MD-2 (Shimazu et al., 1999) triggers the signaling downstream that ultimately leads to a translocation of the transcription factor NF-κB from the cytoplasm to the nucleus.

NF-κB is a family of related proteins. Proteins that belong to this family are RelA (p65), RelB, c-Rel, p50/p105, and p52/p100 (Ghosh et al., 1998) that can form hetero- or homodimers. Each protein of the dimer binds to one half of a specific DNA binding site (Kunsch et al., 1992), and the binding can either activate or block the transcription of a particular gene depending on the combination of that dimer. Generally, the binding of p50/p65, p50/c-rel, p65/p65, or p65/c-rel to their DNA binding sites activates gene transcription, where as the binding of p50 or p52 homodimers to the DNA binding sites represses gene transcription (Hansen et al., 1994a; Hansen et al., 1994b; Kang et al.,

1992; Kunsch et al., 1992; Plaksin et al., 1993). Activation of TLR-4 signaling by LPS initiates the transcription of gene encoding proteins involved in the inflammatory response.

BioMOS® (Alltech, Inc., Nicholasville, KY 40356) is a glucomannoprotein complex isolated from the outer cell wall of the yeast *Saccharomyces cerevisiae*. BioMOS® is one of the potential alternatives to antibiotic growth promotants and has been used as a feed supplement to improve animal health. It has been shown to be able to agglutinate enterobacteria with type-1 fimbriae (Chapter 1) and the agglutination was inhibited by the addition of mannose sugar (Spring et al., 2000). Additionally, BioMOS® reduced the concentration of *S. typhimurium* 29E within the cecum of the birds and was suggested to act as a decoy of the enterobacteria. Moreover, BioMOS® has been shown to increase the number of beneficial bacteria, such as *Enterococcus spp.,* (Fernandez et al., 2002) and reduce the concentration of pathogenic bacteria, such as *C. perfringens,* (Strickling et al., 2000). BioMOS® enhanced immune responses in animals (Kudoh et al., 1999; Swanson et al., 2002). Interestingly, animals receiving BioMOS® in their feed gained significantly more weight than animals fed only a basal diet (Sims et al., 2004; Zdunczyk et al., 2005). The mortality rate of animals fed BioMOS® and challenged with pathogenic bacteria was less than in animals fed no BioMOS® (Spring et al., 2000).

Since BioMOS® is derived from the yeast cell wall, that contains mannan, one might expect that it would be recognized as a pathogen-associated molecular pattern (PAMP) by pattern-recognition receptors (PRRs) such as TLRs or mannose receptor (MR) (Martinez-Pomares and Gordon, 1999; Medzhitov et al., 1997; Park et al., 2005).

Unfortunately, there is no direct evidence to support this hypothesis, but the closest study from which this hypothesis might be drawn was conducted by a group of Japanese investigators (Tada et al., 2002) using a purified neutral fraction (no protein) of mannan prepared from *Saccharomyces cerevisiae*. These investigators showed that mannan induced the secretion of the pro-inflammatory cytokine TNF-α by human monocytes, and this induction was dependent on the presence of LBP, CD14, and TLR-4, similar to the signaling cascade induced by LPS. Other investigators demonstrated that TLR-2 was a pattern-recognition receptor for zymosan (Underhill et al., 1999), a cell wall composed mainly of β-glucan and mannan prepared from *S. cerevisiae* (Levitz, 2004).

In Chapter 2, the Mannan Rich Fraction (MRF), a soluble fraction of BioMOS®, was shown to reduce the activity of an LPS-induced pro-inflamtomatory cytokine, interleukin-6 (IL-6), from chicken macrophage cell lines. Although TNF-α concentration or activity was not quantified in the assay system employed in the earlier study due to lack of adequate avian reagents, observations from the work reported in Chapter 2 were in contrast with those reported by Tada et al. (2002) and Levitz (2004). These results indicated that the structure of an MRF is not the same as that of either neutral fraction mannan or zymosan, and a MRF signaling cascade may also be different from neutral mannan or zymosan.

In the present study an attempt was made to clarify the signaling cascade of MRF via TLR-4 and investigate the possible mechanisms that MRF employs to reduce the inflammatory responses in chicken macrophage cell lines.

Materials and Methods

Cell Line, Medium, and Culture Condition

The cell line used in this study was MQ-NCSU, a chicken macrophage tumor cell line generated from cells isolated from the spleen of Marek's disease virus (MDV)-infected Dekalb XL chicks (Qureshi et al., 1990). MQ-NCSU (North Carolina Agricultural Research Service, Raleigh, NC 27695-7608) was maintained in Leibovitz-McCoy's complete medium (CM) consisting of 38.5% Leibovitz L-15 medium (Sigma, St. Louis, MO), 38.5% McCoy's 5a medium (Sigma), 5% tryptose phosphate broth solution (Sigma, St. Louis, Mo), 8% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 10% chicken serum (Sigma), 1% of 200mM L-glutamine (Sigma), 1% of 100x sodium pyruvate (Sigma), 1% of 100x antibiotic antimycotic solution (Sigma), and 1% of 0.001M 2-mercaptoethanol (2-ME), at 41°C, in a 5% CO_2 humidified incubator.

Cell Treatment and Protein Extraction

MQ-NCSU cells at approximately $3x10^7$ cells/treatment were treated with 10mL CM medium only, 2.5mg/mL Mannan Rich Fraction (MRF) dissolved in 10mL CM medium, 5μg/mL LPS diluted in 10mL CM medium, 2.5mg/mL mannan (Sigma) diluted in 10mL CM medium, a combination of 2.5mg/mL MRF and 5μg/mL LPS in 10mL CM medium, or a combination of 2.5mg/mL mannan and 5μg/mL LPS. Cells were incubated at 41°C, with 5% CO_2 for 0 and 24 hours and harvested by centrifuging at 1500 rpm (400 X g) for 10 minutes at 4°C. Cell pellets were washed twice with PBS. Cells were further processed for protein extraction using the ReadyPrep Protein Extraction Kit (Bio-Rad

Laboratories, Hercules, CA) according to the manufacturer's protocol. Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories) based on the method of Bradford.

Western Blot Analysis

For each sample, a 20μg protein sample was mixed with 6x sodium dodecyl sulfate (SDS) sample buffer (1 part buffer + 4 parts sample). The protein sample was heated at 100°C in the heating box for 5 minutes and immediately chilled on ice. Samples along with the standard, Precision Plus Protein™ Standard (Bio-Rad Laboratories), were loaded into 7.5% Tris-HCl gel (Ready Gel Precast Gel, Bio-Rad Laboratories) and run with constant current at 10mA with 1x Tris/Glycine/SDS (TGS) running buffer (Bio-Rad Laboratories) until all samples lined up at the separating gel. Then the current was changed to 15mA and the gel was run until the loading dye reached the bottom of the gel.

Proteins separated on the gel were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) by running in 1x Tris/Glycine (TG) buffer (Bio-Rad Laboratories) with a constant voltage at 50V for 2 hours. The nitrocellulose membrane was blocked with 5% skim milk dissolved in a Tris Buffered Saline (TBS, Bio-Rad Laboratories) + 0.05% Tween20 (0.05% TBST) at 4°C overnight. The blocking solution was discarded before the addition of 25mL of 1:200 goat anti-human TLR4 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted with 3% skim milk in 0.05% TBST and incubated at 4°C for 18-24 hours. The membrane was washed twice, 15 minutes each, with 0.05% TBST to eliminate nonspecifically and unbound antibodies. The membrane was next incubated with 30mL of a horse radish peroxidase (HRP)

conjugated, mouse anti-goat IgG antibody (Santa Cruz Biotechnology, Inc.) diluted at 1:2000 with 0.05% TBST at room temperature (RT) for 1 hour 30 minutes followed by three washings, 10 minutes each, with 0.05% TBST. An HRP substrate, BM Chemiluminescence Blotting Substrate (POD) from Roche Diagnostics Corporation (Indianapolis, IN), was added and the bands were developed by exposing the membrane to a Kodak X-Omat AR film (Sigma) for 4 minutes. The film was immersed in a GBX developer/replenisher solution (Sigma) for 1 minute, washed two times with running tap water, and fixed with a GBX fixer/replenisher solution (Sigma). The film was dried and the protein bands were examined.

Cell Treatment and Nuclear Content Extraction

Approximately $3x10^7$ MQ-NCSU cells/treatment were treated with 10mL CM medium only, 10mL of 2.5mg/mL MRF dissolved in CM medium, or 10mL of 5μg/mL LPS diluted in CM medium at 41°C, 5% CO_2 incubator for 30 minutes. Cells were harvested by spinning at 1500 rpm (400 X g) for 10 minutes at 4°C. Cell pellets were re-suspended in 1mL medium and centrifuged at 450g for 5 minutes using a refrigerated microcentrifuge. The supernatant was discarded and the cells were kept on ice until ready to be processed. Nuclear contents were extracted from treated cells using CelLytic™ NuCLEAR™ Extraction Kit (N-XTRACT, Sigma) following the instructions provided by the manufacturer. Protein concentration in nuclear contents was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories).

NF-κB Transcription Factor Detection

NF-κB Oligonucleotide Labeling: The oligonucleotide (sequence: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5') that contains DNA binding sites for NF-κB transcription factors (Promega, Madison, WI) was labeled with digoxigenin using the DIG oligonucleotide 3'-end labeling kit, 2nd generation (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's protocol. The efficacy of labeling was determined by making a series of dilutions of labeled samples along with a positive control and 1µl of each sample was applied to a small strip of a positively charged nylon membrane (Roche Diagnostics Corporation). The nucleic acid on the membrane was cross-linked with UV light for 5 minutes. The membrane was blocked with 100mL of 1x blocking solution from a DIG wash and block buffer set (Roche Diagnostics Corporation) at RT for 30 minutes. The blocking solution was discarded followed by the addition of 20mL of an alkaline phosphatase conjugated anti-DIG antibody (anti-DIG-AP) diluted to 1:5000 with blocking buffer and further incubated at RT for an additional 30 minutes. The membrane was washed 2 times each for 15 minutes with 100mL of 1x washing buffer at RT followed by the addition of 50mL of 1x detection buffer. An AP substrate (CDP-*Star*-ready-to-use, Roche Diagnostics Corporation) was added, and the membrane was incubated at 37°C for 10 minutes. The excess substrate was discarded and the membrane was developed as described under Western blot.

Electrophoretic Mobility Shift Assay (EMSA): The EMSA was performed to detect the presence of NF-κB in the nuclear content extracted from treated cells described

above using the reagents from Gel Shift Assay Systems (Promega). Twenty micrograms of nuclear extract, or 0.5µl human NF-κB p50 was mixed with 1µl of 1µg/µl DIG-labeled NF-κB oligonucleotide and 2µl 5x binding buffer. The reaction mixture was adjusted to a total volume of 9µl with nuclease-free water, centrifuged briefly to collect the sample at the bottom of the tube, and incubated at RT for 10 minutes. One microliter of 10x Blue Juice™ Gel Loading Buffer (Invitrogen, Carlsbad, CA) was added to the reaction mixture. The samples were loaded to 5% ready gel TBE gel (Bio-Rad Laboratories) and the gel was run in 0.5x Tris/Boric acid/EDTA (TBE) buffer (Bio-Rad Laboratories) with constant voltage at 100V until the dye reached the bottom of the gel. The samples were transferred from the gel to a positively charged nylon membrane by running in 0.5x TBE buffer with constant voltage at 50V for 2 hours. The membrane was dried, exposed to the UV light for 5 minutes to cross-link the samples, and blocked with blocking solution (DIG wash and block buffer set) at 4°C for 18-24 hours. The blocking solution was discarded followed by the addition of 20mL of an alkaline phosphatase conjugated anti-DIG antibody (anti-DIG-AP) diluted to 1:5000 with blocking buffer and further incubated at RT for an additional of 30 minutes. The membrane was washed 2 times each for 15 minutes each with 100mL of 1x washing buffer at RT followed by the addition of 50mL of 1x detection buffer. An AP substrate (CDP-*Star*-ready-to-use, Roche Diagnostics Corporation) was added and the membrane was incubated at 37°C for 10 minutes. The excess substrate was discarded and the membrane was developed as described under Western blot.

Results

Effect of MRF on TLR-4 expression

Figure 1 shows western blot analysis and table 1 shows the percentages intensity of bands of TLR-4 expression of MQ-NCSUs treated with medium only, 2.5mg/mL MRF, 5μg/mL LPS, or combination of MRF and LPS at 0, 5, and 24 hours. At all time point, the TLR-4 expression of MQ-NCSUs treated with LPS was lower than TLR-4 expression of cells treated with medium alone. The percentages intensity of these bands at 0, 5, and 24 hours were 14%, 25.2% and 5%, respectively, less than the percentage intensity of the band of cells treated with medium only. At 0 and 5 hours, TLR-4 expression of MQ-NCSUs treated with MRF alone was markedly reduced with the percentage intensity of the band less than that of medium-treated cells of 49.4% and 37.4% respectively. TLR-4 expression of MQ-NCSUs treated with MRF and LPS was not detected and showed only 0.7% and 0% intensity of bands at 0 and 5 hours, respectively. By 24 hours, TLR-4 expression of MQ-NCSUs from all treatments was detected and the percentage intensity of the bands cells treated with MRF, LPS, and MRF + LPS were 8.7%, 7.4%, and 5.0%, respectively, less than the percentage intensity of band of cells treated with medium only. The size of TLR-4 bands of chicken macrophage cell line in this study was approximately 60 kDa.

Effect of MRF on NF-κB activation

Figure 2 and table 2 show an EMSA analysis and percentage intensity of the band, respectively, of the nuclear contents extracted from treated MQ-NCSUs. Incubation of labeled-DNA with nuclear extracts from all treatments resulted in a band shift which

indicated the presence of NF-κB in the nucleus of cells. The intensity of the shifted band of cells treated with LPS (23.7%) was stronger than the intensity of the shifted band of cells treated with medium at both 0 (6.3%) and 30 minutes (10.2%). In contrast, the intensity of the shifted band of cells treated with MRF (3.5%) was lower than that band of cells treated with medium only, with an additional band appearing on top of the shifted band. The percentage intensity of the top band of MRF-treated cells (9.4%) was slightly higher than the percentage intensity of band of cells treated with medium at 0h but equivalent to that of cells treated with medium at 30 minutes.

Discussion

Toll-like receptors (TLRs) are a class of proteins that function as pattern-recognition receptors (PRRs) and bind to pathogen-associated molecular patterns (PAMPs), a conserved structure found on a given class of microorganisms (Medzhitov and Janeway, 1997). One TLR or a combination of TLRs can recognize one or more PAMPs (Hayashi et al., 2001; Schwandner et al., 1999; Takeuchi et al., 2001). TLR-4 recognizes LPS, a glycophospholipid found on the outer membrane of Gram-negative bacteria, initiating the signaling cascade that ultimately leads to the translocation of the transcription factor NF-κB from the cytoplasm to the nucleus (Ghosh et al., 1998). The NF-κB binds to a specific gene within the nucleus and, in turn, activates the transcription of that gene (Ghosh et al., 1998). Genes, whose transcriptions are initiated via LPS-TLR4 signal transduction, encode proteins involved in inflammatory responses such as pro-

inflammatory cytokines, nitric oxide synthase (NOS), or adhesion molecules (Muller et al., 1993; Takada et al., 1991; Tamura et al., 1992; Xie et al., 1992).

BioMOS® is a glucomannoprotein isolated from the outer cell wall of the yeast *Saccharomyces cerevisiae*. BioMOS® has been used as a feed supplement to improve animal health and performance. In Chapter II, mannan rich fraction (MRF), which is a soluble fraction of BioMOS®, reduced pro-inflammatory cytokine production and nitric oxide production of chicken macrophage cell lines induced by LPS. In this study, mechanisms involved in the reduction of the LPS-induced inflammatory responses of a chicken macrophage cell line by MRF were investigated.

Since triggering an inflammatory response by LPS requires TLR-4 (Poltorak et al., 1998), MRF was tested to determine whether it reduced an inflammatory response by down-regulating TLR-4 expression. Protein extraction from chicken-macrophage cell line, MQ-NCSUs, treated with medium only, MRF, LPS, or MRF + LPS for 0, 5, and 24 hours were analyzed for TLR-4 expression by western blot analysis. TLR-4 expression of LPS-treated cells was lower than that of medium-treated cell at all time point tested in this experiment. A study by other investigators using flow cytometry showed that a mean fluorescent intensity (MFI) of LPS-induced TLR-4 expression (indicative of receptor number) of MQ-NCSU cell line was significantly increased at 6 and 12 hours, and that expression returned to the basal level by 24 hours (Dil and Qureshi, 2002). Therefore, TLR-4 expression of cells treated with LPS that remained lower than that of cells treated with medium in this study possibly due to the yet expressed at the detectable level at 5 hours and the expression returned to the basal level at 24 hours.

The expression of TLR-4 of cells treated with MRF or MRF + LPS was drastically decreased at 0 hour, and that was not detected at 5 hours post treatment compared to TLR-4 expression of cells treated with medium or LPS alone. To down-regulate any protein expression, its transcription and translation processes have to be reversed. In this experiment, TLR-4 expression was reduced immediately after MRF was added to MQ-NCSU cells (TLR-4 expression at 0h). Thus, it is unlikely that this reduction of TLR-4 expression was due to the down-regulation by MRF. We speculated that MRF probably binds to TLR-4, and when the ligand-binding site of TLR-4 is occupied by MRF, it can no longer be recognized by its putative ligand, an LPS. In this experiment, binding of MRF to TLR-4 may also hinder the binding of TLR4 antibody to TLR-4. Thus, the expression of TLR-4 could not be detected or only small amounts were detected by this western blot analysis at 0 or 5 hours post treatment in cells treated with MRF or MRF + LPS. By 24 hours, TLR-4 expression was detected in all treatments including MRF or MRF + LPS treatments that probably resulted from a newly synthesized TLR-4 that was not bound by MRF or a pre-existing TLR-4 that was released from MRF. Nevertheless, whether MRF actually binds to TLR-4 should be further confirmed. In addition, we found that the size of chicken TLR-4 was smaller (approximately 60 kDa) than the mammalian TLR-4 whose molecular weight is about 90 kDa (Santa Cruz Biotechnolgy, Inc.).

Stimulation of the TLR-4 signaling cascade by LPS resulted in the activation of NF-κB. The influence of MRF on NF-κB activation was studied by EMSA. Normally, transcription factor NF-κB resides within the cytosol in an inactive form. Once cells are

activated by some external stimuli such as LPS, the NF-κB will translocate from the cytoplasm to the nucleus (Ghosh et al., 1998). Thus, the presence of NF-κB in the nucleus indicates the activated state of cells. In this study, an electrophoretic mobility shift assay (EMSA) was used to detect the presence of NF-κB in the nuclear extract of treated MQ-NCSUs. The nuclear content of treated MQ-NCSUs was extracted and subjected to the EMSA. A double-stranded oligonucleotide whose sequence is specific for NF-κB binding was labeled with DIG. If NF-κB is present in the nuclear extract, it will bind to DIG-labeled DNA and form a DNA-protein complex whose size is larger than the size of the DNA alone. When this DNA-protein complex is run on the gel along with labeled-DNA, it will migrate more slowly than labeled-DNA.

As expected, culturing MQ-NCSU cells with LPS resulted in an NF-κB activation as indicated by an increased intensity of the shifted band of cells treated with LPS by at least 10% compared to cells treated with medium only at 30 minutes post treatment. This observation is in accordance with previous report that showed that NF-κB activation measured by flow cytometry of sephadex-elicited macrophages treated with LPS for 30 minutes increased significantly compared with an NF-κB activation of untreated cells (Dil and Qureshi, 2002).

Conversely, an activation of NF-κB was decreased in cells treated with the MRF as the intensity of the shifted band was lower than that found in cells treated with medium and LPS by 6.7% and 20.2% intensity of bands, respectively, indicated lower amount of NF-κB being activated. In addition, there was another band appeared on above this band with a higher percentage intensity (9.4%) than the lower band (3.5%), indicated

competitive binding between these two forms of NF-κB to the same DNA-binding site, as well as MRF and LPS activate different sets of NF-κB dimers. The size of this band is larger than p50, thus is unlikely to represents a homodimer of the transcription factor NF-κB such as a homodimer of p50 or of p52 that are weak or innert gene activators (Plaksin et al., 1993; Siebenlist et al., 1994). Though p100 and p105 are larger than p50, these proteins are restricted to the cytoplasm serving as precursor proteins for p52 and p50, respectively (Henkel et al., 1992). RelB homodimer or RelB/RelA and RelB/Rel heterodimers have not been detected either *in vitro* or *in vivo* (Dobrzanski et al., 1993; Ryseck et al., 1992). NF-κB p65 is larger than p50 and the formation of p65 homodimer has been reported. Thus, this larger band found in the nuclear content extracted from cells treated with MRF possibly represents a homodimer of p65 NF-κB. However, further study should be done to confirm it identity.

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Figure 1. TLR-4 expression of MQ-NCSU cells treated with medium, MRF, LPS, or MRF + LPS at 0, 5, and 24 hours.

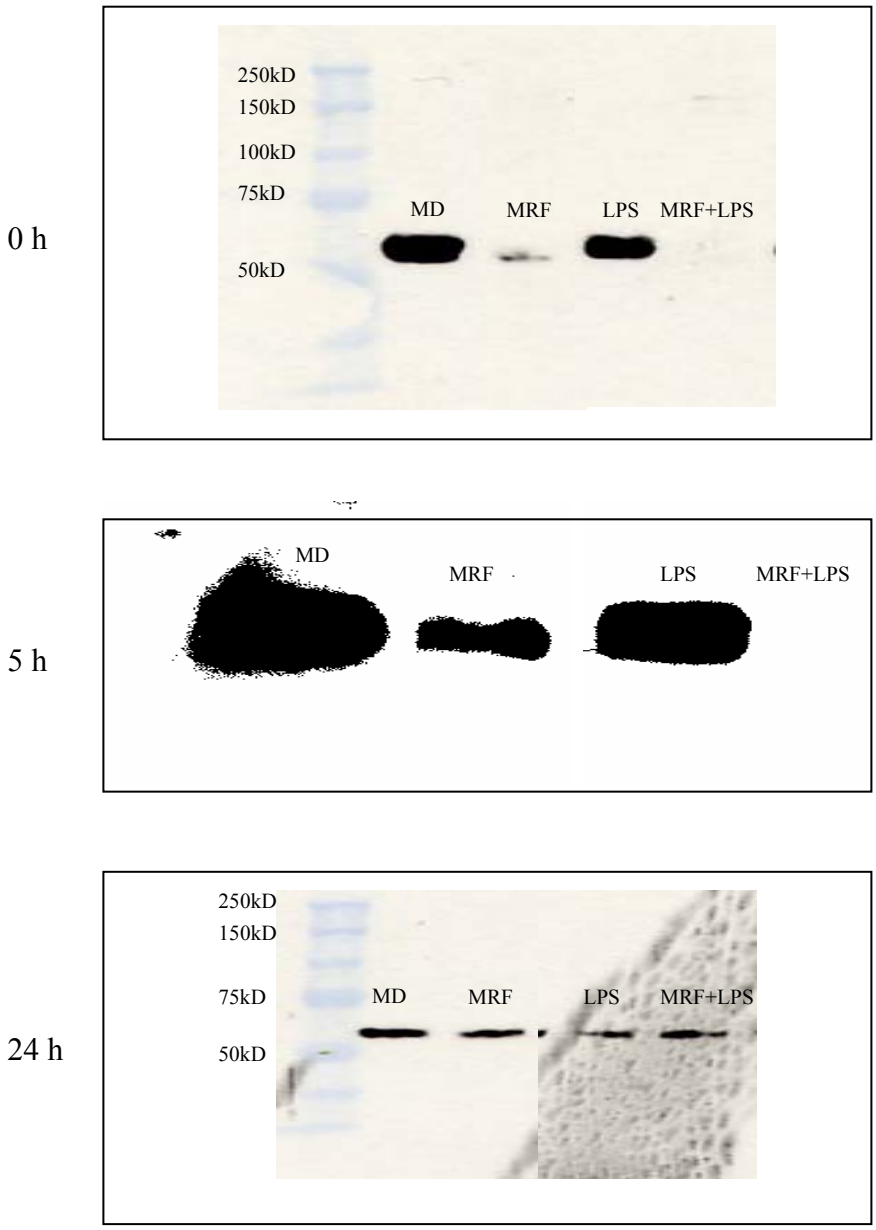
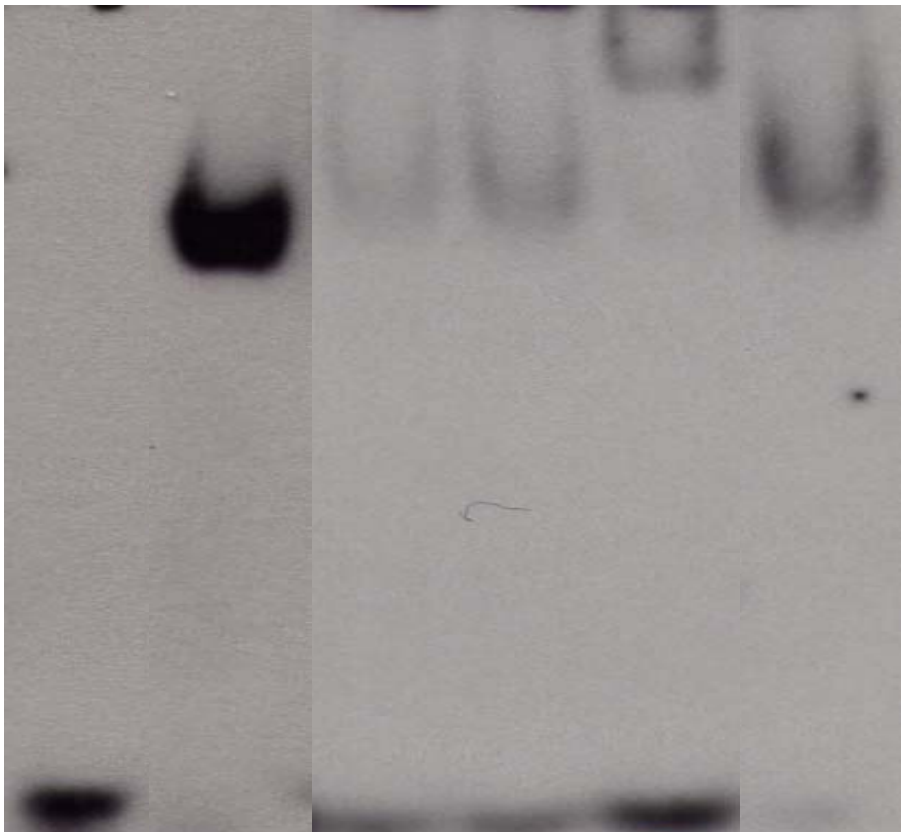


Figure 2. EMSA analyses of nuclear contents extracted from MQ-NCSU cells treated with medium, MRF, or LPS for 30 minutes.



DNA p50 MD0 MD30’ MRF30’ LPS30’

Table 1. Percentage intensity of the bands of TLR-4 expression.

TREATMENT	% INTENSITY		
	0h	5h	24h
Medium	54.2	54.2	30.3
MRF	4.8	16.8	21.6
LPS	40.3	29.0	22.9
MRF + LPS	0.7	0.0	25.3

Table 2. Percentage intensity of the bands of NF-κB activation.

TREATMENT	% INTENSITY
DNA only	2.5
Control p50	44.4
Medium 0min	6.3
Medium 30min	10.2
MRF bottom band 30min	3.5
MRF top band 30min	9.4
LPS 30min	23.7

CHAPTER 4

The Mannan Rich Fraction of *Saccharomyces cerevisiae* Enhances Growth and Regulates Inflammatory Responses of Broiler Chickens

Abstract

Two hundred one-day old broiler chicks divided into four groups received treatments, which included (1) basal diet fed/non-challenged, (2) mannan rich fraction (MRF) fed/non-challenged, (3) basal diet fed/*Escherichia coli*-challenged, or (4) MRF fed/*E. coli*-challenged. Body temperature and body weights of the birds were measured at 1, 2, and 3 weeks of age. At 3 weeks of age, the birds were killed and abdominal exudate cells (AECs), stimulated in response to intra-abdominal Sephadex G50 injection, were collected. The AECs were cultured overnight in supplemented RPMI 1640 at 41°C, with 5% CO_2, and the supernatants were collected for nitric oxide (NO) and interleukin-6 (IL-6) analyses. Some of the AEC from the basal fed, non-challenged birds were used as primary macrophages and were treated with (1) medium only, (2) MRF, (3) lipopolysaccharide (LPS), or (4) MRF + LPS for 18 hours at 41°C, 5% CO_2 and the supernatants were collected for NO and IL-6 analyses. In 3 weeks old broilers, *E. coli*-challenge did not cause a significant increase in body temperature, but *E. coli* challenge decreased body weight. Broiler chickens in both *E. coli*- challenged and non-challenged groups, given MRF supplementation, gained more weight than birds given the non-supplemented basal diet. The LPS-stimulated AECs and primary macrophages showed a significant increase in NO production, but AECs and primary macrophages from MRF-

fed/non-challenged birds produced significantly less NO than AECs from basal-fed, non-challenged birds. The NO production of primary macrophages, isolated from basal-fed, *E. coli*-challenged birds, was significantly greater than the NO production of AECs from basal-fed, non-challenged birds, and primary macrophages from *E. coli*-challenged, MRF-fed birds produced significantly less NO than macrophages from *E. coli*-challenged, basal-fed birds. Primary macrophages from MRF-fed birds had significantly suppressed IL-6-like activity similar to IL-6-like activity of AECs in medium only. LPS stimulation of primary cells significantly elevated IL-6-like activity, but IL-6-like activity was suppressed significantly from LPS stimulated primary cells from MRF-fed broilers. However, in AECs from MRF-fed, nonchallenged broilers IL-6-like activity was increased, but AEC IL-6-like activity was not elevated by *E. coli* challenge from either basal-fed or MRF-fed birds when compared to IL-6-like activity of basal-fed, non-challenged birds.

Introduction

Some of the major causes of economic loss in the poultry industry worldwide are bacteria-associated gastrointestinal diseases that include Salmonellosis, Pullorum Disease, Fowl Typhoid, Paratyphoid Infection, Arizonosis, and Colibacillosis. Birds with these diseases exhibit similar clinical signs which include anorexia, somnolence, diarrhea, droopy wings, and ruffled feathers (Calnek et al., 1997; Jordan and Pattison, 1996). The causative bacterial agents are members of the family *Enterobacteriaceae*, particularly *Escherichia* and *Salmonella,* which are gram-negative, rod-shaped, nonspore-

forming, aerobic or facultative anaerobic organisms. Within the outer membrane of these bacteria there is an endotoxin known as lipopolysaccharide (LPS).

Generally, LPS consists of three main components: an O-antigen, a core region, and lipid A, which is the toxic component of the LPS molecule (Takada and Kotani, 1989). LPS exerts its biological activities by first binding Lipid A specifically to the hepatically-produced serum LPS-binding protein (LBP) to form an LBP-LPS complex (Ramadori et al., 1990). The LBP-LPS complex facilitates the binding of LPS to CD14, a glycoprotein that exists both as soluble and membrane-bound proteins (Haziot et al., 1988). The binding of LPS to CD14 brings the latter molecule closer to its potential receptor complex, Toll-like Receptor-4 (TLR-4), TLR-2, and MD-2. TLR-4 is a type-1 transmembrane protein that contains multiple leucine-rich repeats (LRRs) on the extracellular domain and the Toll/Interleukin-1 Receptor (TIR) domain on its cytoplasmic portion (Hashimoto et al., 1988; Kobe and Deisenhofer, 1995; Medzhitov et al., 1997). MD-2 is a small protein expressed on the surface of several cell types in association with TLR-4 (Shimazu et al., 1999). The binding of LPS to its receptor complex causes the induction of receptor aggregation (Martin and Wesche, 2002) and subsequently triggers the signaling cascade that results in the translocation of the transcription factor nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus. Hanada and Yoshimura (2002) have reported that NF-κB binds to and activates the transcription of certain genes, particularly genes encoding pro-inflammatory cytokines (Interleukin-1, IL-6, and TNF-α) and inducible nitric oxide synthase (iNOS).

Pro-inflammatory cytokines induce several biological activities. These include increasing body temperature (Dinarello, 1996; Dinarello et al., 1986; Kozak et al., 1998; Rothwell et al., 1991), inducing acute-phase protein syntheses by hepatocytes (Castell et al., 1989), inducing of anorexia and somnolence (Plata-Salaman, 1998), and altering nutrient metabolism (Feingold et al., 1992; Filkins and Cornell, 1974). Collectively, the aim of these responses is to help the body to regain homeostasis and to eliminate the invasive pathogens. However, excessive production of pro-inflammatory cytokines causes sepsis, a systemic inflammatory response syndrome (SIRS) characterized by the occurrence of cardiovascular dysfunction, dissemination of intravascular coagulation, and hypotension that lead to inadequate organ perfusion and tissue hypoxia (Parrillo, 1993; Van Amersfoort et al., 2003). The latter can cause multiple organ failure (e.g. kidney and liver), which is usually associated with death of the infected individuals (Brun-Buisson et al., 1995).

Nitric Oxide (NO) is a small reactive nitrogen species that is involved in several cell- and tissue-biological functions in vertebrate animals (Lincoln et al., 1997) including chicken immunologically active cells (Crippen et al., 2003). There have been several studies indicating the association of NO with protective immune responses (James, 1995; Karupiah et al., 1993; Laubach et al., 1995; Lorsbach et al., 1993; MacMicking et al., 1997), but NO also was found to be a key mediator of septic shock (Groeneveld et al., 1994; Parrillo, 1993) due to its ability to act as a potent endogenous vasodilator (Kilbourn et al., 1990).

BioMOS® (Alltech, Inc., Nicholasville, KY 40356) is a glucomannoprotein complex isolated from the outer cell wall of the yeast *Saccharomyces cerevisiae*. BioMOS® is one of the potential alternatives to antibiotics and has been used as a feed supplement to improve animal health. It has been shown to be able to agglutinate enterobacteria with type-1 fimbriae and the agglutination was inhibited by the addition of mannose sugar (Spring et al., 2000). Additionally, the same authors demonstrated that BioMOS® could reduce the concentration of *S. typhimurium* 29E within the cecum of birds and suggested that BioMOS® may act as a decoy of enterobacteria. Moreover, BioMOS® was reported to be able to increase the number of beneficial bacterial microflora such as *Enterococcus spp.* (Fernandez et al., 2002) and reduce the concentration of pathogenic bacteria such as *C. perfringens* (Strickling et al., 2000). BioMOS® can also enhance the immune response in animals (Kudoh et al., 1999; Swanson et al., 2002). Interestingly, animals receiving BioMOS® in their feed gained significantly more weight than the ones receiving only a basal diet (Sims et al., 2004; Zdunczyk et al., 2005). Furthermore, the mortality rate in animals fed BioMOS® and challenged with pathogenic bacteria seems to be lower than in those without BioMOS®. To illustrate, the number of birds that are infected with *Salmonella* was found to be significantly reduced when BioMOS® was given to the birds (Spring et al., 2000).

In a previous chapter, it was shown that the mannan rich fraction (MRF), a soluble fraction of the BioMOS®, decreased inflammatory responses of chicken macrophage cell lines that were exposed to LPS. In this study, we further investigated

whether MRF could reduce inflammatory responses of primary chicken macropphages that were exposed to LPS. In addition, we investigated if MRF could reduce inflammatory responses *in vivo*.

Materials and Methods

Animal Care and Housing

This project was approved and conducted under the supervision of the North Carolina State University Animal Care and Use Committee which has adopted Animal Care and Use Guidelines governing all animal use in experimental procedures. A group of 200 one day old Cobb 500 X Cobb 500 feather sexable broiler chickens with initial average body weight of 41g were used in this trial. Chicks were randomly allotted to each of four treatments in a completely randomized design. Each treatment was assigned to five pens each with 10 chicks providing 0.80 ft^2/bird. The chicks were neck-banded with plastic transdermal tags and placed into two separate rooms of control or challenge with each room containing two batteries of five pens per battery. Brooding temperature and light management within the brooder batteries were consistent with current broiler management practices (Edens et al., 2000). Brooding temperature in the brooders was 95°F (35°C), and this temperature was decreased incrementally (4°C at a time) to 70°F (20°C) by the time the birds were 21 days old. The photoperiod was established at 23 hours light and 1 hour darkness. Birds were allowed *ad libitum* access to feed and water, which were provided in stainless steel pans.

Diets

The basal North Carolina Agricultural Research Service broiler starter diet was provided to the birds. The formulation for the basal diet is presented in Table 1 and provided 3154 kcal/kg ME, 21% CP (1-16 d), 33 IU/kg of vitamin E, and organic selenium (0.3 ppm) as Sel-Plex® (Alltech Biotechnology Center, Nicholasville, KY 40356). The crumbled starter diet was fed to provide 2 pounds per bird. The endpoint for this investigation was body weight, and this was determined at 1, 2 and 3 weeks of age. Mortality was monitored daily.

Bacterial Culture and Oral Challenge

The enteropathogenic *E. coli* (serotype O1; EPEC) was grown overnight at 37^{o}C in LB broth at 37°C with continuous shaking at 150 rpm. The EPEC cells were collected by centrifugation at 1500 rpm for 15 minutes followed by washing twice with sterile 0.85% saline solution and adjusted to 1x10^{8} cells/mL with the same solution. The EPEC challenge dose (10^{6} CFU/mL) was administered by gavage in a volume of 1 mL into the crop of the one-day-old chicks.

Experimental Design

One-day old broiler chicks were divided into four groups of fifty birds each. Group one was a control that received a basal diet without an EPEC *E. coli* challenge. Group two was fed with MRF but not challenged with EPEC *E. coli*. Group three was fed a basal diet and was challenged with EPEC *E. coli*. Group four received MRF and was challenged with EPEC *E. coli*. The EPEC *E. coli* used in this investigation produce type 1 fimbriae that apparently are used as an adhesin to facilitate attachment in the GIT. The

characteristic of type 1 fimbriae production by the EPEC used in this study is important because BioMOS® has been shown to have a strong affinity for bacteria with type 1 fimbriae (Spring et al. 2000). After body weight was measured, the chicks were neck-banded with transdermal plastic tags and 1mL/bird of EPEC *E. coli* was given to the birds assigned to the challenge groups by oral gavaging. At 1, 2, and 3 weeks of age, body weights of all birds were measured. The colonic body temperatures of 10 birds/group at 1 and 2 weeks of age and 2 birds/group at 3 weeks of age were measured with a YSI Precision Thermometer model 4600 (YSI, Inc., Yellow Springs, OH). At 20 days of age, macrophages were isolated from the abdominal exudates from 10 birds/group. Cells from different birds in the same group were pooled and subjected to interleukin-6 (IL-6) and nitric oxide analyses.

Macrophage Isolation

The abdominal exudate cells (AEC) were isolated as described previously (Qureshi et al., 1986). At 20 days of age, 10 birds in each group were weighed and injected intra-abdominally with a 3% (wt./vol.) Sepahdex® G50 (Sigma Chemical Co., St. Louis, MO) suspension at a dose of one mL/100g body weight. Two days later, birds were killed using CO_2 asphyxiation, and the abdominal exudate was collected in siliconized glass tubes from each birds by flushing the abdominal area with sterile, cold 0.85% saline containing 0.5U/mL heparin. Cells were pelleted by centrifugation at 1500 rpm for 20 minutes. The AEC pellets were then re-suspended in RPMI 1640 medium (Sigma) supplemented with 1% antibiotic antimycotic solution (Sigma) and 5% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT).

Macrophage Cultures

The abdominal exudate cells isolated from different groups of birds were adjusted to a concentration of $1x10^6$ cells/mL with supplemented RPMI 1640 medium. Cells were added to a 96-well tissue culture plate (Fisher Scientific, Pittsburgh, PA) at 100μL/well, 10 wells/group, and the plate was incubated overnight at 41°C, 5% CO_2 in a humidified incubator. The supernatants were collected and used for nitric oxide (NO) and IL-6 analyses.

Remaining cells from negative control birds (no MRF, no *E. coli*) were used as the primary cells. These cells were adjusted to a concentration of $1x10^6$ cells/mL with supplemented RPMI 1640 medium and 100μL/well of cells with 10 wells for each treatment were treated with supplemented RPMI 1640 medium only, 2.5mg/mL MRF, 5μg/mL LPS, or 2.5mg/mL + 5μg/mL LPS. Cells were incubated overnight at 41°C, in a 5% CO_2 in humidified incubator prior to the collection of the supernatants. The collected supernatants were analyzed for NO production and IL-6-like activity.

Nitrite Assay

Nitric oxide (NO) has a short half-life. Thus, NO measurement is often carried out using the conventional Griess assay that detects nitrite (NO_2^-), the more stable metabolite of NO (Nathan, 1992; Stuehr and Marletta, 1987a; Stuehr and Marletta, 1987b). The Griess assay uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions to detect NO_2^- by converting it to an azo compound whose absorbency can be detected by spectrophotometrically at 540 nm.

The Griess reagent system (Promega, Madison, WI) was used as the nitrite assay in this study. The standard nitrite solution was prepared according to the protocol provided by the manufacturer to generate a standard reference curve. Fifty microliters of the standard solutions or the supernatants were added in triplicate to the flat-bottom 96-well plate (Immulon® 1B, Thermo Labsystems, Franklin, MA) followed by the addition of 50 μL of the sulfanilamide solution. The plate was incubated at room temperature (RT) for 5-10 minutes in the dark. The NED solution was then added at 50 μL/well and the plate was further incubated for an additional of 5-10 minutes. The absorbency was measured within 30 minutes using a MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA) at 540 nm.

IL-6 Bioassay

The bioactivity of IL-6 was measured by using a B-9 murine tumor B-cell line whose growth depends on the presence of IL-6. The B-9 cell is a murine hybridoma/plasmacytoma B-cell line (DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). It was maintained in RPMI medium supplemented with 10% FBS, 1% antibiotic antimycotic solution, and 25 μL of 20μg/mL recombinant human IL-6 (rhIL-6, Affinity BioReagents™, Golden, CO). The B-9 cells were collected by centrifugation at 1200 rpm for 10 minutes and washed two times with B-9 cell medium without rhIL-6. Cells were re-suspended with a known volume of the B-9 cell medium without rhIL-6. Cells were counted using the trypan blue exclusion method and cell number was adjusted to $1x10^5$ cells/mL concentration. The supernatants were diluted to 1:10 with B-9 cell medium without IL-6. B-9 cells were added, at 100

μL/well, in the 96-well tissue culture plates. Medium without rhIL-6, medium with rhIL-6, or diluted supernatants were added, 100 μl/well, in triplicate into the plates. Plates were incubated at 37°C, 5% CO_2 for 68 hours. The proliferation of B-9 cells was determined by adding 50 μL/well of 1mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) and incubated for an additional 3-4 hours. The viable B-9 cells absorb the MTT, which is then metabolized by mitochondria, yielding a blue crystalline compound. When the plates were removed, 150 μL of the supernatant was discarded followed by the addition of 150 μL of 0.85% acid alcohol (200mL 2-propanol + 1.32mL HCl + 100mL PBS, all chemicals were purchased from Sigma) with thorough mixing to dissolve the crystalline compound. The absorbency of the homogeneous solutions was read at 540 nm. The averages of the absorbency readings were calculated, and the results were reported as the IL-6-like activity.

Data Analysis

Data were analyzed for main effects and their interactions using the general linear model procedure of SAS (SAS Institute, 1996). Means were examined for significance by Least Square Means test at the significance level of $P<0.05$.

Results

Enteropathogenic *E. coli* did not increase body temperature of infected birds

Ten birds at week 1 and 2, and 2 birds at week 3 were randomly selected from each group to measure colonic body temperature. Figure 1 shows the average body temperature of each group of birds at 1, 2, and 3 weeks of age. At no time point

measured, average body temperatures neither of basal-fed nor of MRF-fed EPEC-challenged or control birds were significantly increased.

MRF enhanced growth of broiler chickens

The body weight of all birds was measured at 1 day and at 1, 2 and 3 weeks of age. Mean body weights (MBW) and mean body weight gains (MWG) of each group were determined. Figure 2 shows the mean body weights (MBW) of each group obtained from each week. The initial average body weight was approximately 41g (data not shown). At one week of age, the MBW of the challenged groups were not statistically different from MBW of the basal-fed/non-challenged birds. On the other hand, the MRF-fed/non-challenged birds showed the highest MBW, which was significantly higher than the MBW of the other three groups. At 2 weeks of age, basal-fed/*E. coli* challenged birds showed the lowest MBW, which was significantly lower than the MBW of birds in non-challenged groups and numerically lower than the MBW of the MRF-fed/*E. coli*-challenged birds. The MBW of the MRF-fed/*E. coli*-challenged birds was not different from, but numerically lower than the MBW of the non-challenged birds. By 3 weeks of age, both groups of MRF-fed birds, challenged and non-challenged, showed numerically higher MBWs than that of basal-fed/non-challenged birds. The MBW of basal-fed/*E. coli*-challenged birds was the lowest and was statistically lower than MBW of the other three groups.

Figure 3 illustrates the mean body weight gain (MBG) during the three-week period of birds from the four different treatments. Although the difference between the MBG of basal-fed/non-challenged birds and MRF-fed/non-challenged birds was not

statistic significantly, MBG of MRF-fed/non-challenged birds was numerically higher (24g greater MBG) than the MBG of basal-fed/non-challenged birds. The MBG of basal-fed/*E. coli*-challenged birds was the lowest among all four groups, and this MBG was significantly lower than the MBGs of the other three treatments. Interestingly, MRF-fed/*E. coli*-challenged birds gained the most weight with 3g, 27g, and 91g greater MBG than the MBG of MRF-fed/non-challenged, basal-fed/non-challenged, and basal-fed/ *E. coli*-challenged groups, respectively. However, this MBG was not different from the MBGs of either basal-fed/non-challenged or MRF-fed/non challenged birds.

MRF reduced NO production of LPS-stimulated primary chicken macrophage cells

Figure 4 shows nitrite production, a more stable metabolic product of nitric oxide, in the supernatants collected from treated primary macrophages. Nitrite production of macrophages treated with MRF was the same as nitrite production of cells treated with medium only. When primary macrophage cells were treated with LPS, the nitrite production was significantly increased. When macrophages were treated with LPS in the presence of MRF, the nitrite production was significantly decreased compared to nitrite production of cells treated with LPS alone.

MRF reduced NO production of macrophages from EPEC-challenged broiler chickens

Figure 5 shows nitrite production of macrophages of birds from the four different treatments. The nitrite production of macrophages from MRF-fed/non-challenged birds was significantly lower than nitrite production of macrophages from basal-fed non-challenged birds. Macrophages from basal-fed, *E. coli*-challenged birds had significantly

higher concentrations of nitrite than birds from the other three treatments. However, nitrite production was significantly reduced when *E. coli* challenged birds were fed with MRF.

MRF reduced IL-6-like activity of LPS-stimulated primary chicken macrophages

Figure 6 illustrates the IL-6-like activity of treated primary chicken macrophage cells. The level of IL-6-like activity of cells treated with MRF alone was similar to the level of IL-6-like activity of cells treated with medium only. Primary chicken macrophages treated with LPS had a significantly higher IL-6-like activity than cells from the other three treatments. When MRF was present in the culture of macrophages along with LPS, the IL-6-like activity was significantly reduced compared to the IL-6-like activity of macrophages cultured with LPS only.

MRF did not reduce IL-6-like activity of primary macrophages from *E. coli*-challenged broiler chickens.

Figure 7 shows the IL-6-like activity in the supernatants collected from cultured macrophages obtained from the four different groups of birds. At 3 weeks of age, the IL-6-like activities of both groups of challenged birds were not different from the IL-6-like activity of non-challenged birds fed with only a basal diet. However, the IL-6-like activity of non-challenged, MRF-fed birds was significantly higher than IL-6-like activity of any of the other three treatments.

Discussion

BioMOS®, a glucomanoprotein product derived from the yeast cell walls of a strain of *Saccharomyces cerevisiae* used as a feed supplement to improve animal health and performance due to its ability to agglutinate and reduce the concentration of some pathogenic bacteria within the GIT (Spring et al., 2000), alters the numbers of intestinal microflora (Fernandez et al., 2002; Strickling et al., 2000), and increases an adaptive immune response in animals (Kudoh et al., 1999; Swanson et al., 2002). In this study, the role of the mannan rich fraction (MRF), a soluble fraction of BioMOS®, on some innate immune responses of chickens both *in vitro* and *in vivo* was investigated. These investigations were important because they provided insight into the mechanisms exerted by the MRF in reducing the production of inflammatory cytokines in growing birds and in abdominal exudate macrophages from basal-fed and MRF-fed with and without enteropathogenic *E. coli* (EPEC) challenge.

Several clinical signs develop in chickens during an *E. coli* or *Salmonella* infection and may include fever in response to LPS from the cell walls of the bacteria (Jones et al., 1981 and 1983). Directly injection of LPS to birds induces fever (Ferket et al., 2002). In this study, EPEC *E. coli*-challenged broiler chickens did not develop a febrile condition as evidenced by their stable body temperatures throughout experimental period (Figure 1). With the exception of a few days distress after the *E. coli* oral challenge, birds did not develop diarrhea, droopy wings, ruffled feathers, or show any sign of somnolence. The *E. coli* was given to the birds at the dose of 10^6 cells/bird that is in the range of doses that have been used by several investigators. EPEC E. *coli* does not

generally translocate from the GIT and become systemic (Williams et al., 1997). However, EPEC does affect calcium metabolism in the GIT causing an elevation in intracellular content (Knutton, 1993). If EPEC was to translocate and become systemic, it is possible that other organs would be affected similarly, and in this case it would be possible that body temperature might actually decline in response to elevated intracellular calcium, especially if this occurred in the hypothalamus (Denbow and Edens, 1980). EPEC *E. coli* do cause damage in the GIT and disturb the cellular structure of microvilli on enterocytes thereby reducing the efficiency of nutrient absorption (Williams et al., 1997).

However, challenging the birds with EPEC *E. coli* caused their body weights to decrease significantly at 2 and 3 weeks of age after the challenge compared to the body weight of non-infected birds (Figure 2). When infected birds were fed with MRF, their body weights were maintained close to the level of the body weights of non-infected birds. Furthermore, the body weights (Figure 2) and weight gain (Figure 3) of MRF fed birds were even higher than the body weights of non-infected birds fed with only a basal diet at three weeks of age. These results suggest that feeding birds with MRF, not only improves their health, but also enhances growth over time.

These observations fit well with the hypothesis that BioMOS® (Spring et al., 2000) and possibly the MRF will have a positive influence on the binding of type 1 fimbriae on the EPEC *E. coli* and thereby reduce their influence on inflammation and in the long term improve growth of the chickens given the MRF. However, to determine whether MRF fed birds continued to gain significantly more weight than basal-fed birds,

it is important that follow-up investigations measure body weight gain through market age of the broiler chickens.

The feeding of the MRF reduced production of inflammatory cytokines in birds infected with EPEC *E. coli.* Both nitric oxide and IL-6 productions of the abdominal exudate macrophage cells from MRF-fed broiler chickens were reduced significantly. In non-challenged birds, macrophages isolated from MRF-fed birds produced significantly lower levels of nitric oxide than the macrophages isolated from basal-fed birds (Figure 5). In contrast, nitric oxide production of the macrophages isolated from basal-fed, *E. coli* - challenged birds increased significantly. When MRF was added into the feed of the *E. coli* challenged birds, their macrophage nitric oxide production was reduced significantly.

The abdominal exudate macrophages were used as a source of primary macrophage cells. The nitric oxide production by primary macrophage cells, treated with medium only, MRF, LPS, or MRF + LPS, responded to MRF and LPS (Figure 4) similar to chicken macrophage cell lines (Chapter 3). Although similar observations were made using chicken immortal macrophage cell lines (Chapter 3), there were no data to show that freshly collected macrophages in AECs from chickens fed the MRF would behave the same way. The data shown herein, support the idea that either the MRF or some metabolite from that fraction played a significant role in the modulation of the inflammatory cytokine response even when it is provided in the feed of the chicken. Ostensibly, the type 1 fimbriae on the EPEC were bound by the MRF, and this prevented the bacteria from colonizing in the GIT, but this does not explain the reason why AEC macrophages from MRF-fed chickens also showed decreased production of nitric oxide.

It is hypothesized that the MRF-bacterial complex might be processed by immunologically active cells in the GIT or that this complex might be recognized by M cell macrophages, antigen presenting cells, T-cells, or even heterophils or monocytes in the Peyer's patches in the GIT. In that case, it might be possible to increase the population of macrophages and potential macrophages which had been either exposed or stimulated by the MRF-bacterial complex. Thus, when abdominal Sephadex G50-macrophage recruitment was induced, the AEC population might have been predominately those cells that had been primed by exposure to the MRF-bacterial complex. More research is required to address this potential scenario.

The IL-6 response profile of primary macrophages treated directly with medium only, MRF, LPS, or MRF + LPS (Figure 6) was also similar to the IL-6 and nitric oxide response profiles of chicken macrophage cell lines (Chapter 3). However, the IL-6 response profile of the abdominal exudate macrophages isolated from treated birds was found to be different. Macrophages isolated from the abdominal exudate of the three-week-old, non-challenged, MRF fed birds produced a small, but significantly higher level of IL-6-like activity (Figure 7). The IL-6-like activity of macrophages isolated from both basal-fed and MRF-fed/*E. coli* challenged birds remained at levels similar to the IL-6-like activity of macrophages from basal-fed/non-challenged birds. The reason(s) for this difference is not readily apparent. Mycotic infections, both clinical and subclinical, are opportunistic and common in poultry species (Chute and Richard, 1991). Among the many potential mycotic disease causing agents, one can find *Candida* highly ranked, being found in a great proportion of all poultry species, both healthy and otherwise

(Jordan, 1953). Whether, there was a *Candida* or other yeast infection in the birds in this experiment was not ascertained. However, it has been noted that the cell walls from *Candida* and other fungi can have significant immunological impact in animal models (Vazquez-Torres et al., 1996; Ataoğlu et al., 2000; Vassallo et al., 2001). In rats, mannans from *Candida albicans* and a *Saccharomyces cerevisiae* induced a febrile state, which was attributed to early nitric oxide induction and to direct stimulatory action on prostaglandin secretion (Ataoğlu et al., 2000). The β-D-glucan moiety of the *Pneumocystis carinii* cell wall also had significant influence on macrophage function causing induction of proinflammatory cytokines such as TNF-α and IL-6 (Vassallo et al., 2001). The β-D-glucan moiety of *Candida albacans* also has a property which caused induction of nitric oxide by macrophages and further induced peroxynitrite (Vazquez-Torres et al., 1996). The observation that a small but significant elevation in IL-6-like activity in macrophages from birds fed the MRF and not challenged with the EPEC could be due to experimental error or it could be due to the presence of unrecognized fungal infection(s). However, it is important to understand that the β-D-glucan component in the MRF is very low in comparison with the levels found in traditional sources of *Saccharomyces cerevisiae* yeast cell walls that are used to formulate BioMOS®.

Thus, conducting an *in vivo* kinetic study of IL-6 production in these birds would provide more information. Whether other pro-inflammatory cytokines such as TNF-α or IL-1β are affected by MRF remains to be resolved. Overall, MRF appeared to provide benefits by reducing inflammatory cytokine responses and enhancing growth in the broiler chickens both with and without EPEC challenge.

It is hypothesized that the energy intake in the birds given the MRF might have been repartitioned. Digestible energy, the energy absorbed from the GIT, can be partitioned into maintenance energy, which is used for thermoregulation, molting, tissue repair, immunological responses, etc., and productive energy, which is used for deposition of body proteins and lipids, muscle development, bone growth, egg production, sperm production, feather growth, etc. (Blem, 2000). Based on the data collected in this research, it is hypothesized that the MRF from the *Saccharomyces cerevisiae* yeast strain that is used to formulate BioMOS® can cause repartioning of digestive energy to more productive energy. The mechanism whereby the MRF can do this is to inhibit a significant amount of the secretion of inflammatory cytokines from macrophages. This would result in less damage to cells and tissues that might become inflamed in the normal course of the life of the chicken. With less tissue damage, the requirement for maintenance energy would be reduced and that energy would be repartitioned to productive energy. The data presented for body weight gain clearly showed that MRF feeding had a positive influence on the growth of the birds in this experiment. It has been shown in Chapter 2 and in this chapter that nitric oxide production by macrophages was reduced to levels that were comparable with nitric oxide production by non-stimulated macrophages. Even in this experiment, IL-6-like activity was reduced to very low levels in macrophages either collected from MRF-fed birds with or without EPEC challenge or from macrophages stimulated with LPS.

In conclusion, it can be stated that the MRF from the *Saccharomyces cerevisiae* yeast strain that is used to formulate BioMOS® can act as an immunomodulator in broiler

chickens. In this function, it inhibits full expression of inflammatory cytokines by macrophages and possibly reduces tissue damage that normally ensues from a fully developed inflammatory response in the intestinal tract. With less tissue trauma to repair, the chickens and possibly other food animals would utilize the extra energy for growth rather than repair. As a consequence, improved growth and feed conversions would be expected. In fact, in the commercial sector, improved growth and feed conversion is the norm when BioMOS® has been used in broilers and in turkeys (Parks et al., 2000).

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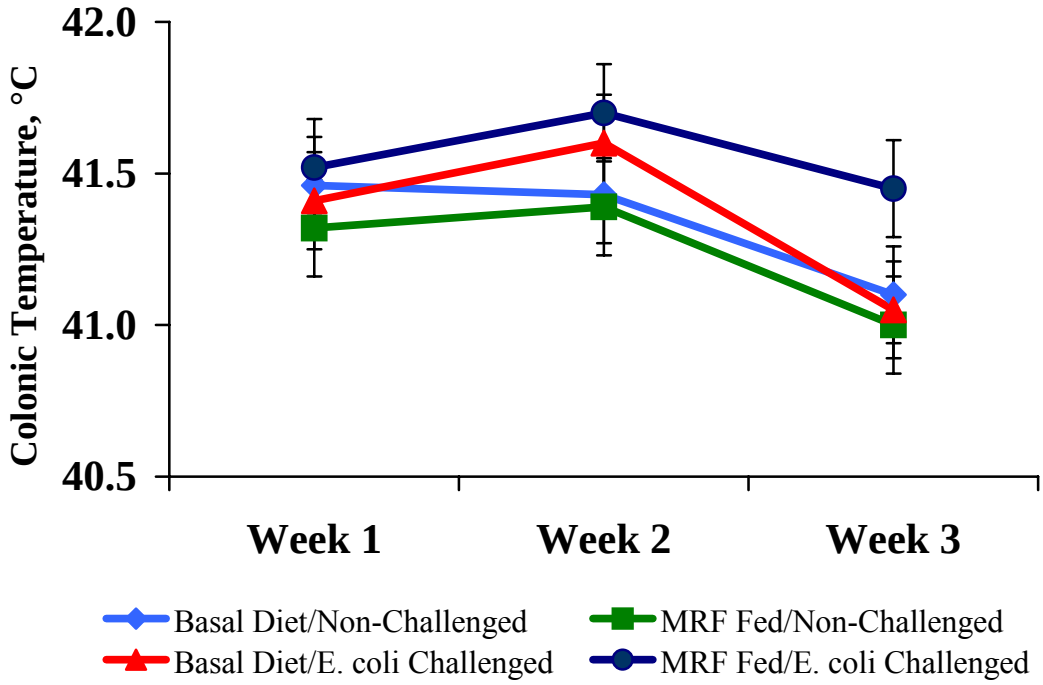
TABLE 1. Composition of North Carolina Agricultural Research Service basal broiler diets.

Dietary Ingredients	Percentage of Dietary Composition
Corn	59.08
Soy	26.95
Limestone	0.70
Dical Phosphate	0.70
Poultry Fat	3.49
Poultry Meal	7.98
DL-Methionine	0.18
Lysine	0.07
Salt	0.40
Choline Chloride	0.20
Minerals[1] (TM-90)	0.20
Vitamins[2] (NCSU-90)	0.05

[1]Trace mineral (TM-90) premix provided in milligrams per kilogram of diet: manganese, 120; zinc, 120; iron, 80; copper, 10; iodine, 2.5; cobalt, 1.0. Selenium premix as organic selenium (Sel-Plex®) was provided to the diet at a level to assure a maximum concentration of 0.3 ppm Se.

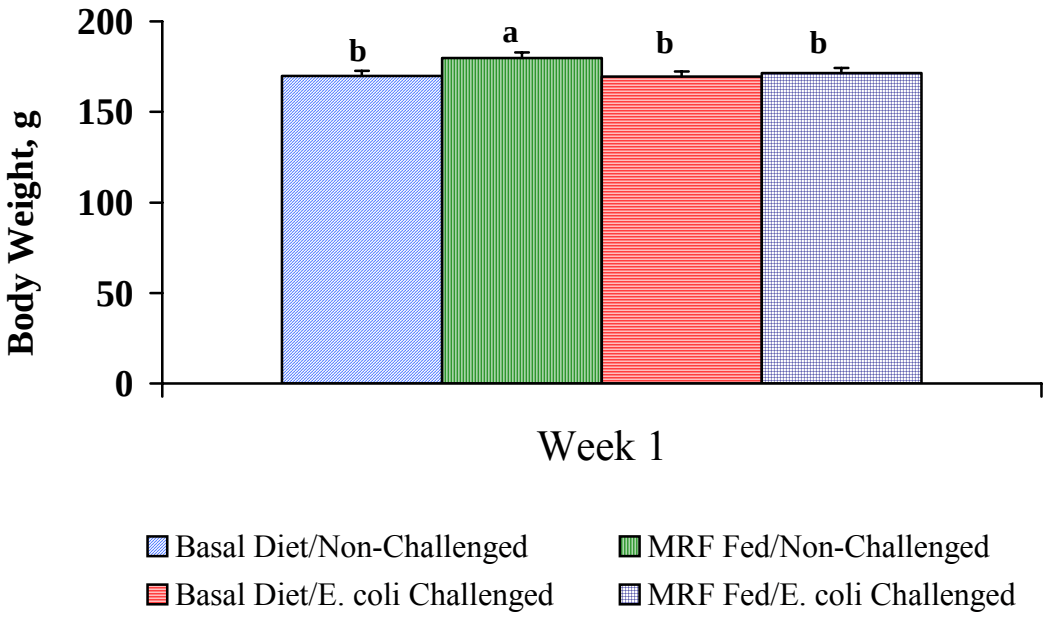
[2]Vitamin premix (NCSU-90) provided per kilogram of diet: Vit. A, 6,600 IU; cholecalciferol, 2,000 IU; Vit. E, 33 IU; Vit. B_{12}, 19.8 μg; riboflavin, 6.6 mg; niacin, 55 mg; pantothenic acid, 11 mg; Vit. K, 2 mg; folic acid, 1.1 mg; thiamine, 2 mg; pyridoxine, 4 mg; biotin, 126 mg.

Figure 1. Colonic temperature (°C) of broiler chickens given either basal or mannan rich fraction from placement through three weeks of age along with or without E. coli challenge at one day of age.

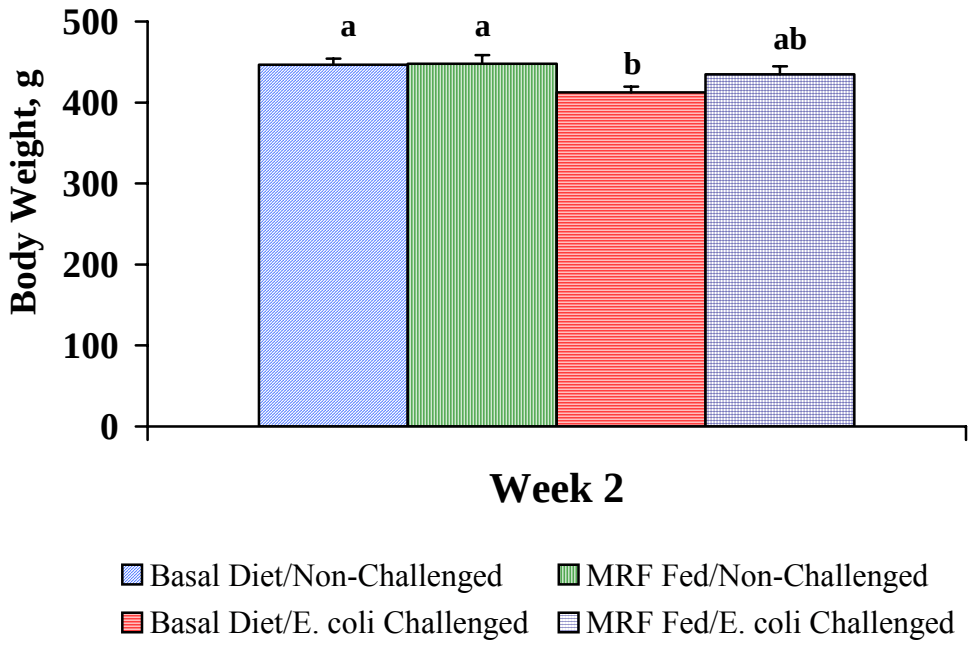


Figures 2A, 2B, and 2C. Mean body weights (grams) of each group of broiler chickens given dietary supplementation of the mannan rich fraction from *Saccharomyces cerevisiae* and subjected to *E. coli* challenge at day of hatch. Unlike lower case letters above bars of the histogram represent significant treatment differences ($P \leq 0.05$).

2A) Week 1



2B) Week 2


500
400
300
200
100
0
Body Weight, g
a
a
b
ab
Week 2
Basal Diet/Non-Challenged
MRF Fed/Non-Challenged
Basal Diet/E. coli Challenged
MRF Fed/E. coli Challenged

2C) Week 3

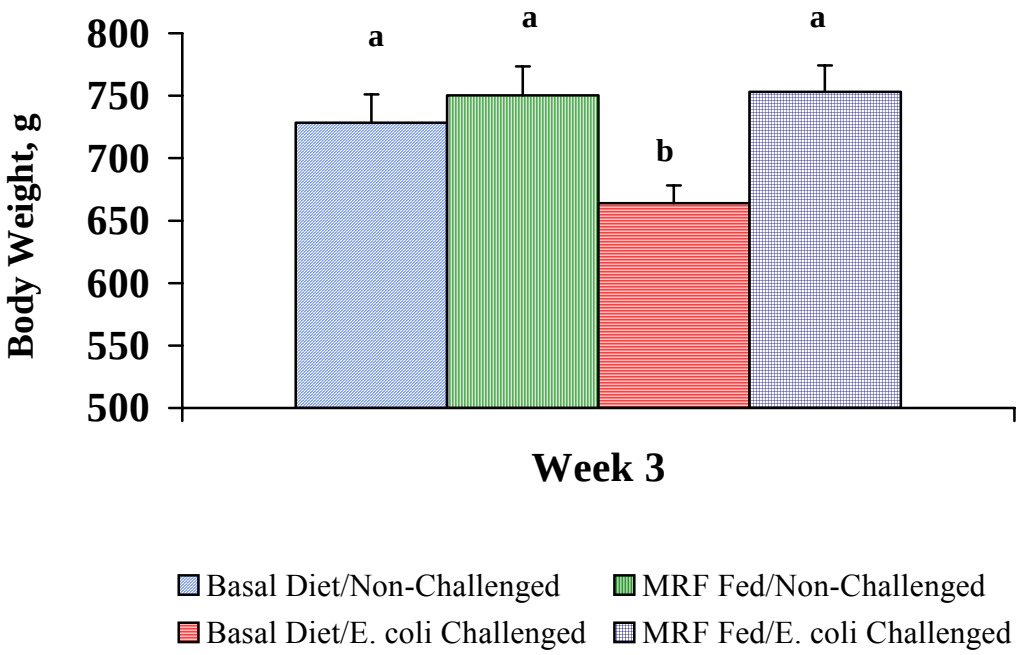

800
750
700
650
600
550
500
Body Weight, g
a
a
b
a
Week 3
Basal Diet/Non-Challenged
MRF Fed/Non-Challenged
Basal Diet/E. coli Challenged
MRF Fed/E. coli Challenged

Figure 3. Mean body weight gains of different groups of broiler chickens given dietary supplementation of the mannan rich fraction from *Saccharomyces cerevisiae* and subjected to *E. coli* challenge at day of hatch. Unlike lower case letters above bars of the histogram represent significant treatment differences ($P \leq 0.05$).

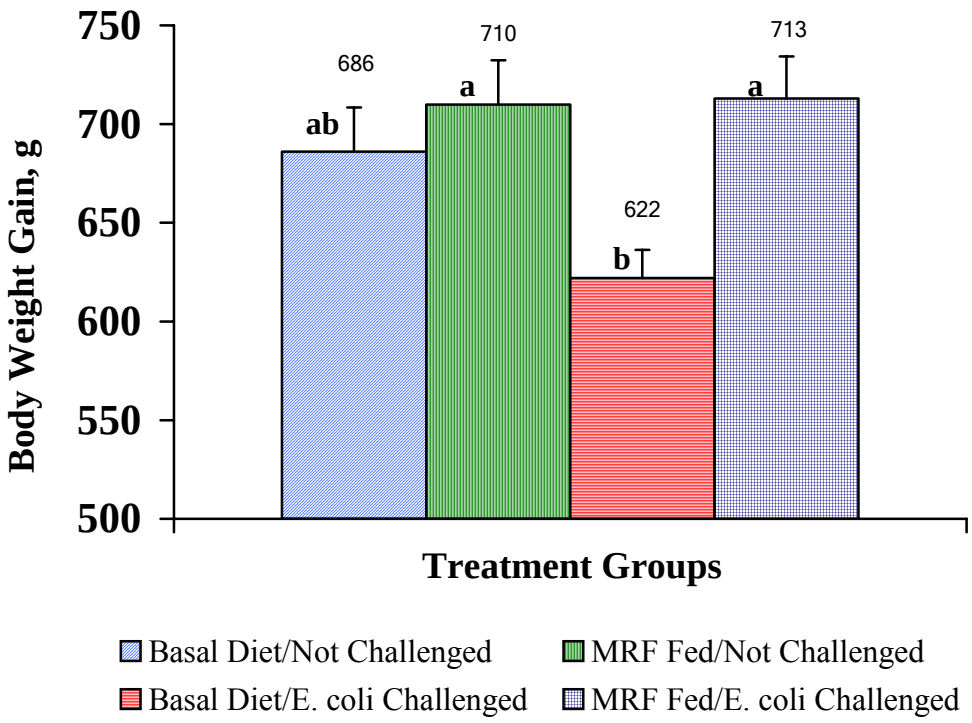


Figure 4. Nitrite production of primary chicken macrophage cells (abdominal exudate cells) treated with medium, MRF, LPS, or MRF + LPS. Unlike lower case letters above bars of the histogram represent significant treatment differences ($P \leq 0.05$).

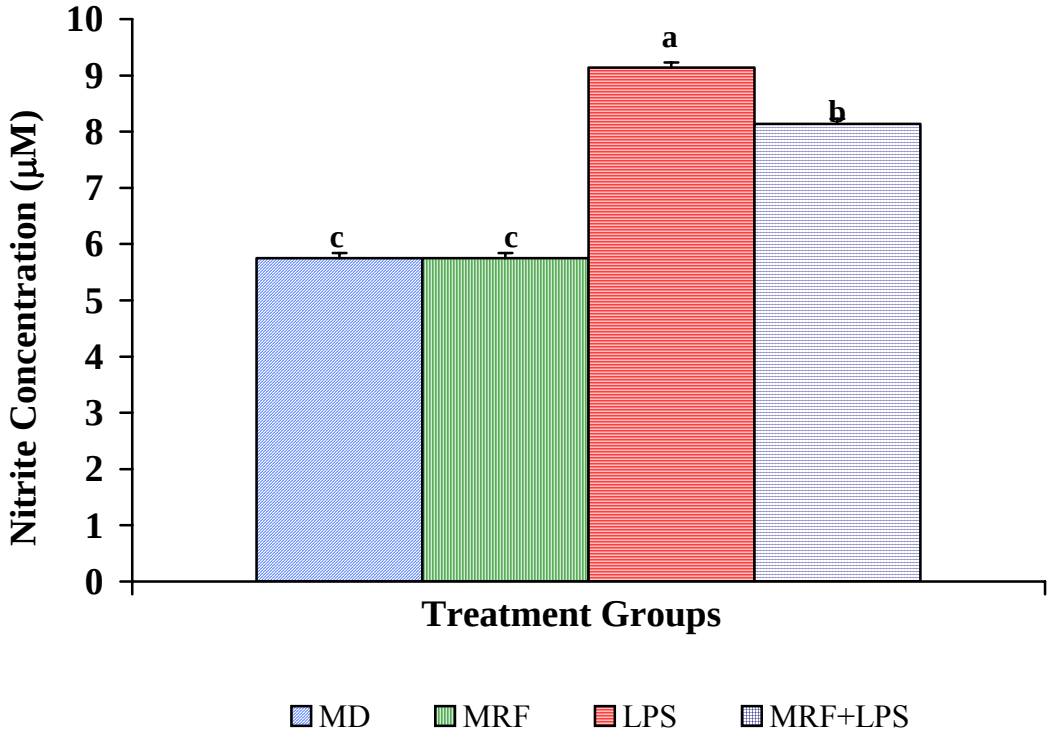


Figure 5. Nitrite production of the abdominal exudate macrophage cells isolated from four different groups of three-week-old broiler chickens. Unlike lower case letters above bars of the histogram represent significant treatment differences ($P \leq 0.05$).

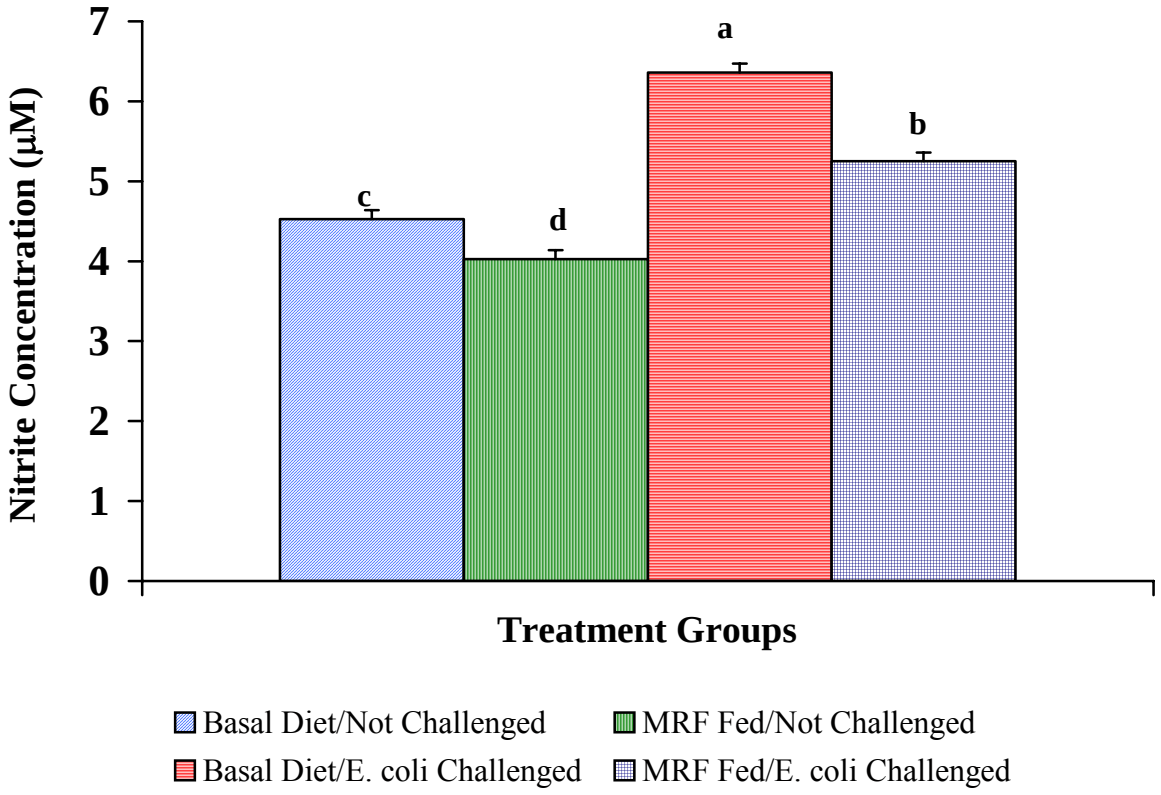


Figure 6. IL-6-like activity of primary chicken macrophage cells treated with medium, MRF, LPS, or MRF + LPS. Unlike lower case letters above bars of the histogram represent significant treatment differences ($P \leq 0.05$).

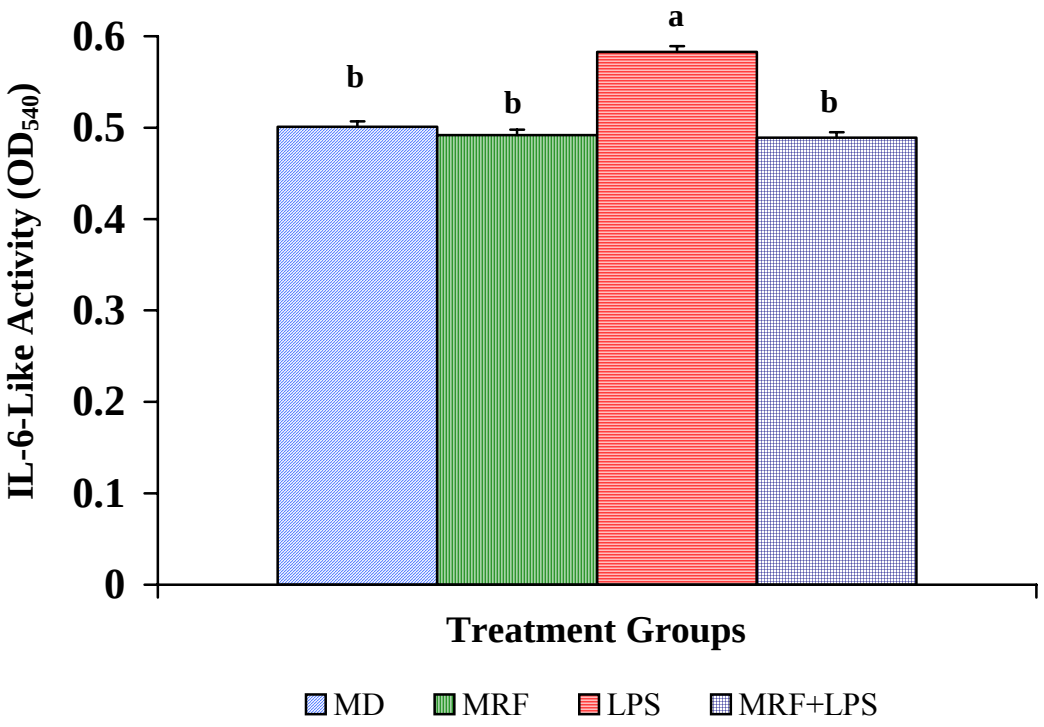
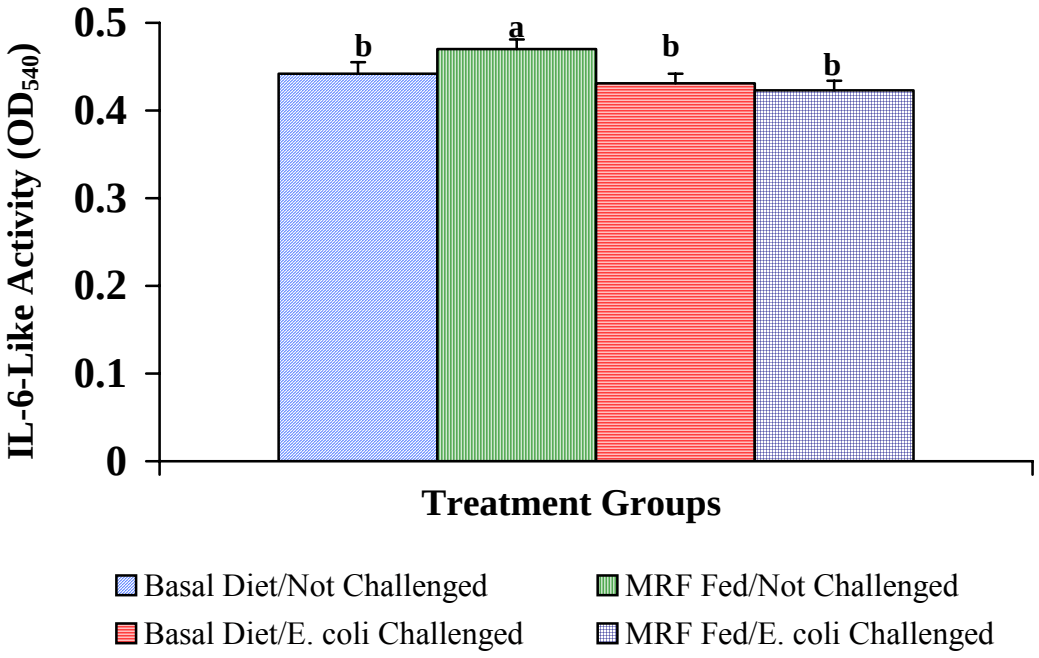


Figure 7. IL-6-like activity of the abdominal exudate macrophage cells isolated from four different groups of three-week-old broiler chickens. Unlike lower case letters above bars of the histogram represent significant treatment differences ($P \leq 0.05$).



SUMMARY AND CONCLUSION

Mannanoligosaccharide (MOS), a glucomannoprotein complex isolated from the outer cell wall, is used as a feed additive to improve animal growth and performance. There are several mechanisms by which MOS promotes the growth and performance of animals one of which is to enhance the immune responses of animals (Kudoh et al., 1999; Swanson et al., 2002). In this study I investigated whether the mannan rich fraction (MRF), a soluble fraction of BioMOS® isolated from the outer cell wall of yeast strain *Saccharomyces cerevisiae* (Alltech Inc., Nicholasvile, KY), improved the performance of broiler chickens by reducing an inflammatory response.

First, I examined the ability of Product F, Product D, Product B, Product E, Product A, and Product C yeast cell wall products to agglutinate *E. coli* and *S. typhimurium*. The results indicated that in all products tested, their ability to agglutinate *S. typhimurium* was slightly lower than their ability to agglutinate *E. coli*. For these products to agglutinate different bacteria with differential activity is possibly due to the variation of the composition of type-1 fimbriae, a structure expressed on some bacterial cell surfaces that mediates the attachment of bacteria to target cells. In addition, different yeast products agglutinated the same bacteria with different percentages of agglutination. Product B, Product E, and Product A agglutinated over 70% of *E. coli* cells. Product F and Product C agglutinated *E. coli* with percentages of aggregated cells of 68.5% and 60.27%, respectively. Product D had only 45.69% aggregated *E. coli* cells. Product E and Product A agglutinated nearly 70% (69.60% for Product E and 68.93% for Product A) *S. typhimurium* cells. Product F, Product B and Product C exhibited lower agglutination-

inducing activity than Product E and Product A with percentage of aggregated cells of 61.67%, 57.69% and 58.91%, respectively. As seen in the agglutination assay with *E. coli*, Product D was the product with the lowest agglutination-inducing activity that only 45.90% *S. typhimumrium* cells were aggregated. The variation of percentages of aggregated cells among different yeast products was due, in part, to the differences of percentages of mannan that each product contains.

Next, I determined whether MRF reduced an inflammatory response of the chicken macrophage cell lines exposed to LPS. Two chicken macrophage cell lines, MQ-NCSU and HTC, were cultured with medium, MRF, LPS, or MRF + LPS and their nitrite and IL-6 production as well as *iNOS* and *IL-6* gene expression were determined. The nitrite and IL-6 production of both cell lines treated with MRF did not exceed that of cells treated with medium alone indicating a non-pathogenic nature of MRF. In contrast, cells treated with LPS produced significantly higher amounts of nitrite and IL-6 production than cells cultured with medium alone. When cells were cultured with LPS in the presence of MRF, their nitrite and IL-6 production was significantly reduced. RT-PCR analysis also showed the reduction of *iNOS* and *IL-6* gene expression of cells treated with MRF + LPS compared to cells treated with LPS alone. These results indicated that MRF acts as an anti-inflammatory agent and the reduction also occured at the molecular level.

Based on the finding that MRF reduced the inflammatory responses at the transcriptional level, I asked whether MRF reduces those inflammatory responses by modifying some signaling molecules of an LPS signaling cascade. First I determined the effect of MRF on an expression of LPS receptor, TLR-4, utilizing a western blot

technique. My results indicated that at 0 and 5 hours post treatment, TLR-4 expression of the MQ-NCSU cell line treated with either MRF alone or MRF and LPS was markedly reduced or not detected compared to TLR-4 expression of cells treated with medium or LPS only. The reduction of TLR-4 expression that occurred also at 0 hour reflected the binding of MRF to TLR-4 that inhibited the binding of TLR-4 to its own ligand, a LPS, as well as interfered with TLR-4 antibody to bind to TLR-4 rather than the down-regulation of TLR-4 expression by MRF. By 24 hours, TLR-4 expression of cells from all treatments was detected, and the levels of TLR-4 expression among treatments were not drastically different. A newly synthesized or pre-existing TLR-4 released from MRF is possibly responsible for the detectable level of TLR-4 expression at 24 hours. The other molecule in an LPS signaling cascade that might be modified by an MRF is NF-κB. A gel shift analysis showed an increased level of NF-κB activation of MQ-NCSU cells treated with LPS as previously shown (Dil and Qureshi, 2002). Conversely, an activation of the same NF-κB dimer was markedly reduced in cells treated with MRF. In addition, a different set of NF-κB dimers with a larger size which was believed to be a p65/p65 homodimer was activated by MRF. This set of transcription factors may compete with a conventional NF-κB (p50/p65) to bind to the same DNA and blocked the transcription of that particular gene. Additionally, this NF-κB may bind to and activate different sets of genes whose products act to down-regulate inflammatory responses.

Frequently, an *in vitro* finding does not always translate to an *in vivo* system. Therefore, I conducted an *in vivo* experiment to test whether MRF reduces inflammatory responses in broiler chickens challenged with enteropathogenic (EPEC) *E. coli*. Broiler

chickens were divided into four groups. Two groups of birds were fed with a basal diet and one of these two groups was challenged with EPEC *E. coli*. The other two groups were fed with MRF and one of them was challenged with EPEC *E. coli*. Body weights and body temperature were measured weekly for three weeks. At three weeks of age, birds were killed 24 hours after Sephadex G50 recruitment of macrophages was initiated, and their abdominal macrophages were collected for nitrite and IL-6 production analyses. Body temperatures of birds challenged with EPEC *E. coli* were not significantly increased from non-challenged birds. In addition, clinical signs such as diarrhea or somnolence was not observed in challenged birds. There were two explanations for these results; 1) the number of bacteria given to the birds was too low or 2) this strain of *E. coli* did not cause an infection within the GIT. However, I find that the body weights of birds challenged with *E. coli* and received only a basal diet were reduced significantly signifying that GIT damage associated with the EPEC had occurred and that the EPEC challenge was in fact sufficient to affect the birds. Feeding birds with MRF was beneficial to them as indicated by a significantly higher body weights of challenged birds compared with the body weights of challenged birds that were fed with only a basal diet. In addition, nitrite production of Sephadex-elicited macrophages from non-challenged birds fed with MRF was significantly lower than nitrite production of macrophages isolated from non-challenged birds that were fed with only a basal diet. In contrast, the IL-6 production of macrophages isolated from non-challenged birds fed with MRF was slightly but significantly higher than that of the other three groups of birds. However, sephadex-elicited macrophages isolated from non-challenged birds that were fed with

only a basal diet treated with medium, MRF, LPS, or MRF and LPS exhibited cytokine responses similar to responses of chicken macrophage cell lines in terms of both nitrite and IL-6 production.

In conclusion, MRF is able to reduce inflammatory responses of macrophages elicited in response to LPS exposure or *E. coli* challenge both *in vitro* and *in vivo*. However, a time course response of IL-6 production as well as other pro-inflammatory cytokines of macrophages from birds should be investigated further to gain an insight of the effect of MRF on these pro-inflammatory cytokines and on the performance of birds.

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