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Actigen® Influence on the Gene Expression of Heat Shock Proteins in Ross 708 Broiler Chickens

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Abstract: In field trials, heat-exposed chickens given Actigen®, a second generation mannan oligosaccharide (MOS) from *Saccharomyces cerevisiae*, maintained good intestinal health and performance. This investigation explored the influence of Actigen® on heat shock protein (HSP) responses in Ross 708 broiler chickens. Gender-segregated broilers were given either a control or Actigen®-supplemented (800 g/ton in starter, 400 g/ton in grower and 200 g/ton in finisher) diet over a 6 week growing period. At 3 and 6 weeks of age, broilers of each gender on each diet were exposed to 41°C for 1 h in a temperature-controlled chamber while controls were maintained at 24°C. After heat exposure, liver and ileum tissues were collected and preserved in RNAlater for determination of gene expression via Real Time PCR. Significant differences in mRNA expression for HSP90A, HSP90AA and HSP90B due to gender were found in the ileum, but no gender-related differences for these HSPs were found in the liver. In all heat-exposed birds, gene expression was elevated for HSP90A, HSP90AA, HSP90B, HSP70 and HSP60 in both liver and ileum with males at 3 and 6 weeks of age showing the greater HSP response. Lower Actigen®-related HSP90AA and HSP90B mRNA expression in the liver suggested that Actigen® potentially modified HSP expression outside the intestinal tract. Actigen® mechanism (s) of action outside the intestine are equivocal, but they might be indirect. Lower HSP mRNA expression in Actigen®-fed birds indicated that the supplement can modify the HSP response while allowing continued good performance during heat-exposure.

Key words: Actigen®, heat shock protein, gene expression, chicken

INTRODUCTION

In the poultry industry, heat stress has long been a serious problem associated with decreased egg production, shell quality, body weight gain, feed conversion, increased susceptibility to disease and mortality and overall performance of (Gonzalez-Esquerra and Leeson, 2005; Rozenboim *et al*., 2007; Quinteiro-Filho *et al*., 2010; Soleimani *et al*., 2011). Extensive studies have demonstrated the many negative effects of exposure to high environmental temperature (Edens, 1976, 1977; Teeter and Smith, 1986; Cahaner *et al*., 1995). Attempts to ameliorate the effects of heat stress have largely focused on modifying the living environment of the birds. Furthermore, several attempts have been made to ameliorate heat stress via nutritional protocols and a variety of nutrients have been used to achieve that goal. Among the many nutritional approaches to reduce the effects of heat stress are supplementation of dietary vitamin C (Pardue *et al*., 1985; Mahmoud *et al*., 2003, 2004a; Attia *et al*., 2009), betaine (Attia *et al*., 2009); potassium chloride in drinking water (Ait-Boulahsen *et al*., 1995; Ahmad *et al*., 2008; Roussan *et al*., 2008), maintenance of dietary phosphorus (Mahmoud *et al*., 2004b), supplementation of organic selenium (Mahmoud and Edens, 2003, 2005), decreasing dietary calcium and fasting (McCormick *et al*., 1980; Ait-Boulahsen *et al*., 1993), protein (Cahaner *et al*., 1995), dietary vitamin E supplementation (Bollengier-Lee *et al*., 1998; Niu *et al*., 2009ab), vitamin and electrolyte supplementation via the drinking water (Ferket and Qureshi, 1992; Ahmad and Sarwar, 2006), carbonated water (Bottje and Harrison, 1985), betaine (Attia *et al*., 2009), aspirin and sodium bicarbonate (Roussan *et al*., 2008) and others with varying degrees of success. Thus, previous research has demonstrated that nutritional and dietary approaches to alleviate heat stress in chickens is feasible provided that the heat stressor is not of the lethal nature. Mechanisms associated with the many nutritional approaches to heat stress amelioration are not well understood.

Recently, anecdotal information from university trials (S. Collett, personal communication) with Actigen® (Alltech, Inc., Nicholasville, Kentucky 40356), a second generation yeast cell wall derivative from *Saccharomyces cerevisiae* that provides 2.5 fold more concentrated mannnan oligosaccharide (MOS) than its predecessor, BioMos® (Hooge and Connolly, 2011), suggested that broilers treated with Actigen® during a hot summer growing season maintained good performance and had fewer mortalities than did broilers not given Actigen® in their diet. Hooge and Connolly (2011) performed a meta-analysis of Actigen® effects in broiler

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chickens and reported that the feed supplement significantly increased 42 day body weight, improved feed conversion ratio or feed/gain ratio and decreased mortality. The improvement in performance data of Actigen®-fed broilers was not significantly different than performance of antibiotic-fed broilers of the same age. MOS has been shown to improve gut health in broilers, which was indicated by a significant increase in poult body weight (Bradley *et al*., 1994). MOS has been shown to improve body weights in laying hens subjected to chronic high ambient temperatures (Bozkurt *et al*., 2012). It has been reported that MOS in the gastrointestinal tract has the ability to bind to pathogenic bacteria since the mannose in the mannan oligosaccharide acts as a ligand that binds the type 1 fimbrae on certain pathogenic bacteria preventing their colonization and resultant damage in the intestine (Eshdat *et al*., 1978; Spring *et al*., 2000). Inhibition of colonization by pathogenic bacteria in the gastrointestinal tract will result in improved gut health and function in growing animals (Ferket *et al*., 2005). Collett (2005) reported that MOS, as a dietary additive, enhanced the protective antibody response yielding enhanced disease resistance and suppressed the acute phase (fever) response to bacterial challenge. Thus, in the healthy gastrointestinal tract, MOS will reduce the ever-present potential risk of bacterial infection. As a result, performance of broilers consuming MOS improves in the absence of stressors and maintains good performance in the presence of potential intestinal bacterial infections.

Stress is a condition induced by numerous types of stressors, including exposure to high temperature environments both acutely and chronically. One well documented response to exposure to high temperature is the induction of the heat shock response (Ritossa, 1962), which is characterized by rapid induction and expression of highly conserved polypeptides called heat shock proteins (HSP; Lindquist, 1986) ranging in molecular weight from 10-110 kDa.

Among the HSPs, the HSP70, HSP90 and HSP60 families of proteins have received the most interest in poultry species (Wang, 1992; Mahmoud, 2000). The HSP70 group aids in importation of several crucial proteins into the endoplasmic reticulum and nucleus (Schlesinger, 1990). The HSP70 proteins appear to be the most responsive to heat stress within an organism (Barral *et al*., 2004) and HSP70 has been shown to be very important to the development of thermotolerance in vertebrate animals including chickens (Wang, 1992; Mahmoud, 2000). The HSP90 family of proteins has two major functions, cell signaling and de-novo synthesis of certain proteins (Nathan *et al*., 1997). HSP90 exists in several forms HSP90A and HSP90B, which is an inducible form of HSP90 in chickens (Mahmoud, 2000). The HSP90 proteins function downstream to the HSP60 and HSP70 proteins (Miksa, 2005; Barral *et al*., 2004; Lin *et al*., 2007). HSP90 interacts with steroid receptor pathways (Hightower, 1991; Borrelli *et al*., 1996), especially in the glucocorticoid receptor pathway (Pratt and Toff, 1997). The HSP 60 family shields newly synthesized proteins during conformational folding (Bukau and Horwich, 1998). Some primary functions of the HSPs are service as molecular chaperones, aiding in the tertiary folding of the newly synthesized polypeptide chains, refolding of mis-folded proteins and aiding in the degradation of denatured proteins (Hightower, 1991; Barral *et al*., 2004). It has been suggested that the HSP60, HSP70 and HSP90 families of proteins interact to carry out their various cellular functions (Hendrick and Hartl, 1993; Morimoto *et al*., 1994).

The induction of HSPs in heat exposed chickens and turkeys has been studied extensively in our group. Acquisition of thermotolerance related to induction of HSP70 and HSP90 in association with heat conditioning was demonstrated in both chickens and turkeys (Wang and Edens, 1994, 1998). Thermotolerance was evidenced by HSP production plateau and then decreased HSP70 production after sequential exposures to high ambient temperature episodes, which signaled a decreased need for the HSP70 protection after heat conditioning (Wang and Edens, 1998). Since Actigen® appears to play some role in maintenance of chicken performance, during growth in a hot summer environment via an undefined mechanism, the idea, that the Actigen® feed additive might affect on a molecular basis the expression of HSPs, was addressed. This study focused on the effect of dietary Actigen® supplementation on expression of HSP 70, HSP 90 and HSP 60 induction in the ileum and liver in response to an acute, mild heat stressor.

MATERIALS AND METHODS

Animal welfare: 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.

Animals and husbandry: At hatch, 160 Ross 708 broiler chicks were obtained from a commercial hatchery (Mountaire Hatchery, Siler City, NC). The day old chicks were sorted by gender by feather sexing providing 80 males and 80 females. The chicks were weighed, neck banded and placed into separate pens by treatment group. There were 8 pens with 10 chicks per pen per room consisting of two male control diet pens, two female control diet pens, two male Actigen® diet pens and two female Actigen® diet pens.

Treatment pens were assigned randomly within each of the two identical environmentally-controlled rooms. Each

room contained 8 (4'X3') pens with a 250 watt heat lamp shared between 2 pens and had approximately 5 inches of new pine wood shavings as bedding. The rooms were kept at a constant 30°C for the first week, reduced to 28°C for the second week, reduced to 26°C for the third week and reduced to 24°C for the remainder of the trial. The chicks were kept on 24 h of incandescent light for the first 3 weeks and then were placed on 23 h of light and 1 h dark for the last 3 weeks. Feed was dispensed from a single hanging tube feeder and water was provided in a single plastic water trough in each pen.

Diets: The birds were fed 3 different diets consisting of the North Carolina Agriculture Research Service starter (3035 kcal/kg, 22.855% CP; 2 pounds per bird), grower (3100 kcal/kg, 20% CP; 6 pounds per bird) and finisher (3150 kcal/kg, 18% CP; 8 pounds per bird) that met all nutritional requirements for the Ross 708 broiler. To the experimental groups, Actigen® (Alltech Inc. Nicholasville, KY 40356) was supplemented at the level of 800 g/ton to the starter, 400 g/ton to the grower and 200 g/ton to the finisher.

Sampling: At 3 and 6 weeks of age, 12 birds, taken randomly from the treatment pens were then randomly placed into groups of 6 for either non-heat-exposed control or heat exposed groups. The non-heat-exposed control and heat-exposed birds were placed into a coop without feed or water. The heat-exposed birds then were placed in the heat chamber that had a preset temperature of 41°C for a period of 1 h and the non-heat exposed groups were held in the coops at 24°C for 1 h. After the heat exposure, birds were euthanized via cervical dislocation, which was followed by collection of liver and ileum tissue samples. Tissue samples were saved in 5 mL of RNAlater (Life Technologies, Grand Island, NY) for mRNA extraction.

Messenger RNA extraction: Approximately 0.1 g of each tissue sample was homogenized by bead beating using a mini bead-beater (Biospec, Bartlesville, OK) and RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Germantown, MA) according to manufacturer protocol. Briefly, tissue was homogenized in kit Buffer RLT and then centrifuged to pellet any remaining tissue debri. Supernatant was mixed with 70% ethanol and loaded on to the RNeasy column. The column was subsequently washed with kit buffers RW1 and RPE. The mRNA samples were eluted from the column in 50 µL of nuclease-free water and were scanned at 460 nm and 480 nm wavelengths (Nano-Drop 2000 Spectrophotometer, Thermo-Scientific) to determine the concentration and purity of the extracted mRNA.

A quantity of 500ng of each RNA sample was denatured in a 96 well Thermocycler (Applied Biosystems, Carlsbad, CA) at 65°C for 15 min and run on a 1% agarose gel containing ethidium bromide. The gel was then visualized on a UV light box to evaluate RNA quality by the appearance of the intact bands of the 28S and 18S ribosomal subunits. Non-degraded RNA was then used to synthesize cDNA for use in real time PCR.

Table 1: Primers used for real time PCR and gene expression

Primer	Sequence
HSP70 forward	GCGGAGCGAGTGGCTGACTG
HSP70 reverse	CGGTTCCCCTGGTCGTTGGC
HSP90A forward	CCTCCTCCATACGTGATGTGTCA
HSP90A reverse	GCCTGGGCATTGATGAAGATG
HSP90B forward	GGCTCTGGCATGCACGCTTC
HSP90B reverse	TCTTCCACGGTCGCATCCACA
HSP90AA forward	CCCCAGACACATGCCAACCGC
HSP90AA reverse	AGGACTGGCCTCCTCAGCAGC
HSP60 forward	GCAGATGCCGTAGCTGTCACCA
HSP60 reverse	TGGGACTCCCCCAGCTTTGTT

cDNA: A quantity of 1ug of each RNA sample was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster city, CA). The 20 µL cDNA synthesis reaction contained in addition to the RNA template, 2 µL of 10x RT buffer, 0.8 µL of 25XdNTP mix, 2 µL 10x RT Random Primers and 1 µL MulitScribe Reverse Transcriptase. A volume of 10 µL of nuclease-free water was added bring the reaction up to final volume.

Real time PCR: The 6 cDNA samples from each treatment group were combined in one of two pools, each pool containing 3 samples. Pools were diluted 1:20 with nuclease free water for use as template in real time PCR reactions.

Primer pairs for five HSP genes were designed for use in real time PCR (Table 1). All real time reactions consisted of 1 µL of cDNA, 1 µL of primer, 10 µL of Power SYBR green mastermix (Applied Biosystems, Foster city, CA) and 8 µL nuclease-free water. Initial denaturation of 95°C for 7 min followed by 40 cycles of 95°C denature for 30 sec, (empirically determined) annealing temperature for 30 sec and 72°C extension for 30 sec, a final 72°C extension for 5 min and a melt curve consisting of 95°C for 1 min followed by 55°C initially for 1 min with the temperature increasing by 0.5°C each cycle for 80 cycles.

Statistics: Gene expression values, reported as threshold cycle (CT), were subjected to multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA), respectively, to examine the main effects of Actigen® diet, age, heat exposure, gender and interactions of main effects using the Statistical Analysis System-JMP® 10 program (2012; SAS, Cary, NC). Individual contrasts were made via comparison of means using the Tukey-Kramer procedure in JMP® 10 (2012; SAS, Cary, NC). Significance levels were set at $p = 0.05$.

RESULTS

Ileum heat shock protein responses

Diet effect: Actigen® supplementation to the diet did not influence HSP mRNA expression for any HSP under study in the ileum of the Ross 708 broilers (Table 2). Yet there were significant diet X gender interactions detected for HSP90AA and HSP60 (Table 2). The significant HSP90AA interaction was attributed to the control-fed females having lower mRNA expression than control-fed males while there was no difference between genders in the Actigen®-fed groups and this was due to control-fed females at 6 weeks of age having the lowest mRNA HSP90AA expression among all groups. The significant HSP60 diet X gender interaction was due to 3 weeks old control-fed males expressing greater HSP60 mRNA levels than all other birds among the various treatment groups.

Age effect: A significant age effect was noted for HSP90AA, HSP60, HSP70 and HSP90A responses in the ileum. For HSP90AA mRNA levels, the 3 weeks old birds had the greater levels compared with the 6 weeks old birds (Tables 2 and 3), which was apparent in the males compared with the females. The HSP60 age effect was attributed to the 3 weeks old birds expressing more HSP60 mRNA than the 6 weeks old birds (Table 2 and 3), which was a male response. The HSP70 mRNA levels were significantly greater in the 3 weeks old birds than in the 6 weeks old birds (Table 2 and 3), which was also due to males at both ages. The 3 weeks old birds had greater HSP90A mRNA levels than did the 6 weeks old birds (Table 2 and 3), which was due to males having the greater expression levels at 3 weeks of age compared with females.

Temperature effect: A significant temperature exposure effect was found in HSP mRNA expression levels (Table 2). Heat exposure induced significantly greater mRNA expression for HSP90B, HSP90AA, HSP60, HSP70 and HSP90A (Table 2 and 3).

Gender effect: A significant gender effect was found for mRNA expression for HSP90B, HSP90AA and HSP90A (Table 2). Males expressed more mRNA for HSP90B, HSP90AA and HSP90A at both 3 and 6 weeks of age than did the females (Table 3).

Liver heat shock protein responses

Diet effect: A significant diet effect was found for liver mRNA HSP90B, HSP90AA, HSP60 and HSP70.

Table 2: Multivariate analysis of variance (MANOVA) p-values* of HSP expression in the ileum of Ross 708 broiler chickens

	HSP90B	HSP90AA	HSP60	HSP70	HSP90A
Diet (Actigen®)	-	-	-	-	-
Age	-	0.0010*	0.0259*	0.0097*	0.0302*
Heat/No heat	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
Gender	0.0002*	0.0014*	-	-	<.0001*
Diet X Heat	-	-	-	-	-
Diet X Gender	-	<.0001*	0.0411*	-	-
Diet X Gender X Heat	-	-	-	-	-

*p-values from MANOVA analysis of HSP expression in the ileum of 3 and 6 week broilers. Lack of a p-value (-) denotes no significant difference

Table 3: Treatment means (CT) and pooled standard error of means (SEM) for the ileum HSP90B, HSP90AA, HSP60, HSP70 and HSP90A gene expression for 3 and 6 weeks old male and female Ross 708 broiler chickens fed Actigen® and exposed acutely to high ambient temperature (41°C) for 1 h

Treatment group	Age in weeks	HSP90B	HSP90AA	HSP60	HSP70	HSP90A
FAH[1]	3	10.13$^{abcd, 2}$	9.70^{e}	13.89^{ef}	11.69^{cd}	7.05^{c}
FAN	3	10.67^{abc}	13.37^{ab}	15.20^{ab}	16.45^{ab}	10.85^{a}
FCH	3	10.05^{bcd}	9.44^{ef}	14.21^{cde}	9.04^{de}	6.80^{c}
FCN	3	10.84^{ab}	13.57^{a}	15.43^{a}	16.77^{ab}	10.53^{ab}
MAH	3	10.02^{bcd}	9.99^{e}	14.45^{bcde}	10.66^{cde}	6.77^{c}
MAN	3	10.39^{abc}	12.94^{abc}	15.01^{abc}	16.49^{ab}	9.61^{b}
MCH	3	9.43^{d}	8.77^{f}	13.28^{f}	7.43^{e}	5.45^{d}
MCN	3	10.49^{abc}	12.55^{c}	14.85^{abcd}	17.25^{a}	9.85^{ab}
FAH	6	10.07^{bcd}	9.95^{e}	14.09^{def}	10.92^{cd}	7.14^{c}
FAN	6	10.32^{abcd}	12.69^{bc}	15.44^{a}	16.20^{ab}	10.07^{ab}
FCH	6	10.43^{abc}	11.30^{d}	14.46^{bcde}	13.49^{bc}	7.86^{c}
FCN	6	11.02^{a}	13.35^{ab}	15.16^{ab}	16.83^{a}	10.26^{ab}
MAH	6	9.84^{cd}	10.10^{e}	13.87^{ef}	10.90^{cd}	7.41^{c}
MAN	6	10.24^{abcd}	13.17^{abc}	15.43^{a}	16.83^{a}	9.97^{ab}
MCH	6	10.02^{bcd}	10.17^{e}	14.54^{bcde}	11.55^{cd}	7.21^{c}
MCN	6	10.32^{abcd}	12.61^{bc}	15.08^{abc}	16.91^{a}	9.44^{b}
POOLED SEM		0.17	0.16	0.17	0.65	0.33

[1]; a,b,c,d,e,f In a column, means not connected by a common lower case superscript letter differ significantly (p = 0.05)

[2]FAH-female, Actigen®-fed, heat-exposed
FAN-female, Actigen®-fed, no heat exposure
FCH-female, control diet, heat-exposed
FCN-female, control diet, no heat exposure
MAH-male, Actigen®-fed, heat-exposed
MAN-male, Actigen®-fed, no heat exposure
MCH-male, control diet, heat-exposed
MCN-male, control diet, no heat exposure

Table 4: Multivariate analysis of variance (MANOVA) p-values* of HSP expression in the liver of Ross 708 broiler chickens

	HSP 90B	HSP 90AA	HSP 60	HSP 70	HSP 90A
Diet (Actigen)	0.0038*	0.0040*	0.0353*	0.0211*	-
Age	<.0001*	-	-	-	-
Heat/ No heat	-	<.0001*	0.0002*	<.0001*	<.0001*
Gender	-	-	-	-	-
Diet X Heat	-	-	-	-	-
Diet X Gender	0.0361*	-	-	0.0360*	-
Diet X Gender X Heat	-	-	-	0.0023*	-

*p-values from MANOVA analysis of HSP expression in the ileum of 3 and 6 week broilers. Lack of a p-value denotes no significant difference

Table 5: Treatment means (CT) and pooled standard error of means (SEM) for the liver HSP90B, HSP90AA, HSP60, HSP70 and HSP90A gene expression for 3 and 6 weeks old male and female Ross 708 broiler chickens fed Actigen® and exposed acutely to high ambient temperature (41°C) for 1 h

Treatment group	Age in weeks	HSP90B	HSP90AA	HSP60	HSP70	HSP90A
FAH[1]	3	14.83^{a,2}	10.10^{f}	11.36^{de}	9.36^{bc}	6.27^{c}
FAN	3	13.86^{ab}	13.53^{abcd}	12.33^{abcde}	15.15^{a}	9.43^{a}
FCH	3	12.20^{bc}	9.71^{f}	11.55^{cde}	7.68^{c}	6.68^{bc}
FCN	3	10.75^{cd}	12.43^{a}	11.88^{bcde}	15.02^{a}	8.56^{a}
MAH	3	11.58^{cd}	11.57^{de}	12.85^{abcd}	9.09^{bc}	6.26^{c}
MAN	3	11.73^{cd}	13.74^{a}	13.28^{ab}	15.65^{a}	8.92^{a}
MCH	3	11.48^{cd}	10.11^{ef}	12.23^{abcde}	7.35^{c}	6.15^{c}
MCN	3	11.33^{cd}	12.00^{bcd}	11.98^{abcde}	14.92^{a}	8.13^{ab}
FAH	6	10.14^{d}	9.76^{e}	12.13^{abcde}	8.92^{bc}	6.18^{c}
FAN	6	10.94^{cd}	12.48^{abcd}	12.58^{abcde}	13.86^{a}	8.60^{a}
FCH	6	10.08^{d}	9.76^{e}	11.63^{bcde}	10.14^{b}	5.80^{c}
FCN	6	11.34^{cd}	11.59^{cd}	12.43^{abcde}	14.49^{a}	9.56^{a}
MAH	6	10.94^{cd}	9.79^{f}	11.19^{e}	9.02^{bc}	5.91^{c}
MAN	6	11.75^{cd}	13.06^{abc}	13.08^{abc}	15.49^{a}	9.31^{a}
MCH	6	10.44^{cd}	8.98^{f}	11.03^{e}	7.39^{c}	5.30^{c}
MCN	6	11.70^{cd}	12.18^{abcd}	13.65^{a}	15.42^{a}	9.04^{a}
POOLED SEM		0.38	0.30	0.32	0.43	0.33

[1]: a,b,c,d,e In a column, means not connected by a common lower case superscript letter differ significantly (p = 0.05)

[2]FAH-female, Actigen®-fed, heat-exposed
FAN-female, Actigen®-fed, no heat exposure
FCH-female, control diet, heat-exposed
FCN-female, control diet, no heat exposure
MAH-male, Actigen®-fed, heat-exposed
MAN-male, Actigen®-fed, no heat exposure
MCH-male, control diet, heat-exposed
MCN-male, control diet, no heat exposure

Actigen®-fed broilers had significantly lower mRNA expression for these HSPs compared with control-fed birds (Table 4 and 5).

Additionally, there were significant diet X gender interactions for liver HSP90B and HSP70 mRNA expression levels (Table 4). The interaction for HSP90B was attributed to Actigen® -fed male broilers having greater HSP90B mRNA levels than females, which had the least HSP90B mRNA levels among all groups. The HSP70 diet X gender interaction was attributed to Actigen®-fed males having less HSP70 mRNA expression than the females and control-fed birds (Table 5).

Age effect: A significant age effect on HSP mRNA expression in the liver was found only for HSP90B (Table 4 and 5). The 6 weeks old broilers had significantly greater HSP90B mRNA expression than did the 3 weeks old broilers (Table 5) and this difference was due to 3 weeks old females having significantly lower HSP90B expression than found in all other groups.

Temperature effect: A significant temperature exposure effect was found for mRNA expression levels for HSP90AA, HSP60, HSP70 and HSP90A (Table 4). In each instance, heat exposure induced a significant increase in the mRNA expression for each HSP family of proteins (Table 5).

Gender effect: There were no gender main effect differences for any of the liver HSP families of proteins (Table 4). However, there was a diet X gender X temperature interaction for liver HSP70 mRNA expression (Table 4). In both Actigen®-fed and control-fed broilers, heat exposure induced a significant increase in liver HSP70 mRNA expression, but Actigen®-fed broilers had lower heat-induced HSP70 mRNA than did heat-exposed control-fed broilers, which was especially noticeable in male Actigen®-fed broilers exposed to heat than in the control-fed heat-exposed males, while there was no difference between Actigen®-fed and control-fed female broilers exposed to high temperature.

DISCUSSION

Heat stress is a common, unavoidable problem in commercial poultry production on a worldwide basis and has received an extraordinary amount of study directed at development of managerial and nutritional protocols

to alleviate the problem in broilers and other classes of commercial poultry (McCormick *et al*., 1980; Bottje and Harrison, 1985; Pardue *et al*., 1985; Ferket and Qureshi, 1992; Ait-Boulahsen *et al*., 1993, 1995; Cahaner *et al*., 1995; Bollengier-Lee *et al*., 1998; Mahmoud and Edens, 2003, 2005; Mahmoud *et al*., 2003, 2004ab; Ahmad and Sarwar, 2006; Ahmad *et al*., 2008; Roussan *et al*., 2008; Attia *et al*., 2009; Niu *et al*., 2009ab). Results from the many studies dealing with acute and chronic heat exposures have continued to reveal the many intricacies of heat distress in poultry and have led to development of many strategies to help in the struggle to maintain good performance in those animals subject to high temperature exposure. However, the fundamental basis of heat stress still eludes scientists around the world.

Heat stressors are classical inducers of non-specific physiological responses in birds and other animals causing depression or inhibition of growth, production and livability. There are many physiological changes that occur in a bird experiencing heat stress (Edens, 1977; Yahav *et al*., 1995). Among the physiological responses associated with acute and chronic heat distress are hormonal responses, which predominantly affect the adrenal (Edens and Siegel, 1975, 1976) and thyroid (Fox, 1980; May, 1982; May *et al*., 1986) functions. A large body of evidence clearly indicates that there is an intimate relationship between HSP90 and steroid hormone receptor functions (Pratt and Toth, 1997), but the relationship of these hormone receptors with heat shock proteins is not limited to HSP90 since HSP70, HSP60 and possibly others are involved with assembly of the receptors and their components (Pratt and Toft, 1997). Indeed, HSP genes are sensitive to various stimuli such as light and even thyroid hormones (Graham *et al*., 2009). Graham *et al*. (2009) observed that photo-stimulation and the thyroid hormone responsive HSP90B1 gene, which encodes HSP108, are involved in photoperiodic signaling in chickens. Thus, the idea that several HSPs are involved in facilitating homeostatic processes in normal and stressed cells is a powerful concept.

It is well documented that a thermal stressor induces in chickens rapid synthesis of HSPs in all tissues (Wang, 1992; Wang and Edens, 1998, 2008; Leiw *et al*., 2003, Yu *et al*., 2008). In this current investigation, we have demonstrated that a short exposure of only 1 h to a 41°C challenge was sufficient to induce significant elevations in the heat shock proteins in the liver and the ileum. There were significant increases in the expression of HSP genes (HSP60, HSP70, HSP90, HSP90A, HSP90AA and HSP90B) in the ileum and liver due to heating. This is in agreement with past publications on HSPs, which demonstrated significantly increased expression of HSPs in acutely heat-exposed animals attempting to protect the organism and its genome from the thermal stressor (Pauli and Tissieres, 1990; Fayet *et al*., 1989; Wang and Edens, 1998, 1994, 2008). Mahmoud (2000) reported that HSP90B is normally found at higher levels than most HSPs at normal temperature and only slightly induced by heat exposure and results from our study demonstrated a similar response in the liver of heated chickens. However, for all the other HSPs, the relative increase in mRNA expression was greater in the ileum than in the liver (Table 3 and 5).

Earlier research suggested that gender plays a role in many aspects of an organism's response to stressors. This sexual dimorphic difference has been attributed to glucocorticoid and steroid hormones (Wang and Edens, 2008). HSPs have been found to interact with the hormone binding domain (HBD) of the nuclear receptors and the HSPs aid in the folding of the HBD to form a conformation that has a very high binding affinity for steroid hormone nuclear receptors (Pratt and Toft, 1997). Male broilers showed a significant increase in the expression levels of HSP90A, HSP90B and HSP90AA in the ileum, which differed from the female heat shock responses. Wang (1992) demonstrated that there were significant interactions between steroid hormones and HSPs and that both testosterone and estrogen levels played significant roles in HSP production in male and female broilers, respectively, when they were subjected to a heat stressor. A number of studies have shown that the HSP90 family is intimately involved with steroid hormone receptors in animals (Wang and Edens, 2008; Lin *et al*., 2007; Barral *et al*., 2004; Schlesinger, 1990; Hightower, 1991).

Actigen® is a second generation MOS product derived from yeast outer cell walls from *Saccharomyces cerevisiae*, which constitutes the first generation BioMos® (Hooge and Connolly, 2011). BioMos® has been shown to improve gut health in broilers, illustrated by a significant increase in turkey poult body weight associated with increased villi length and area and decreased number of goblet cells (Bradley *et al*., 1994). BioMos® has also been shown to help ameliorate some of the oxidative damage that results from heat stress in broiler chicks (Sohail *et al*., 2011). The mechanism of this beneficial effect is not yet clear, but it is believed to be associated with MOS-associated increased numbers of probiotic gut microbes, which potentially release bioactive substances that could prevent oxidative damage (Sohail *et al*., 2011). However, in this current research, a biological challenge was not imposed on the broilers, but we still observed a significant influence of Actigen® on either the induction or suppression of various HSPs. There was significantly lower HSP90B, HSP90AA and HSP70 mRNA expression in the livers related to dietary Actigen® supplementation, which is very surprising since mannan from MOS is not absorbed efficiently in chickens (Collett, 2005). Nevertheless, one can not discount the possibly of partial absorption of the oligosaccharides, which can result in local and systemic

responses (Seifert and Watzl, 2007) as suggested in this investigation by the HSP responses in the liver.

In the ileum, there were no significant HSP responses associated with dietary Actigen® supplementation. However, HSP expression levels in the ileum tended to be higher for all of the HSP genes compared with that found in the liver (Table 3 and 5 for ileum and liver, respectively) and the heat-induced HSP response in the ileum was relatively less than in the liver. These observations suggest that Actigen® may be able to lower the maximal expression levels of HSPs in poultry that undergo exposure to a heat stressor while still allowing for the development of thermotolerance, which is associated with heat-induced HSP expression.

An explanation for these observations may be that the higher constitutive HSP mRNA in the ileum is due to the fact that the microenvironment in the lumen of the ileum is inherently more stressful than in the microenvironment in liver tissue. Wang (1992) has shown that there is a negative effect of high constitutive HSP expression on the induced HSP due to exposure to a stressor. Therefore, the relatively small Actigen®-related changes in the HSP responses in the ileum might be indicative of a negative feedback on HSP mRNA expression and conversely, the relatively higher changes in the HSP responses in the liver may also be reflecting a less stressful status in the liver of non-heated exposed broilers. It has been reported that MOS affects many pathways where multiple heat-related changes could occur (Xiao *et al*., 2012). This change might be related to secondary effects in the liver, which allowed for decreased Actigen®-related HSP production in the liver but not in the ileum.

There were significant differences in gene expression of HSP90AA, HSP60 and HSP70 in the ileum and HSP90B and HSP90AA in the liver due to an age difference with the 3 week birds having the highest expression levels of HSP mRNA. This could be due to 3 week old birds being less developed and having less cellular stress memory than older birds. Conversely, Wang and Edens (1998) suggest that as broilers and turkeys undergo repeated exposures to a stressor, the animals become conditioned to the stressor and require less induction of HSP. Thus, older birds may mount less of a HSP response. Mahmoud (2000) stated that an animal's heat shock response is based upon several factors, one being the age at which the animal is exposed to the stressor. Early exposure to a stressor may provide enhanced heat shock responsiveness (Wang and Edens, 1998).

The mechanism(s) played by dietary Actigen® in thermotolerance in heat exposed poultry still requires elucidation. Xiao *et al*. (2012) examined microarray data from the jejunum of MOS-fed broilers and determined that MOS had a major role in regulating genes associated with protein synthesis. This is a relevant observation as it applies to this current investigation. Wang (1992) observed that induction of high level of HSP expression due to heat exposure caused a near complete cessation of other cellular protein synthesis. In our study, we noted that heat exposure caused an increase in HSP induction in the liver, but Actigen® supplementation acted to prevent maximal liver HSP expressions compared with control-fed broilers. This observation would appear to contribute to the improved performance of Actigen®-supplemented broilers exposed to a thermal stressor. However the pathways affected by Actigen® supplementation encompass many different physiological functions of the bird making it difficult to establish a single Actigen®-associated mechanism that leads to improved performance (Xiao *et al*., 2012). The fact that BioMos® improves gut integrity and function (Bradely *et al*., 1994) via a prebiotic control of potentially pathogenic bacteria (Baurhoo *et al*., 2007; Sims *et al*., 2004), it is reasonable to assume that reduced stress in the gastrointestinal tract might be translated into decreased stress at the whole organism level. From the results of this study, it is evident that there is interaction between dietary Actigen® supplementation and lower HSP induction in the liver as noted in this investigation. Since stressors of various origins can induce the different HSP families of proteins (Lindquist, 1986; Schlesinger, 1990; Hightower, 1991), it is possible that local interaction of potentially pathogenic bacteria at the level of intestinal enterocyte receptor complexes might be sufficient to cause a state of alarm that could result in a system heat shock response, especially if bacterial toxins become systemic thereby inducing several HSP inductions. Actigen® supplementation, acting to bind bacterial mannose receptors (Spring *et al*., 2000), would effectively reduce the bacterial interaction on the enterocyte resulting in a decreased systemic stress response as suggested by lower constitutive HSP in non-heated Actigen®-fed broilers and less induction in the liver of Actigen®-fed broilers subjected to a heat stressor compared with control-fed heat exposed broilers.

Conclusion and recommendation: Dietary Actigen® supplementation is a good managerial aid in reducing the effects of high temperature exposure in broiler chickens. Actigen® has many beneficial influences and among these is the modulation of the heat shock response so that maximal/over expression of the HSPs does not become a critical problem that could result in inhibition of cellular synthesis of other proteins. The beneficial effects of Actigen® probably are not due to a direct anti-stress effect on body tissues but might in fact be due to its ability to reduce/prevent colonization by potential pathogens within the gastrointestinal tract,

which then results in less local stress that does not translate as a non-specific systemic stress characterized by the very basic cellular heat shock response.

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