Egg shell membrane improves immunity of post hatch poultry: a paradigm for nutritional immunomodulation

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Introduction

- The main purpose of antibiotic alternatives (ATA) are to protect against opportunistic pathogens that may otherwise cause infection, prevent growth, productivity, and make food unsafe
- ATA functional categories (a) which provide transient protection and (2) those which provide long lasting protection
- This later category can be further divided into 2 subcategories with respect to their protective action; enhancing specific or overall immunity ..

Egg shell membranes (ESM)

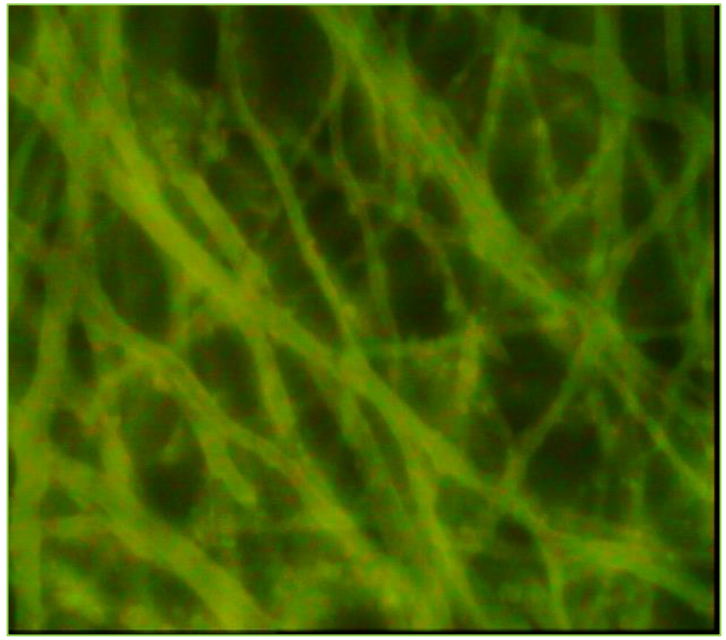
- ~90 billion eggs produced annually in USA, the shell byproducts are largely wasted

Fresh egg shell membrane

Hatchery egg shell membrane (HESM)

Egg shell membrane

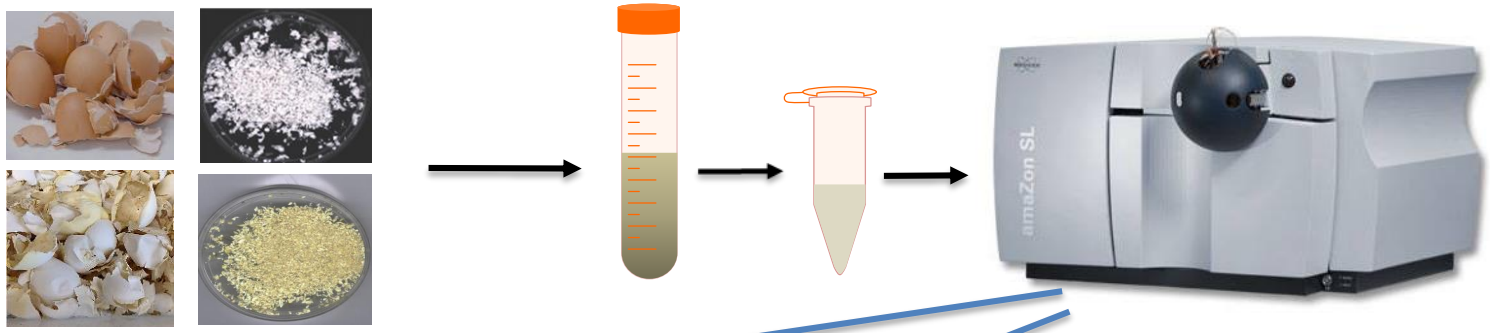
Fresh membrane yield/egg: 0.2-0.25 g
88% of dry weight of ESM is protein
Membranes are largely trypsin resistant

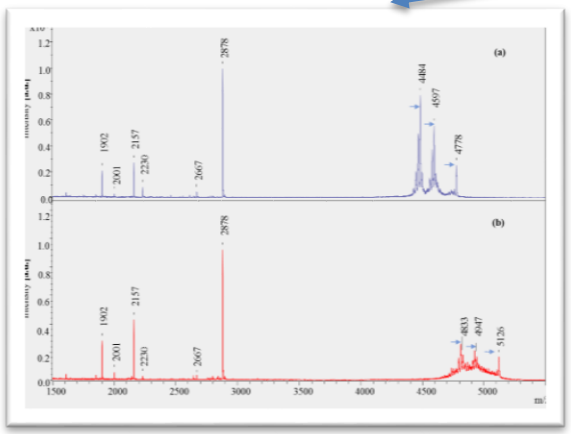


FITC-stained egg shell membrane

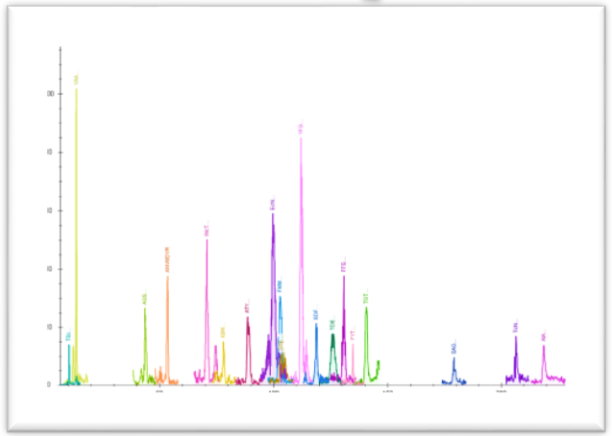
Powdered ESM flakes

Mass spectrometry analysis of proteins in the shell membranes

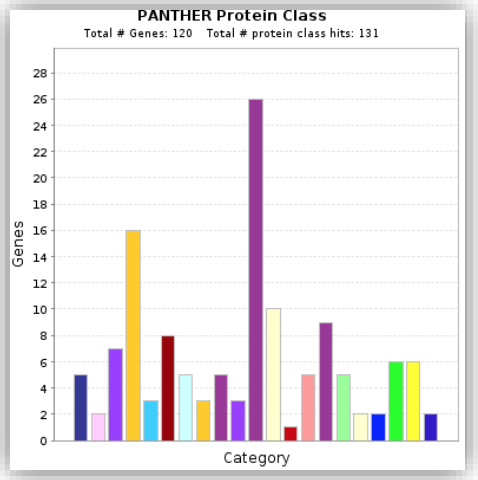




MALDI-MS of Methanol Extract



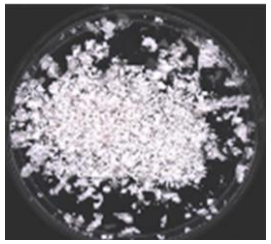
LC-Tandem mass spectrometry of tryptic digest of extracted proteins



Bioinormatics

Proteins and peptides of ESM

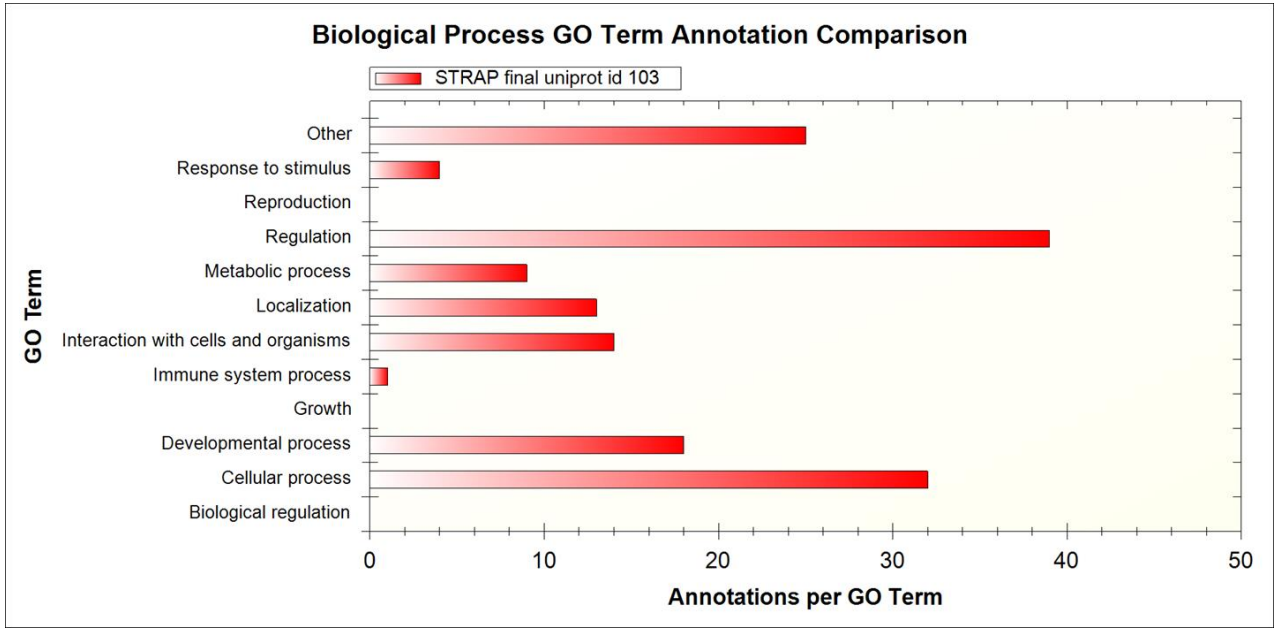
Fresh membrane



Total # Proteins identified => 250

Hatchery shell membranes

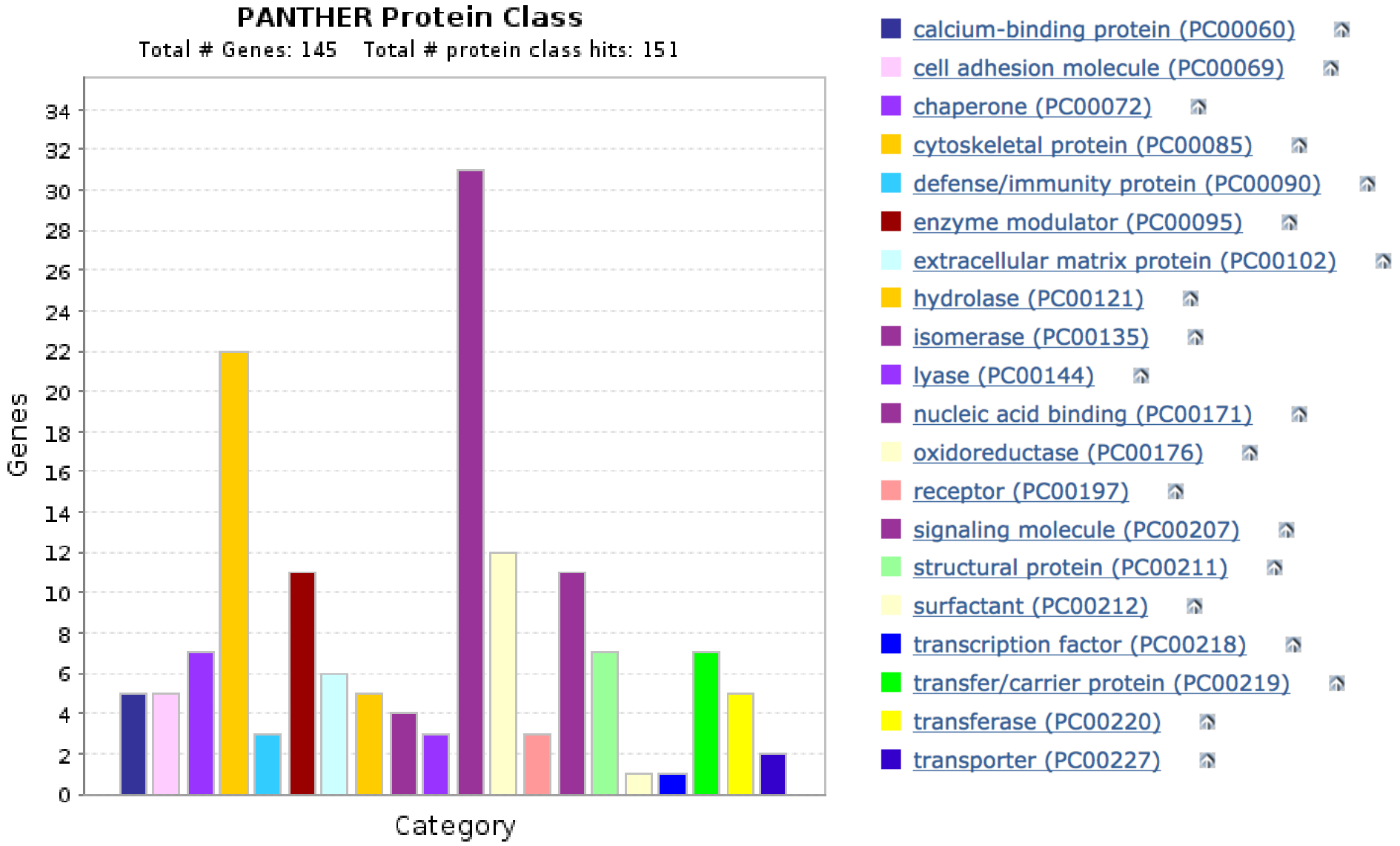
Total # Proteins identified = 167



→ Cellular regulation and developmental related proteins dominate

Makkar et al., 2015, J Agric Food Chem, 63:9888-98

HESM Proteins



Functional annotation of proteins by PANTHER identified by protein class

Proteome Science, Submitted

Examples of some major egg shell membrane associated proteins and peptides

- **Antimicrobial proteins and peptides:** ovoransferin, gallinacin, lysozyme, ovocleidin, ovomucin, keratin peptides, gallin (ESM)….
- **Cytoskeletal andextracellular matrix Proteins:** actin and its binding cognate proteins, collagens, aggrecan, keratins
- **Motor proteins**: Titin and Titin-like motor complex, dynein, nebulin
- **Heat shock and adhesion proteins:** HSP90, HSP70; cadherin
- **Carbohydrate and nucleic acid metabolism enzymes and proteins:** Ribonucleoproteins, histones, elongation factors, ribosomal proteins
- **Enzymes and enzyme inhibitors** signal peptidase, orsomucoid
- **Polyubiquitin-B Ubiquitin , Protein degrading enzymes** (HESM)
- **Blood associated proteins and peptides:** (HESM), α-D-globin
- **Growth factors:** FGF, thymosin β4

Gallus and bacterial proteins identified in HESM extract

- **Total # guanidine HCl extracted proteins=167**
- **Methanol extracted proteins and peptides identified =42, 1 uncharacterized**
- **Bacterial proteins identified =50**
- *Candidatus, Pseudomonas, Enterococci, Yersinia, and Butyrivibrio, and several phytobacteria (Sphingomonas taxi), Nocardioide (soil bacteria), and marine bacteria (hyphomonas, marinobacter adhaerens), and*
- *Apicomplexan specific protein from Eimeria*

Experiment 1

2 weeks

Control Diet
or
Diet + ESM (0.2 or 0.4%)

1 week

Control Diet

Growth parameters
Hematology
Clinical chemistry
Serum IgG and IgM
Corticosterone

Makkar et al. Poult Sci. 2015 94:1184-9

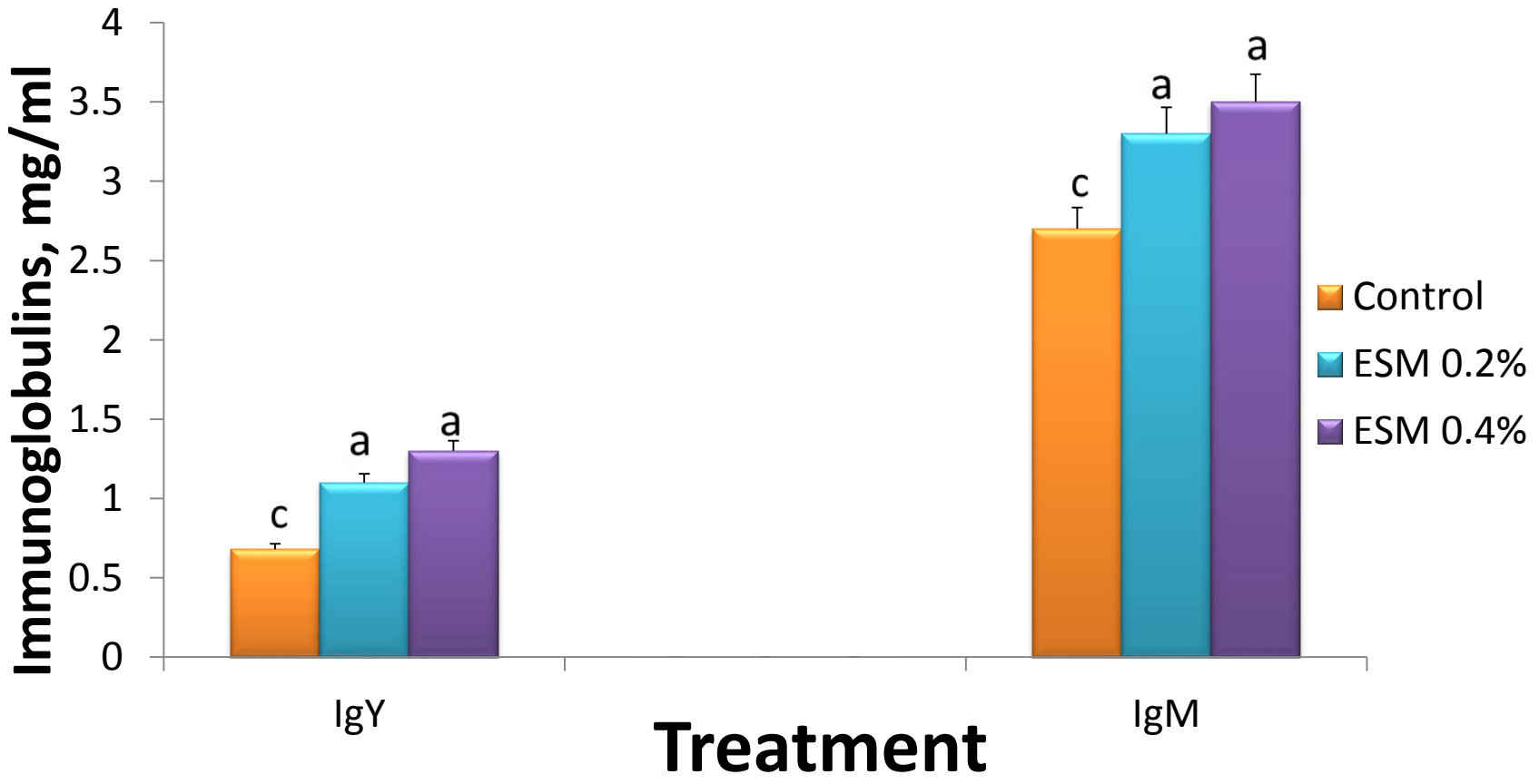
BW and the relative organ weights of chicken fed diets with and without eggshell membrane (ESM) (n = 16)

Parameters	Control	+ESM 0.2%	+ESM 0.4%
Body weight (g)	905.38 ± 18.35^{b}	967.50 ± 12.97^{a}	958.00 ± 16.89^{a}
Relative heart weight (%)	0.50 ± 0.01^{a}	0.54 ± 0.02^{a}	0.54 ± 0.01^{a}
Relative liver weight (%)	2.21 ± 0.07^{a}	2.46 ± 0.20^{a}	2.25 ± 0.05^{a}
Relative spleen weight (%)	0.08 ± 0.01^{a}	0.10 ± 0.01^{a}	0.11 ± 0.01^{a}
Relative bursa weight (%)	0.16 ± 0.01^{a}	0.17 ± 0.01^{a}	0.20 ± 0.01^{a}

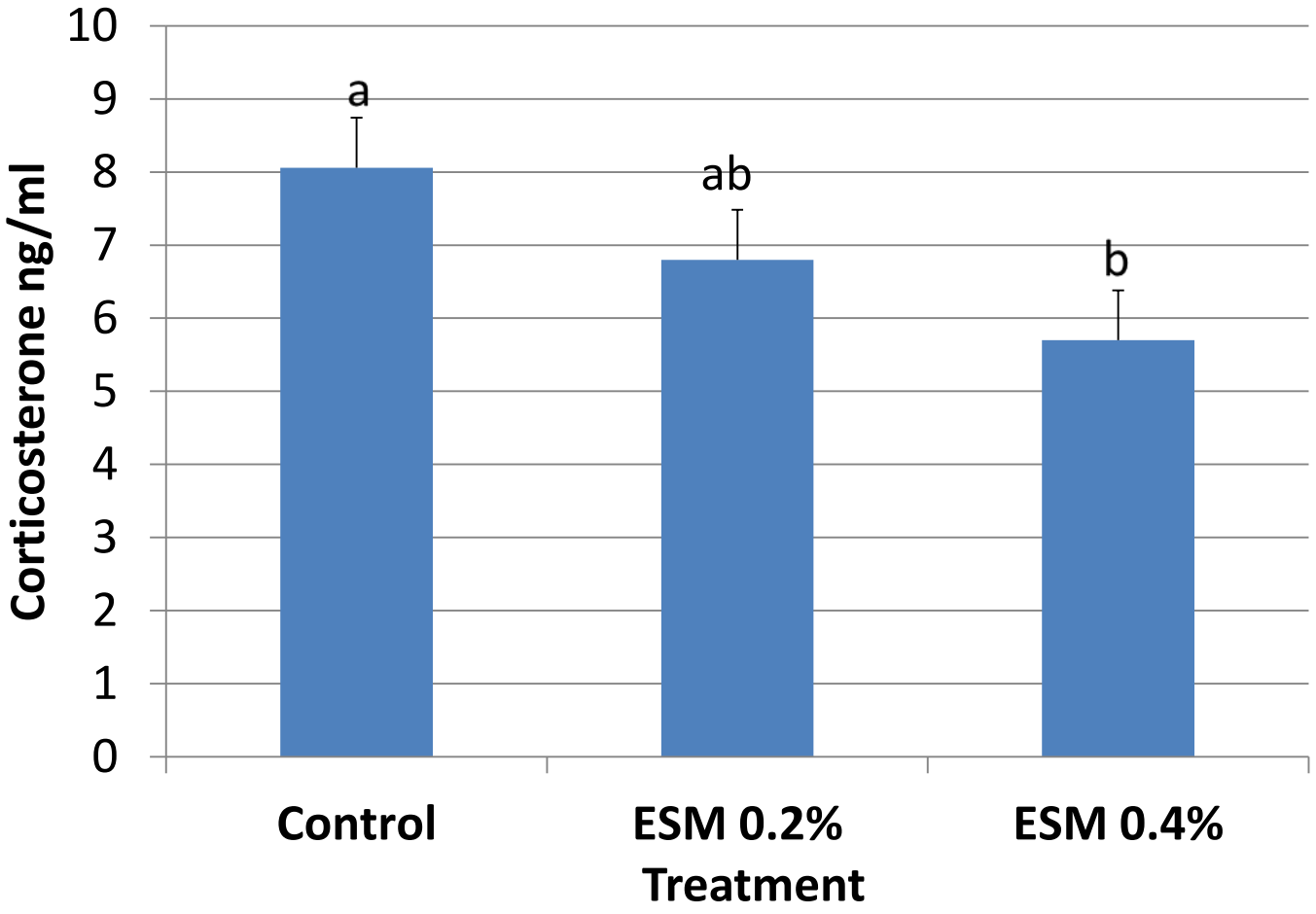
Hematology changes (n=12/group)

Variables	Control	+ESM 0.2%	+ESM 0.4%
White blood cell (×10^3/µL)	28.61 ± 2.87^{b}	35.45 ± 2.25^{a,b}	37.50 ± 2.30^{a}
Heterophil (%)	14.83 ± 1.30^{a}	11.96 ± 1.22^{a,b}	11.20 ± 1.19^{a,b}
Lymphocyte (%)	74.81 ± 2.02^{b}	80.0 ± 1.77^{a,b}	81.60 ± 1.55^{a}
Monocyte (%)	8.02 ± 0.84^{a}	5.53 ± 0.70^{b}	4.95 ± 0.37^{b}
Eosinophil (%)	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.00^{a}
Basophil (%)	2.34 ± 0.12^{a}	2.50 ± 0.14^{a}	2.17 ± 0.18^{a}
Red blood cell (× 10^6/µL)	2.08 ± 0.02^{b}	2.09 ± 0.03^{b}	2.20 ± 0.03^{a}
Thrombocyte (k/µL)	13.36 ± 0.65^{a}	11.81 ± 0.70^{a,b}	9.93 ± 0.39^{b}
Heterophil-to-lymphocyte ratio	0.21 ± 0.03^{a}	0.16 ± 0.02^{a}	0.14 ± 0.02^{a,b}
Hemoglobin (g/dL)	7.00 ± 0.10^{a}	6.92 ± 0.09^{a}	7.06 ± 0.08^{a}
Hematocrit (%)	52.78 ± 0.67^{b}	52.65 ± 0.77^{b}	55.67 ± 0.68^{a}
Mean corpuscular volume (fL)	254.06 ± 1.66^{a}	252.33 ± 0.82^{a}	257.40 ± 1.68^{a}
Red cell distribution width (%)	11.76 ± 0.16^{a}	11.79 ± 0.11^{a}	11.91 ± 0.14^{a}

Immunoglobulin concentrations of Control and +ESM diet fed chickens at 3 week of age (n=12)



Serum corticosterone levels, n=12



Summary

- ESM provides growth improvement, reduces stress variables, and elevates immunoglobulin levels at 3 weeks of age

Effect of Hatchery shell membrane

- HESM was prepared by grinding in blander then sterilized by wetting with 90% ethanol, dried then mixed with feed at the level of 0.5%

Experimental schedule

Day-old chicks

Control
Weeks 1-2
+ HESM feed,

Control feed
Weeks 3-5
Control feed

Day 34
(weigh)

Saline, 4h/24h

LPS, 1mg/kg, 4/24 h

Saline, 4h/24h

LPS, 1mg/kg, 4/24 h

24 h effect

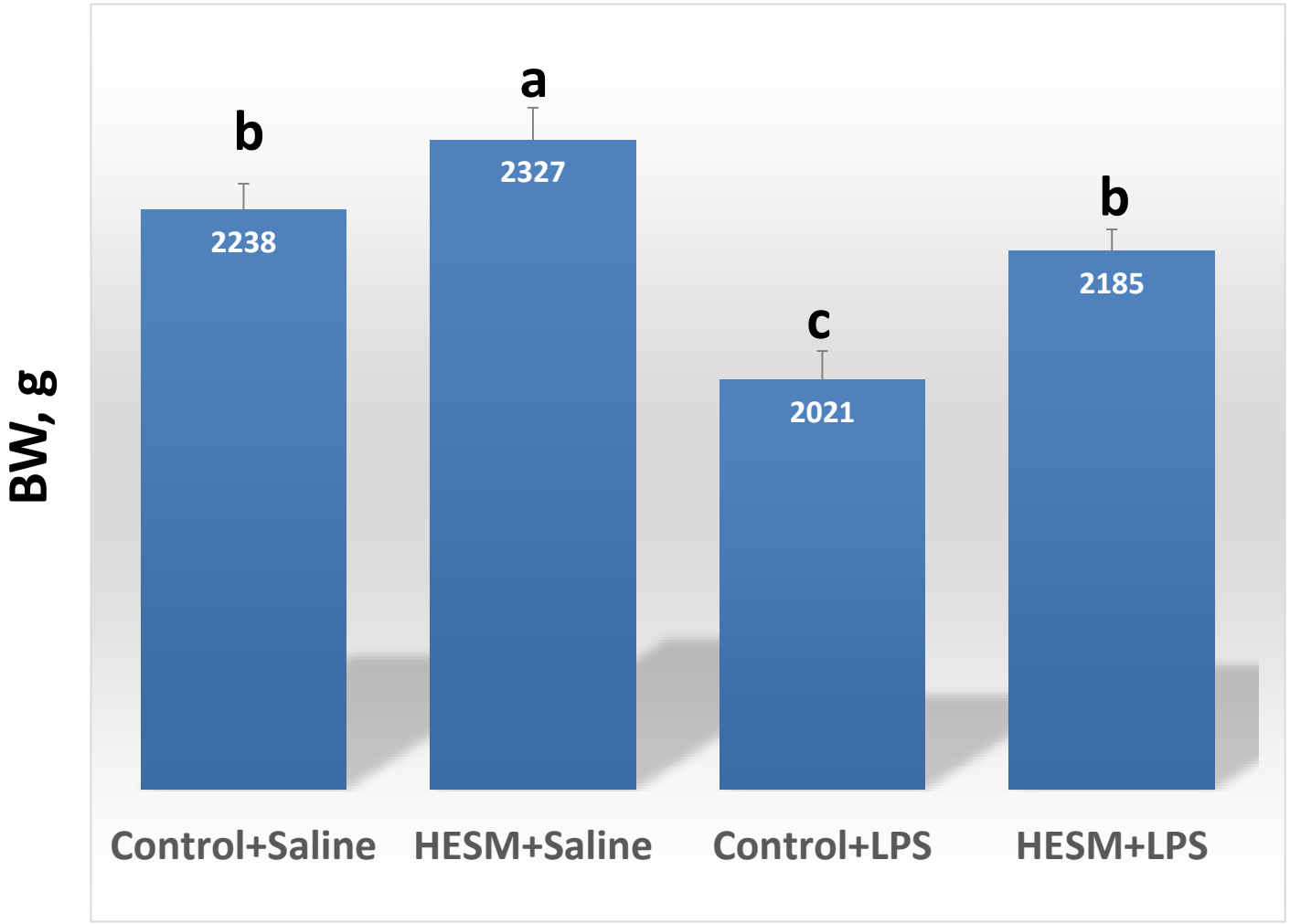
Mortality, Body weight
Hematology
Clinical chemistry
Serum immunoglobulin
Serum corticosterone
Intestine histology

4 h effect

RT-PCR: Splenic gene expression
IL-1,4, 6, 10, 12, IFN-g,TGF β, VEGF

Makkar, S. et al., PLoS One. 2016, 11(7):e0159433

Body weight changes due to HESM at 5 week of age and 24 h LPS treatment (n=32-36)



Hematology profiles of chickens fed control or diets containing HESM supplement and treated with LPS

	Saline		LPS	
Parameters	**Control**	**HESM**	**Control**	**HESM**
WBC (10^3/µL)	49.76±1.42^a	46.01±1.75^b	54.09± 3.12^a	45.60±3.0^b
Heterophil (H) (%)	11.44±0.36^c	12.62±0.46^c	30.85±2.35^a	22.94±3.14^b
Lymphocyte (L) (%)	83.81±0.65^a	82.31±0.70^a	61.80±2.22^c	69.92±3.10^b
Heterophil/ Lymphocyte (H/L)	0.14±0.01^c	0.15±0.01^c	0.50±0.06^a	0.32±0.05^b
Monocyte (M) (%)	2.32±0.18^b	2.75±0.28^b	4.31±0.33^a	4.01±0.26^a
Eosinophil (E) (%)	0.02±0.01^a	0.01±0.00^a	0.02±0.00^a	0.02±0.00^a
Basophil (B) (%)	1.99±0.17^b	2.3±0.18^b	2.99±0.19^a	3.03±0.17^a
Red blood cell (× 10^6/µL)	2.18±0.04^a	2.18±0.04^a	2.24±0.03^a	2.33±0.02^a
Hemoglobin (g/dL)	6.77±0.09^b	6.80±0.085^b	6.92±0.062^b	7.17±0.073^a
Hematocrit (%)	59.26 ±1.15^b	60.27±0.98^b	60.11±0.68^b	63.02±0.65^a
Mean corpuscular volume (MCV)(fL)	271.23±1.73^b	276.16±1.69^a	267.32±1.58^b	271.07±1.16^b
Thrombocyte (k/µL)	0.03±0.03^a	0.00±0.00^a	0.64±0.44^a	0.003±0.00^a

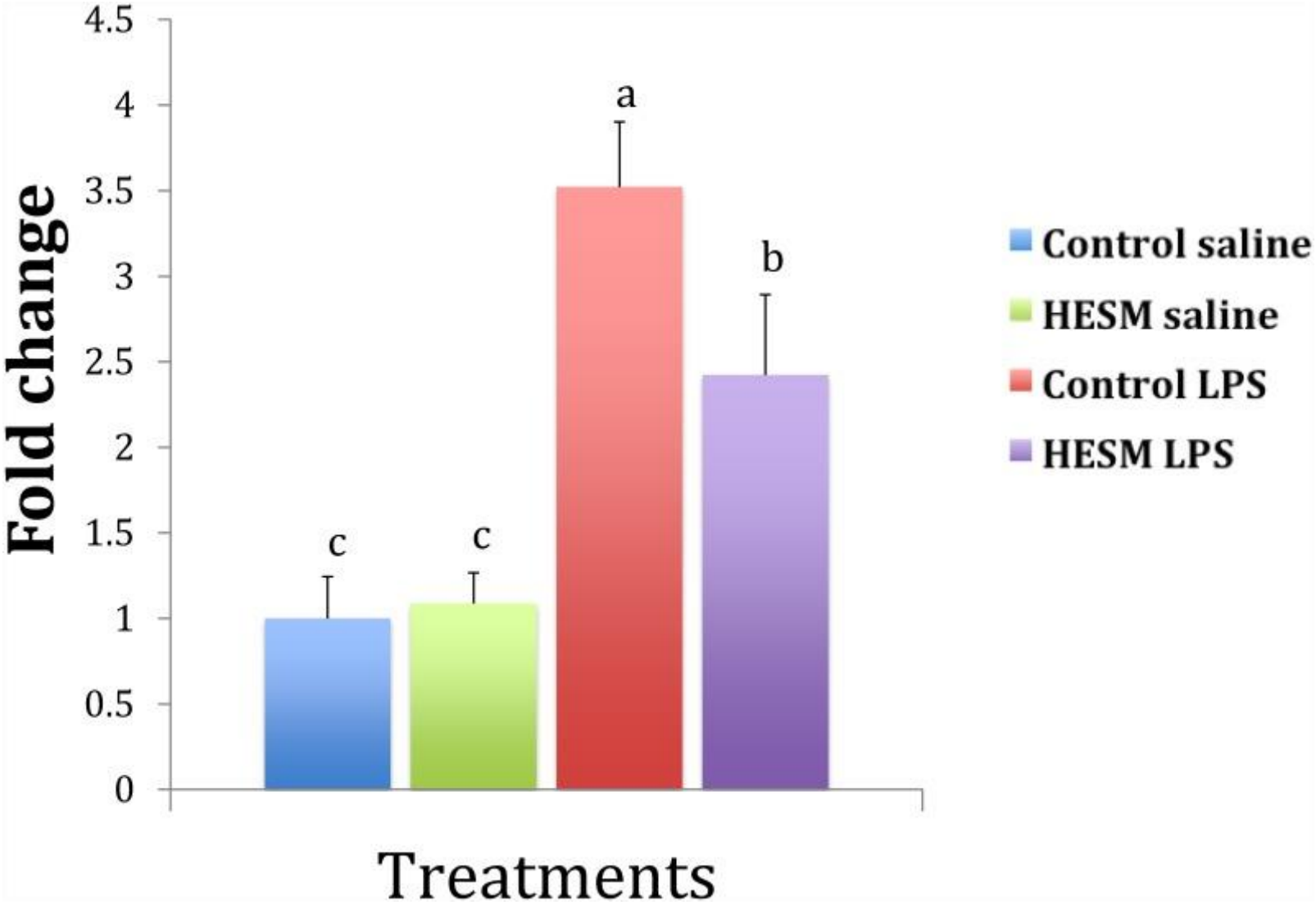
Serum concentration of IgG, IgM, and Corticosterone of 5 week-old chickens (n=12)

Variable	Control feed	+ HESM feed
IgG (mg/ml)	1.20±0.07[a]	0.82±0.07[b]
IgM (mg/ml)	2.69±0.24[a]	2.60±0.22[a]
IgM (mg/ml	0.56±0.15[a]	0.81±0.39[a]
Corticosterone (ng/ml	5.7±0.77[a]	3.81±0.41[b]

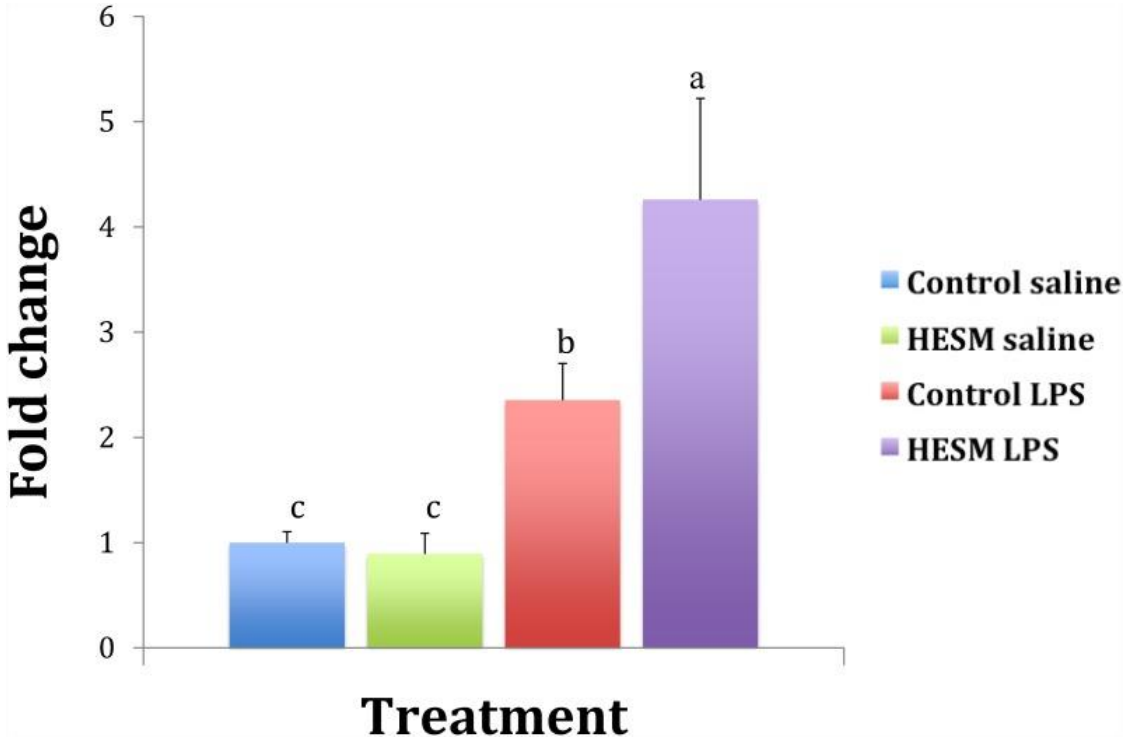
EFFECT OF LPS ON HEMATOLOGY STRESS VARIABLES AT 24 HOURS (n=12/group)

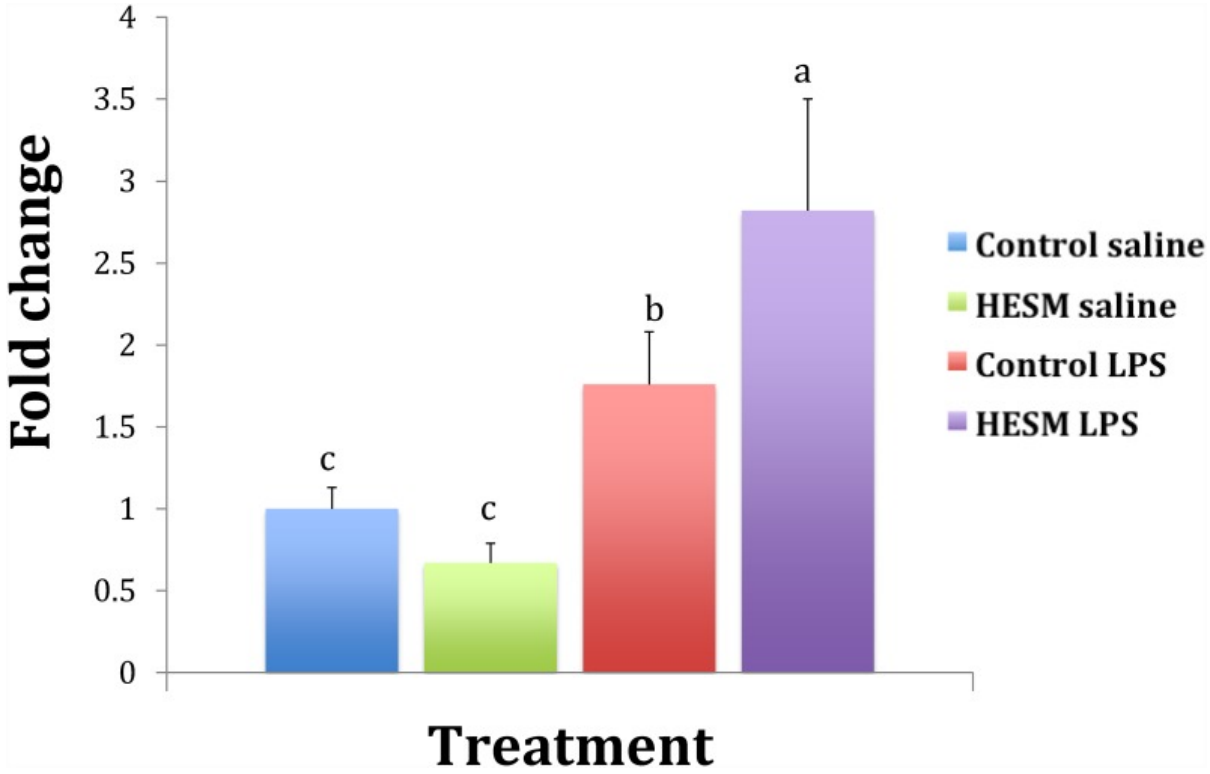
	Control feed		+HESM (0.5%) feed	
Variables	**Saline**	**LPS**	**Saline**	**LPS**
Heterophil	11.44 ± 0.36^{c}	30.85 ± 2.35^{a}	12.62 ± 0.46^{c}	22.94 ± 3.14^{b}
Heterophil/ Lymphocyte	0.14 ± 0.01^{c}	0.50 ± 0.06^{a}	0.15 ± 0.02^{a}	0.32 ± 0.05^{b}
Corticosterone (ng/mL)	7.74 ± 0.95^{a}	$6.70 \pm 0.60^{a,b}$	5.01 ± 0.53^{b}	$6.58 \pm 0.96^{a,b}$

Splenic expression of Interleukin-6 gene

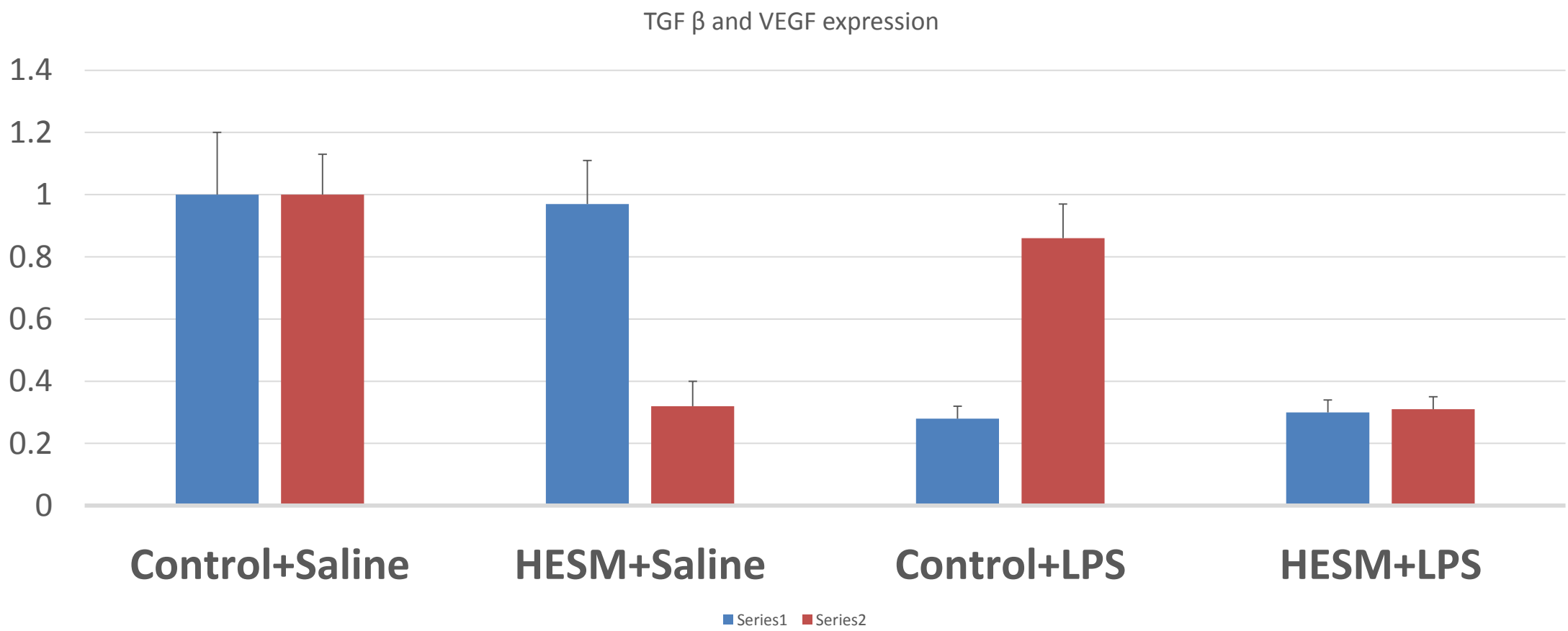


Expression of Interleukins 4 and 10 genes





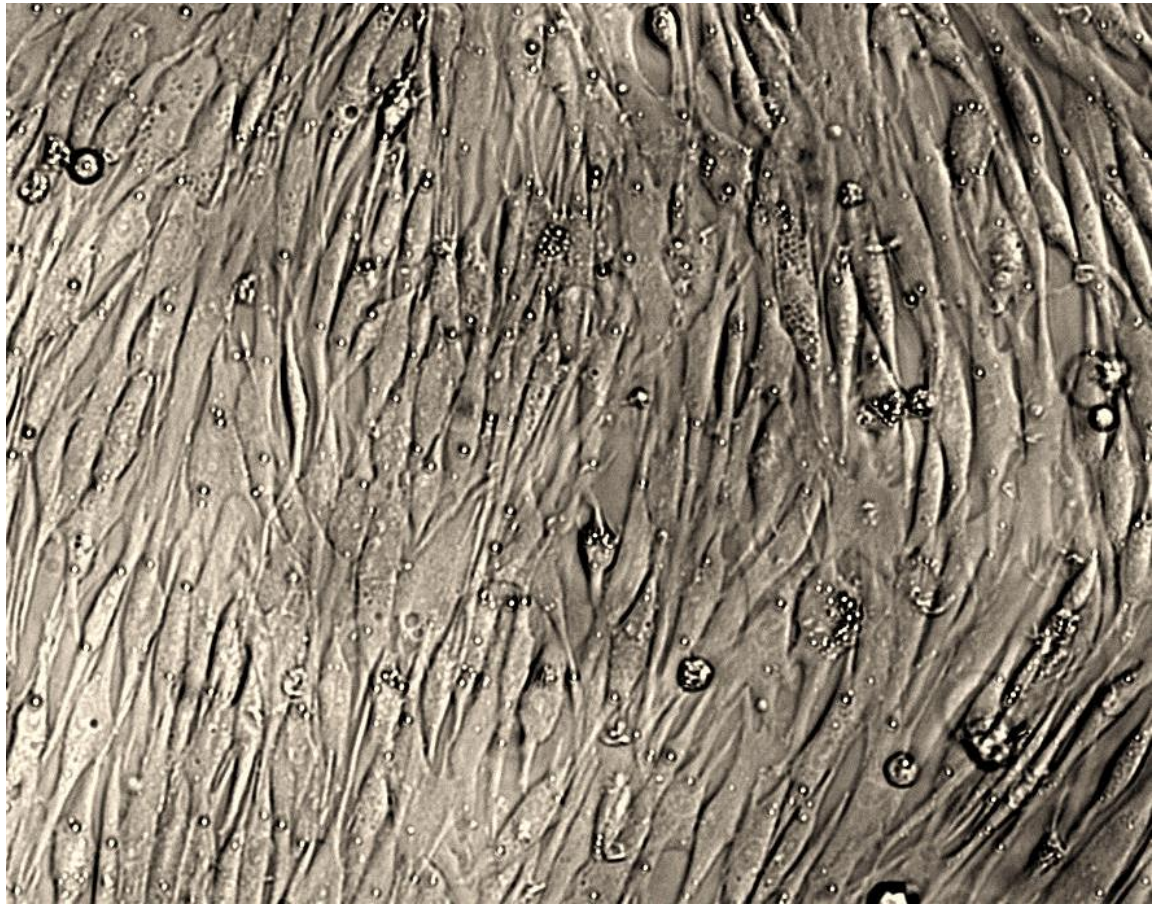
Expression of TGFβ and VEGF

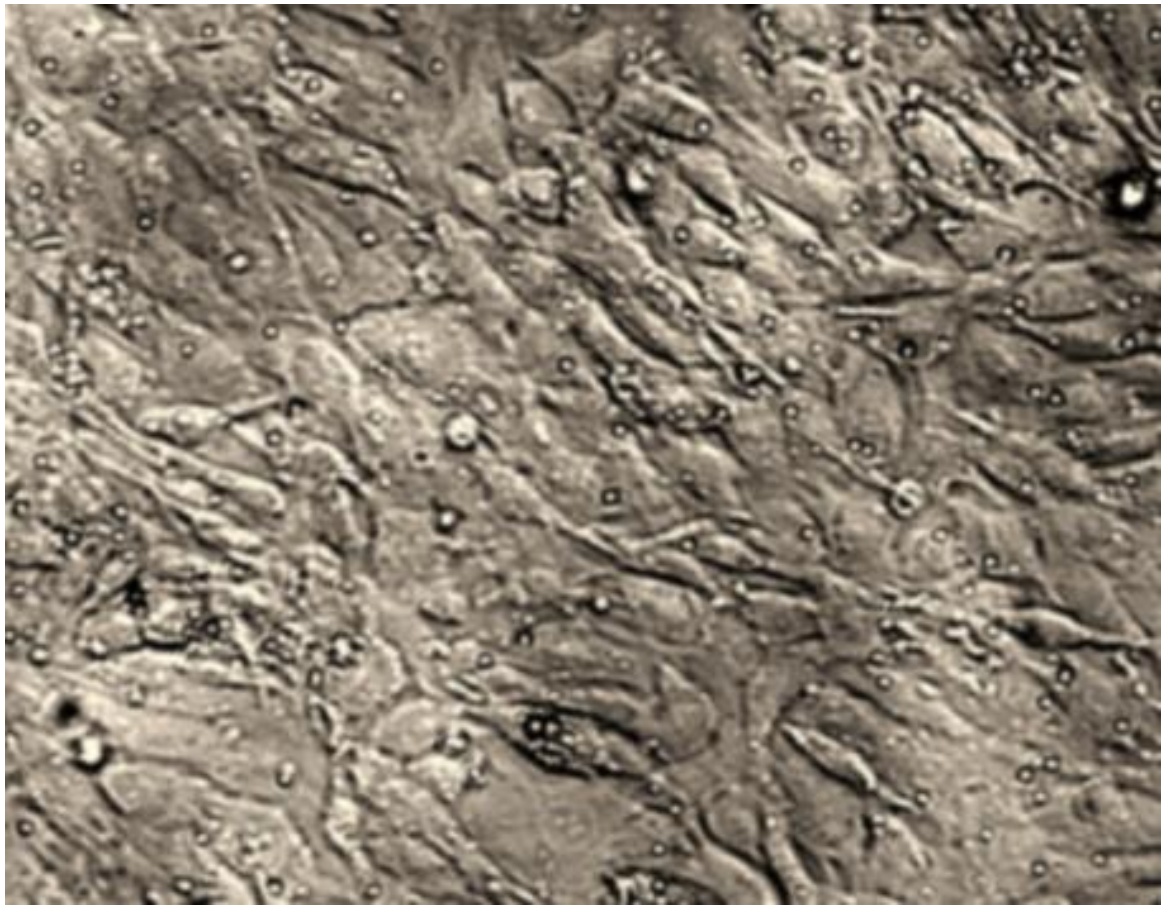


Summary

- Both ESM and HESM show moderate but statistical improvement in growth
- At 3 weeks there is increased levels of IgG and IgM due to ESM whereas HESM did not show any change measured at 5 weeks.
- HESM treatment induced resistance to endotoxin induced changes
- Improve splenic expression of anti-inflammatory and decrease the expression of pro-inflammatory genes
- Reduce stress variables such as corticosterone (3 and 5 week) and heterophil to lymphocyte ratios.

Trophic effect of 0.1% HESM extract on chicken enterocytes (48h)





Conclusion

- The bioactive proteins and peptides which include many antimicrobial and cell regulatory proteins, present in the shell membranes, are likely responsible for the development of better physiological and immune competence in poultry
- With appropriate enrichment, the membranes could be used as carriers for different microbial proteins that may aid in the development of mucosal immunity against pathogens and provide long lasting immunity

Some afterthought

Diets of baby birds

Final conclusion

- Identify other allogeneic and xenogeneic sources of nutritional supplements that may improve natural immunity against disease circumventing the use of antibiotics

Acknowledgment

- Drs. S. Makkar and B. Packialakshmi, graduate students
- Sonia Tsai, Scott Zornes, and Wally McDonner for Clinical chemistry and animal protocols