UNIVERSITI PUTRA MALAYSIA

EFFECTS OF SUPPLEMENTING PROTEASE IN LOW PROTEIN AND LOW ENERGY DIETS ON GROWTH PERFORMANCE AND PHYSIOLOGICAL RESPONSES OF BROILER CHICKEN UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

ELIZABETH LAW FANG LIN

IPTSM 2018 3

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By

ELIZABETH LAW FANG LIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

July 2018

DEDICATION

With appreciation and respect,
this thesis is dedicated to

This work is dedicated to my late mother
Mdm. Goh Choo Moi who is my inspiration forever
To my beloved son, Cheng Wen Jie and Cheng Wen Zhen
who supported and inspired me with confidence and ambitions

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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July 2018

Chairman : Professor Zulkifli Idrus, PhD
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Protein and energy are two important cost determinants in poultry diet's formulation. There is a great of interest in the use of low crude protein (CP) and/or metabolisable energy (ME) diets for feed cost saving. However, the diet may negatively affect the broilers performance. Proteases have been proven to improve nutrient digestibility and performance of broilers fed low CP diets. However, the ability of nutrient digestibility of proteases is inconsistence and depends on types of proteases used. Furthermorre, none of the study reported protease supplementation improved nutrient digestibility and performance of broilers fed low-CP and/or low-ME diets under stressful condition. Hence, four studies were conducted to access the effects of two different proteases (EA and EB) on nutrient digestibility of low CP feed and to evaluate the effects of CP and or ME reduction with supplemental protease on growth performance and physiological responses of broilers under natural environment, heat stress (HS) and high stocking density (SD) conditions.

The objective of experiment one (Chapter 3) was to evaluate effects of two commercial endopeptidase proteases (EA and EB) on apparent ileal digestible energy (AIDE), CP and amino acids (AA) of diet in broilers fed on either recommended-CP (CP19) or low-CP (CP16) diets. Diets with recommended-CP had higher AIDE ($P<0.001$), CP ($P<0.001$) and AA ($P<0.05$) digestibility than those of low-CP diets irrespective of protease supplementation. Proteases EA (Cibenza^{TM} DP100; Novus International Inc., USA) improved AIDE ($P=0.003$), CP ($P=0.004$), and majority of AA ($P<0.005$) digestibility in broiler chickens but not protease EB (Ronozyme®ProAct; DSM Nutritional

Products Ltd, Switzerland) compared to control regardless of dietary protein levels. Hence, protease EA was used for the following experiments (Chapter 4, 5 and 6).

Experiment 2 (Chapter 4) was conducted to investigate the effects of reducing CP (21.0%,19.7%, 18.5% and 17.2% from 1-21 days and 19.0%, 17.9%, 16.7%,and 15.6% from 22 to 35 days, respectively) with endopeptidase protease EA supplementation on growth performance, serum metabolites, carcass traits, small intestinal morphology and endogenous protease activity in broiler chickens under the natural tropical environment. Reducing CP linearly reduced weight gain (WG) ($P<0.001$), serum albumin ($P<0.001$), total protein ($P<0.001$) and carcass traits ($P<0.005$) and increased feed conversion ratio (FCR) ($P<0.001$), serum triglycerides ($P<0.005$) and adipose fat ($P<0.001$). There was no adverse effect of reducing dietary CP on morphological parameters of the intestine ($P>0.005$) and on the pancreatic ($P>0.005$) and small intestinal ($P>0.005$) digesta endogenous protease activity. Protease supplementation improved FCR ($P<0.005$), WG ($P<0.005$), carcass yield ($P<0.005$) and intestinal absorptive surface area ($P<0.005$). Following the optimization of nutritional factor in previous two experiment (chapter 3 and 4), it is necessary to answer the question whether the same nutritional condition is affected by various environmental factors such as high ambient temperature (Chapter 5) and high stocking density (Chapter 6).

Experiment 3 (Chapter 5) was conducted to investigate the effect of low CP and/or ME with supplemental protease on the growth performance, corticosterone (CORT), acute phase proteins (APP), heat shock protein (HSP) 70, adipose fat and breast meat yield of broilers under low and HS conditions. Regardless of protease supplementation, both dietary CP and ME could be reduced to 18.5% and 2985 kcal/kg, respectively without any adverse effects on feed intake (FI) ($P>0.005$), WG ($P>0.005$) and mortality in broilers during starter (d 1-21) period. However, either CP or ME could be reduced during finisher (d 22-42) period. Reduction of both dietary CP and ME was detrimental to FCR ($P<0.005$) although breast meat yield ($P>0.005$) was not affected. Irrespective of dietary CP and ME, supplementation of protease had negligible influence on growth performance ($P>0.005$). Protease reduced adipose fat in broilers fed low-ME diets. Heat stress adversely affects the growth performance ($P<0.005$), breast meat yield ($P<0.005$), serum metabolites ($P<0.001$), CORT ($P<0.001$) and APP ($P<0.005$) of broiler chickens regardless of protease supplementation, dietary CP or ME.

Experiment 4 (Chapter 6) was conducted to investigate the effects of protease supplementation in low-CP diet during the finisher period on growth performance, pododermatitis, immune response, physiological stress responses and cecal microfloral counts in broiler chickens under normal and high stocking densities. Results demonstrated that low-CP and low-ME diets

could be fed to broilers during the starter period without detrimental effects on FCR (P>0.005) but negatively affected the antibody production against ND vaccination (P<0.001) later in life. Supplementing protease to low-CP diet during the finisher period had only a negligible effect on the performance (P>0.005) and immunity (P>0.005) of broiler chickens. However, cecal *Clostridium* population was reduced (P<0.001) following protease supplementation. High-SD was detrimental to growth performance (P<0.005), antibody production (P<0.001) and pododermatitis (P<0.001) in broiler chickens.

In conclusion, dietary protein can be reduced to 18.5% and 16.7% during starter and finisher periods, respectively. However, ME could only be reduced to 2985 kcal/kgduring starter period but not during finisher period. There was little evidence that dietary addition of protease was consistently beneficial to broiler chickens fed low CP diets. Endopeptidase protease and dietary protein levels can independently affect the AIDE, CP, and AA digestibility in broiler chickens. Overall, the effects of endopeptidase protease suplementation in broilers fed low-CP diets were significant during the starter period. However, the significant effects were not appearant during the finisher period. In addition, growth performance and physiological reponses of broiler chickens can be affected by reducing CP and ME (nutritional factors) and environment challenges (high ambient temperature and high stocking density).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN PEMBERIAN PROTEASE DALAM DIET RENDAH PROTEIN DAN TENAGA TERHADAP PRESTASI DAN TINDAK BALAS FISIOLOGIKAL AYAM PEDAGING DI DALAM KEADAAN PERSEKITARAN YANG BERBEZA

Oleh

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Julai 2018

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Protein dan tenaga merupakan dua penentu kos penting dalam formulasi makanan ayam pedaging. Diet rendah protein kasar (CP) dan/atau nilai tenaga (ME) telah menjadi tumpuan disebabkan ia dapat membantu dalam penjimatan kos makanan. Tetapi diet ini mungkin akan menjejaskan prestasi pertumbuhan ayam pedaging. Pemberian protease di dalam diet ayam pedaging yang rendah CP dan ME telah dibuktikan dapat meningkatkan penghadaman nutrien dan prestasi ayam. Tetapi, tahap pengahadaman nutrien bergantung kepada jenis protease dan tiada pengajian yang menunjukkan penambahan protease dalam rendah CP and rendah ME diet terhadap meningkatkan pengahadaman nutrien dan prestasi pertumbuhan ayam pedaging di bawah keadaan yang tegang. Kajian-kajian berikutnya telah dijalankan untuk mengenalpasti kesan protease terhadap penghadaman nutrient dan diet rendah CP dan/atau ME terhadap ayam pedaging di bawah iklim tropika semulajadi, suhu ambien yang tinggi (HS) dan kepadatan stok tinggi (SD).

Eksperimen pertama (Bab 3) telah dijalankan untuk menyiasat keberkesanan dua jenis komersial endopeptidase protease (EA dan EB) terhadap penghadaman ME, CP dan amino asid (AA) terhadap ayam pedaging yang diberikan diet normal (19% CP) and rendah CP (16% CP). Keputusan menunjukkan diet yang diformulasi dengan CP pada tahap normal mempunyai nilai penghadaman yang lebih tinggi terhadap tenaga, CP dan AA jika dibandingkan dengan diet rendah CP. Protease EA (Cibenza™ DP100; Novus International Inc., USA) dapat meningkatkan penghadaman nutrien seperti

tenaga, CP, dan majority AA jika dibandingkan dengan protease EB (Ronozyme® ProAct; DSM Nutritional Products Ltd, Switzerland) and diet kawalantanpamengiratahap protein di diet. Oleh itu, protease EA telah digunakan untuk eksperimen berikutnya (Bab 4, 5, dan 6).

Eksperimen kedua (Bab 4) dijalankan untuk menyiasat kesan pengurangan CP (21.0%,19.7%, 18.5% 17.2% pada umur 1-21 hari dan 19.0%, 17.9%, 16.7% dan 15.6% pada umur 22-35 hari) dengan protease endopeptidase EA terhadap prestasi pertumbuhan, metabolit darah, nilai karkas, morfologi usus dan aktiviti protease endogen dalam ayam pedaging di bawah alam semulajadi tropika. Pengurangan CP telah merencatkan kadar penambahan berat badan ayam (WG), albumin and jumlah protein dalam serum, nilai karkas dan meningkatkan nisbah penukaranmakanan (FCR), trigliserida dalam serum dan lemak pada bahagian abdomen. Pengurangan CP tidak mendatangkan kesan negative terhadap morfologi usus kecil and aktiviti protease endogen di pancreas. Penambahan protease di dalam diet ayam pedaging mendatangkan kesan positif terhapdap FCR, WG, nilai karkas dan luas permukaan penyerapan usus. Melalui pengoptimuman faktor pemakanan yang dijalankan di eksperimen 1 (Bab 3) dan 2 (Bab 4), eksperimen seterusnya dijalankan untuk mengenalpasti sama ada faktor persekitaran seperti suhu tinggi ambien (Bab 5) dan kepadatan stok yang tinggi (Bab 6) akan memberikan kesan terhadap faktor pemakanan.

Eksperimen 3 (Bab 5) menganalisis kesan pengurangan kedua-dua CP dan/atau ME dengan panambahan protease terhadap prestasi pertumbuhan, corticosterone (CORT), acute phase protein (APP), heat shock protein (HSP) 70, lemak pada bahagian abdomen dan bahagian daging dada ayam di bawah keaadan tidak panas dan HS. Tanpa mengambilkira penambahan protease, CP dan ME pada tahap 18.5% dan 2985 kcal/kg tidak mendatangkan kesan negative keatas pengambilan makanan (FI), WG dan kadar kematian pada fasa pemula (d 1-21). Walaubagaimanapun, hanya CP atau ME boleh dikurangkan pada fasa pengemuk (d 22-42). Pengurangan kedua-duanya akan menjejaskan FCR walaupun tidak mendatangkan kesan pada bahagian daging dada ayam. Panambahan protease mendatangkan kesan yang kecil terhadap prestasi pertumbuhan ayam tidak mengira tahap CP dan ME di diet tetapi dapat mengurangkan lemak pada bahagian abdomen ayam pedaging yang diberikan diet rendah ME. Tekanan suhu ambien yang tinggi akan menjejaskan prestasi pertumbuhan, bahagian daging dada, metabolit dalam serum, CORT dan APP tanpa mengirakira protease, CP atau ME di diet.

Eksperimen 4 (Bab 6) bertujuan untuk mengkaji kesan-kesan panambahan protease dalam diet rendah-CP pada fasa pengemuk terhadap prestasi pertumbuhan, pododermatitis, tindakbalas tehadap imunisasi, fisiologi dan microfloral di dalam usus ayam padaging di bawah kepadatan stok yang nomal dan tinggi. Keputusan menunjukkan bahawa diet rendah CP dan

rendah ME pada fasa pemula tidak menjejaskan FCR ayam pedaging. Walaubagaimanapun, diet ini menjejaskan pengeluaran antibody terhadap vaksinasi Newcastle disease (ND). Di samping itu, panambahan protease di diet rendah CP pada fasa pengemuk hanya memberikan kesan yang minimal pada prestasi pertumbuhan dan imunisasi terhadap ayam pedaging. Walaubagaimanapun, ia berkesan dalam mengurangkan populasi Clostridium. Prestasi pertumbuhan, pengeluaran antibodi dan pododermatitis ayam pedaging telah terjejas akibat daripada kepadatan stok yang tinggi

Kesimpulannya, keputusan menunjukkan penghadaman nutrien seperti CP. ME dan AA bergantung kepada jenis protease dan tahap CP dalam diet. Selain daripada itu, tahap CP diet ayam pedaging boleh dikurangkan kepada 18.5% pada fasa pemula dan 16.7% pada fasa pengemuk tetapi ME hanya boleh dikurangkan kepada 2985 kca/kg pada fasa pemula dan tidak boleh dikurangkan pada fasa pengemuk. Kekurangan bukti menunjukkan bahawa penambahan protease di dalam diet akan menyumbangkan manfaat secara konsisten terhadap ayam pedaging terutama terhadap diet yang rendah CP. Pencernaan AIDE, CP, dan AA dalam ayam pedaging dipengaruhi oleh protease endopeptidase dan tahap CP. Secara keseluruhannya, endopeptidase protease memberi kesan terhadap ayam pedaging pasa fasa pemula tetapi tidak pada fasa pengemuk. Tambahannya, prestasi pertumbuhan dan tindak balas fisiologi ayam pedaging dipengaruhi oleh pengurangan CP dan ME (faktor pemakanan) dan cabaran alam sekitar (suhu ambien yang tinggi dan ketumpatan stok yang tinggi).

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I certify that a Thesis Examination Committee has met on 5 July 2018 to conduct the final examination of Elizabeth Law Fang Lin on her thesis entitled "Effects of Supplementing Protease in Low Protein and Low Energy Diets on Growth Performance and Physiological Responses of Broiler Chicken under Different Environmental Conditions" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

AA	Amino Acids
AABA	L-alpha-Amino-n-Butyric Acid
AGP	Alpha-1-acid Glycoprotein
AID	Apparent Ileal Digestibility
AIDE	Apparent Ileal Digestibility Energy
ALB	Serum Albumin
AME	Apparent Metabolizable Energy
AME_n	Apparent Metabolizable Energy Corrected to Zero Nitrogen
ANOVA	Analysis of Variance
AOAC	Association Official Agricultural Chemists
APP	Acute Phase Proteins
ARG	Arginine
ASA	Absorptive Surface Area
BW	Body Weight
CD	Crypt Depth
CK	Serum Creatine Kinase
cm	Centimeter
CORT	Corticosterone
CP	Crude Protein
CPN	Ceruloplasmin
CRD	Completely Randomized Design
Cys	Cysteine

d	Day
DM	Dry Matter
EB	Ronozyme®ProAct
FCR	Feed Conversion Ratio
Ft^2	Square Feet
g	Gram
g	Gravity
GE	Gross Energy
Glu	Glutamine
Gly	Glycine
h	Hour
HCl	Hydrochloride Acid
His	Histidine
HS	Heat Stress
H_2SO_4	Sulphuric Acid
HSP	Heat Shock Protein
Ile	Isoleucine
IU	International Unit
kcal	Kilo Calories
kg	Kilogram
Leu	Leucine
M	Molar
Lys	Lysine
M^2	Square Meter

ME	Metabolisable Energy
Met	Methionine
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimetre
mM	Mille mole
ND	Newcastle Disease
NE	Net Energy
nmol/L	Nanomoles per Liter
NRC	National Research Council
NSP	Non Starch Polysaccharide
OVT	Ovotransferrin
PBS	Potassium Buffer Solution
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration
Phe	Phenylalanine
ppm	Parts Per Million
RH	Relative Humidity
rpm	Revolutions Per Minute
s	Seconds
SA	Surface Area
SAS	Statistical Analysis System
SEM	Standard Error of Mean

Ser	Serine
SI	Small Intestinal
spp	Species
TG	Triglycerides
Thr	Threonine
TiO_2	Titanium Dioxide
TP	Serum Total Protein
Trp	Tryptophan
UA	Serum Uric Acid
USDA	United States Department of Agriculture
Val	Valine
WG	Weight gain
wk	Week
VH	Villus Height
vs	Versus
VW	Villus Width
lb	Pound
µg	Microgram
µl	Microlitre
µmol/g	Micromoles per Gram
%	Percent
ºC	Degree Celsius

CHAPTER 1

INTRODUCTION

Protein and energy are two main cost determinants in poultry feed (Kamran et al., 2008; Dairo et al., 2010). They are required for growth, maintenance, production and metabolic function (NRC, 1994, Hada et al., 2013). However, the prices of protein and energy ingredients (mainly corn and soybean meal) have been on the rise due to higher global demand and competition with other industries such as biofuel production (Infante‐Rodriguez et al., 2016). It is a common practice to formulate commercial broiler chicken diets with crude protein (CP) and metabolisable energy (ME) levels according to the recommendations of the respective breeding companies. However, earlier studies (Nguyen and Bunchasak, 2005; Dairo et al., 2010) have shown that dietary CP and/or ME can be reduced without any detrimental effect on growth performance by maintaining amino acids (AA) within the recommended requirement. However, not all AA are commercially available. Furthermore, the antinutritive value in plant protein source may impair the action of digestive enzymes, decrease digestive flow and absorption of nutrients, and subsequently growth will be adversely affected (Bedford, 1995). Alternatively, enzyme supplementation may allow feeding of lower protein diets to broiler chickens without affecting performance in a sustainable and economic manner (Simbaya, *et* al., 1996; Yu, *et al*., 2007; Freitas, *et al*., 2011; Fru-Nji, *et al*., 2011; Cowieson, *et al*., 2006a).

Enzyme supplementation may improve the production efficiency of poultry by enhancing digestion and reducing nutrient loss through excreta in the poultry. Consequently, this may allow the levels of certain nutrients to be reduced in the diet thus being considered an economic advantage (Romero et al., 2013; Nahm, 2007). More particularly, protease was shown to improve nutrient digestibility and performance of broilers fed low-CP (Angel et al., 2011; Fru-Nji et al., 2011) and/or low-ME (Freitas et al., 2011) diets. The benefits of protease supplementation in poultry can be associated with improved intestinal morphology (Xu et al., 2017) and gut microbiota (Barekatain et al., 2013). Kamel et al. (2015) further reported that protease supplementation in diets with varied CP and ME levels had somehow reduced total colony count of ileal C*lostridium* spp.

Most of the earlier studies in poultry used protease in combination with carbohydrases (Mahagna et al., 1995; Barekatain et al., 2013; Flores et al., 2016). There is little information on the effect of single exogenous protease supplementation on broiler chickens. Given the adverse effects of stress on digestion and nutrient absorption (Zuprizal et al., 1993; Soleimani et al., 2010), it is important to determine the effects of protease supplementation on poultry under stressful environment. Proteases as stand-alone enzymes are relatively

new to the feed enzyme market compared to non-starch polysaccharides (NSP) degrading enzymes or phytases. The applications of proteases in feed have thus far only been the focus of specific marketing and research attention for the past 5 to 10 years (Adeola and Cowieson, 2011; Fru-Nji et al.,2011; Rehman et al., 2017). There is no documented report on the implications of protease supplementation towards environmentally challenged chickens.

In general, the rearing environment of a chicken is a composite of interacting between external (temperature, light, social, human-animal interactions) and internal (disease organisms, toxins) stressors conditons. The bird's capability in coping with the surrounding environment depends largely on the severity of the stressors and its physiological competence to respond properly. Heat stress (HS) is a major constraint for optimum production of poultry in the tropical environment. The adverse effects of a hot environment towards growth performance, immunity and well-being of broiler chickens are well documented (Gonzalez-Esquerra and Leeson, 2006; Lilian et al., 2016). The fast growing commercial broilers are particularly susceptible to HS-related problems because metabolic heat production increases along with growth rate but not heat dissipation (Nwe Nwe Htin et al., 2006). Feeding broiler chickens with diet high in CP is a costly metabolic process as excess AA in the feed must be catabolised (Musharaf and Latshaw, 1999). Cahaner et al. (1995) and Cheng et al. (1997) reported that excess fed CP reduced the growth rate of heat-stressed commercially fast growing broiler chickens. It is well established also that CP has the highest heat increment in the fed broiler chickens due to higher metabolic heat production causing extra load for heat dissipation as compared to fat and carbohydrate metabolism (Mickelberry et al., 1966; Musharaf and Latshaw, 1999). Previous studies showed that lower-CP diets while maintaining AA within the recommended requirements may alleviate the detrimental effects of HS (Lin et al., 2006) and improve protein efficiency (Awad et al., 2014a,b). On the contrary, other studies have recommended higher dietary protein to compensate the reduced protein intake in view of decreased feed consumption associated to HS (Temim et al., 1999; Temim et al., 2000; Gonzalez-Esquerra and Leeson, 2005). These inconsistencies in findings could be attributed to factors such as age, breed, dietary energy level, duration of heat exposure, and AA composition (Cahaner et al.. 1995; Gonzalez-Esquerra and Leeson, 2005; Zaman et al., 2008). Many studies have also examined the effect of stocking density on growth performance and carcass quality of broiler chickens (Puron and Santamaria, 1995; Estevez, 2007; Shakeri et al., 2014). Although overcrowding may compromise the welfare of poultry, some studies have actually failed to observe significant changes in physiological stress indicators such as plasma levels of corticosterone (CORT) (Thaxton et al., 2006), heterophil/lymphocyte ratios (Cravener et al., 1992) and humoral immune response (Heckert et al., 2002). Houshmand et al. (2012) studied the relationship between dietary protein and stocking density (SD) in broiler chickens. The authors noted significant dietary protein level x SD interactions for body weight (BW) but not for physiological stress indicators such as heterophil/lymphocyte ratios and CORT. Shakeri et

al. (2014) and Najafi et al. (2015) reported that overcrowding elevates the circulating concentrations of acute phase proteins (APP) and heat shock protein (HSP) 70 in broiler chickens.

It is hypothesed that the supplementation of endopeptidase protease can enhance the growth performance of broiler chickens fed low dietary CP and ME diets under high ambient temperature or high SD. Therefore, the general objectives of current studies were designed to ascertain the effects of protease supplementation in low CP and ME diets towards environmentally stressed broiler chickens on growth performance and physiological responses.

The specific objectives were

(i) To study the effects of two commercial endopeptidase proteases (EA and EB) on apparent ileal digestible energy (AIDE), CP and AA of diet in broilers fed on either recommended-CP (CP19) or low-CP (CP16) diets
(ii) To examine the effect of endopeptidase protease supplementation on growth performance, blood metabolites, carcass traits and small intestinal (SI) morphology in broiler chicken that were fed low-CP diet and reared under natural hot and humid tropical climate
(iii) To investigate the effects of feeding recommended or low CP and recommended or low ME with or without supplemental protease on growth performance, carcass characteristics and physiological responses of broiler chickens raised under cyclic heat stressed conditions
(iv) To evaluate the effects of protease supplementation in low-CP diet during the finisher period on growth performance, incidence of pododermatitis, immune response, physiological stress responses and cecal microflora counts in broiler chickens reared under normal and high stocking densities

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