



UNIVERSITI PUTRA MALAYSIA

**EFFECTS OF *Lactobacillus* PROBIOTIC ON GROWTH PERFORMANCE
AND COCCIDIOSIS IN BROILER CHICKENS CHALLENGED WITH
*Eimeria tenella***

WAN NUR FADHILAH BINTI SHAMSUDIN

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By

WAN NUR FADHILAH BINTI SHAMSUDIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Science**

February 2019

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Master of Science

EFFECTS OF *Lactobacillus* PROBIOTIC ON GROWTH PERFORMANCE AND COCCIDIOSIS IN BROILER CHICKENS CHALLENGED WITH *Eimeria tenella*

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WAN NUR FADHILAH BINTI SHAMSUDIN

February 2019

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Avian coccidiosis is an intestinal disease caused by several species of *Eimeria* (family Eimeriidae) of the phylum Apicomplexa. Probiotics had been evaluated as an alternative to anticoccidial drugs, and although the resistance of birds to coccidiosis was enhanced, but the protective effects were not always associated with an improved growth performance. Therefore, it was necessary to find probiotic species with anti-coccidiosis activity and at the same time improved broilers growth performance when challenged with *E. tenella*. Five out of 46 lactic acid bacteria (LAB) isolates from chicken intestine demonstrated good tolerance to acidic pH 3, 0.3% bile salts and of these, three isolates showed strong adhesion to Caco-2 cells. These three isolates also showed antagonistic activity against pathogenic *E. coli* (078:K80), *Salmonella enterica serovar Typhimurium*, *C. perfringens* (Type A), *Salmonella enterica serovar Enteritidis*, *E. faecium* (FM3), *E. faecalis* (E227). All three isolates had high inhibition zone (> 6mm) against *E. coli* 078:K80 and *Salmonella enterica serovar Typhimurium* and were susceptible to antibiotics gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol and ampicillin. These isolates were identified by using 16S rRNA sequencing technique and identified as two *L. salivarius* and one *L. reuteri* with accession number MH375403, MH375402 and MH375404, respectively. The LAB isolates were evaluated for their efficacy as anticoccidiosis alternatives in broilers challenged with *E. tenella*. Four dietary treatments: untreated, unchallenged broilers fed basal diet (BD); treated with anti-coccidial drug (EimeriaX 12%) and challenged (ANTI); untreated and challenged (COCCID); and treated with probiotic (*Lactobacilli* mixture at 5×10^8 CFU/kg) and challenged (PRO); were used in a feeding trial of 42 days with 40 chicks (Cobb 500) per treatment divided into 5 replicates. All groups, except BD, were challenged with 2×10^4 /mL of *E. tenella* oocysts on day 14 of broilers age. The results showed that broilers fed PRO diet had comparable body weight and feed conversion ratio (FCR) compared to BD and ANTI groups. Broilers of COCCID group had significantly lower ($P < 0.05$) body weight and higher FCR. The lesion score was only detected in both infected chickens of PRO and COCCID groups at day 21 and was not detected at day 42. Oocyst count was significantly higher ($P < 0.05$) in the COCCID group, compared to PRO

and ANTI groups. The population of *Lactobacillus* spp. and *Bifidobacterium* spp., as well as villus and crypt depth were significantly higher ($P < 0.05$) in the PRO group, while the populations of *E. coli* and *C. perfringens* were lower. In conclusion, the isolated LAB probiotics could improve the growth performance and alleviate the effects of coccidiosis infection when challenged with *E. tenella*, comparable to broilers treated with anticoccidial drug.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

KESAN PROBIOTIK *Lactobacillus* TERHADAP PRESTASI PERTUMBUHAN DAN KOKSIDIOSIS DALAM AYAM PEDAGING YANG DI CABAR DENGAN *Eimeria tenella*

Oleh

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Koksidiosis poltri ialah penyakit usus yang disebabkan oleh beberapa spesis *Eimeria* (keluarga Eimeriidae) filum Apikompleksa. *Eimeria tenella* adalah paling patogenik. Antikoksidiosis koksidiosis, koksidostat dan ionofor digunakan untuk mengawal koksidiosis. Kesedaran mengenai keselamatan kesihatan pengguna yang dikaitkan dengan residu ubat di dalam rangkai makanan mencetuskan larangan dan penggantian ubatan sintetik kepada makanan tambahan seperti probiotik. Sebagai alternatif kepada ubatan antikoksid, probiotik dapat meningkatkan rintangan burung kepada koksidiosis dengan berkesan, tetapi kesannya tidak sentiasa berkait dengan prestasi penumbuhan. Lantaran itu, adalah perlu untuk mengasingkan probiotik baru yang menunjukkan aktiviti antikoksidiosis dan berkesan positif terhadap pertumbuhan ayam pedaging yang dijangkiti *E. tenella*. Lima daripada 46 isolat bakteria asid laktik yang diasingkan daripada usus ayam menunjukkan toleransi baik terhadap pH 3, 0.3% garam hempedu, dan tiga daripadanya menunjukkan lekatan tertinggi kepada sel Caco-2. Tiga isolat ini juga menunjukkan aktiviti antagonistik terhadap patogen *E. coli* (078:K80), *Salmonella enterica serovar Typhimurium*, *C. perfringens* (Type A), *Salmonella enterica serovar Enteritidis*, *E. faecium* (FM3), *E. faecalis* (E227). Semua ketiga isolat ini memberikan kesan perencatan yang tinggi (nilai >6mm) terhadap *E. coli* (078:K80) dan *Salmonella enterica serovar Typhimurium* juga rentan terhadap antibiotik gentamisin, kanamisin, streptomisin, tetrasiklin, eritromisin, klindamisin, kloramfenikol dan ampisilin. Isolat ini dikenalpasti melalui jujukan 16S rRNA sebagai dua *L. salivarius* dan satu *L. reuteri* dengan nombor kemasukan masing-masing MH375403, MH375402 dan MH375404. Bakteria asid laktik dinilai keberkesanan sebagai alternatif antikoksidiosis kepada ayam pedaging yang dicabar *E. tenella*. Empat rawatan pemakanan: ayam pedaging tidak dirawat atau dicabar (BD); dirawat dengan antikoksid (*EimeriaX* 12%) dan dicabar (ANTI); tidak dirawat dan dicabar (COCCID); dirawat dengan probiotik (campuran *Lactobacillus* isolat pada kadar 5×10^8 CFU/kg) dan dicabar (PRO), digunakan dalam kajian selama 42 hari dengan 40 ekor anak (Cobb 500) setiap kumpulan dibahagi kepada 5 replikat. Setiap kumpulan, kecuali BD dicabar dengan 2×10^4 /mL oosis *E. tenella* pada umur 14 hari. Keputusan percubaan menunjukkan ayam yang diberi probiotik

mempunyai berat badan dan kadar penukaran makanan (FCR) yang tinggi berbanding kumpulan BD dan ANTI. Ayam kumpulan COCCID menunjukkan berat badan yang paling rendah dan FCR tertinggi. Skor luka hanya dikesan pada kedua-dua kumpulan PRO dan COCCID pada umur 21 hari tetapi tidak pada umur 42 hari. Kiraan oosis tinggi secara ketara ($P < 0.05$) pada kumpulan COCCID dibandingkan dengan PRO dan ANTI. Populasi *Lactobacillus* spp. dan *Bifidobacterium* spp. vilus dan kedalaman *crypt* adalah tinggi secara ketara ($P < 0.05$) pada kumpulan PRO manakala populasi *E. coli* dan *C. perfringens* rendah. Sebagai kesimpulan, probiotik bakteria asid laktik boleh menambahbaik prestasi pertumbuhan ayam pedaging dan mengurangkan kesan jangkitan koksidirosis apabila dicabar dengan *E. tenella* setanding dengan ayam pedaging yang diberi ubat antikoksid.



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*“The best things in life are the people you love, the places you’ve seen,
and the memories you’ve made along the way...”*

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AGP	Antibiotic Growth Promoter
AME	Apparent metabolisable energy
ATCC	American Type Culture Collection
AST	Aspartate Aminotransferase
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BW	Body weight
BWG	Body weight gain
Ca	Calcium
CaCl ₂	Calcium chloride
CFU	Colony forming unit
Cl	Chloride
cm	Centimeter
Co	Cobalt
Cu	Copper
DMEM	Dulbecco's Modified Eagle Medium
DVS	Department of Veterinary Services
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FBAs	Free Bile Acids
FBS	Fetal bovine serum
Fe	Iron
FCR	Feed conversion ratio
FDA	Food and Drug Administration
g	Grams
GE	Gross energy
GIT	Gastrointestinal tract
h	Hour
Hb	Hemoglobin
H ₂ O	Water
HCl	Hydrogen chloride
HDL	High-density Lipoprotein
H:L	Heterophils:Lymphocytes
IDF	International Dairy Federation

IgA	Immunoglobulin A
ISO	International Organization for Standardization
IST	ISO-Sensitest
K	Potassium
Kcal	Kilocalories
KCl	Potassium chloride
kg	Kilogram
LAB	Lactic Acid Bacteria
LDL	Low-density Lipoprotein
LIVES	Laboratory Of Vaccines and Immunotherapeutics
LSM	Lactic Acid Bacteria Susceptibility Test Medium
MARDI	Malaysian Agricultural Research and Development Institute
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
Mg	Magnesium
MgSO ₄	Magnesium sulphate
MICs	Minimal Inhibitory Concentrations
MIU	Million international units
min	Minutes
mg	Milligram
mL	Milliliter
mm	Millimeter
Mn	Manganese
MRS	De Man, Rogosa and Sharpe
N	Nitrogen
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydrochloride
NCBI	National Center for Biotechnology Information
ND	No detected
NRC	National Research Council
nm	Nanometer
OD	Optical density
P	Phosphorus
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PCV	Packed Cell Volume

PenStrep	Penicillin-Streptomycin
pH	Potential of Hydrogen
PKE	Palm Kernel Expeller
RBC	Red Blood Cell
rpm	Ram per minutes
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	Seconds
Se	Selenium
sp.	Species
SPSS	Statistical package for the Social Science
UPM	Universiti Putra Malaysia
USA	United State of America
UV	Ultraviolet
v/v	Volume per volume
WBC	White blood cell
WHO	World Health Organization
w/v	Weight per volume
vol	Volume
Zn	Zinc
µg	Microgram
µL	Microliter
x g	Gravity force

CHAPTER 1

INTRODUCTION

The demand for poultry meat and poultry products is increasing, due to the increase in population density and consumption of poultry products as the primary protein source (The Poultry Site, 2014). Malaysia is considered self-sufficient in poultry meat and eggs, where 90% of the production is in Peninsular Malaysia, and the rest in East Malaysia.

In poultry rearing, the high-density stocking rate requires proper management to alleviate problems related to the stressful conditions which may cause problems associated to infectious disease that may affect productivity (Baracho et al., 2006). Infectious disease can be caused by pathogens consisting of bacteria, viruses and protozoa (Elmusharaf and Beynen, 2007; Alexander et al., 2008; Ricke, 2014). Pathogenic *E. coli* causes a variety of diseases known as colibacillosis, which causes enteric disease and increases mortality rate in chickens, and results in economic losses in the poultry industry (Kumar et al., 2004). Salmonellae infections constitute a serious health, breeding and economic problem especially in poultry farms. Coccidiosis is another disease caused by protozoan parasite which infects the intestinal system of poultry (Tewari and Maharana, 2011). Current approaches to counter avian coccidiosis involve the use of anticoccidial chemicals, vaccines and natural products. Anticoccidial chemicals, coccidiocides, coccidiostats, and ionophores, have long been used as a thought strategy to manage avian coccidiosis in current poultry production (Chapman et al., 2010). However, *Eimeria* species have developed resistance to both chemical and ionophore drugs over time (Stringfellow et al., 2011). As such, the employment of live vaccines to manage coccidiosis has greatly raised. However, early and uniform administration of live oocysts as vaccine results in a low-level infection, necessary for immunity development, but it can cause an early reduction in growth and may increase the chick's susceptibility to secondary infections, such as necrotic enteritis.

The use of antibiotics is practised to ensure health and growth performance of the birds. The use of antibiotic as growth promoters and infection control in animal feed has been reported for about 50 years ago (Hao et al., 2014). In Malaysia specifically, the Department of Veterinary Services (DVS) produced specific guidelines on concentration of drugs or antibiotics that can be used by the local farmers (DVS, 2006). Although poultry production is well managed, but the concern about antibiotic residues in the poultry products as well as the emergence of antibiotic resistance genes in the pathogens have become a great concern worldwide (Castanon, 2007). There are reports showing that the residue of the antibiotic growth promoters in poultry products may cause harm to the consumers (Chapman et al., 2013; Reid et al., 2014). These phenomena have trigger serious efforts by researchers to find safe alternatives to avoid dependency on harmful drugs for enhancing poultry production (Floros et al., 2010).

The Food and Drug Administration (FDA) has projected a ban on using antibiotics at subtherapeutic levels in feed attributable to the potential for compromising the health of humans. However, a large phase of the regulated trade, as well as farmers and ranchers, has contended that in nearly thirty years of use, antibiotics at subtherapeutic levels in

animals haven't compromised human or animal health or influenced the medical aid of human sickness (Be'er et al., 2009). The first country to ban the use of nontherapeutic antibiotic was Sweden in 1986 as requested by the Federation of Swedish Farmers and continue to stop all prophylactic medication in 1988. Denmark restricts direct sale of therapeutic antimicrobials from veterinarians and limits veterinary profits from antimicrobial sales in 1994 and Germany bans the use of avoparcin in 1996. European Union (EU) strictly bans use of all antibiotic growth promoters in 2006 (Cogliani et al., 2011).

Coccidiosis, the most common parasitic disease in poultry, is known to develop through a combination of several different species of the parasites as each species has its own specific location in the intestine (Elmusharaf and Beynen, 2007). So far, nine *Eimeria* species, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. mitis*, *E. tenella*, *E. mivati*, and *E. hagani*, have been identified from chickens (Joyner and Long, 1974). The *E. tenella* is found to be one of the most pathogenic species as it can survive outside of the intestine and infect others in the same flock, thus spreading the disease (Tewari and Maharana, 2011). To date, the occurrence of coccidiosis has resulted in great losses in poultry production (Giannenas et al., 2012).

The increasing demand by customers for drug residue free poultry and poultry products has led to the event of other ways for the treatment and management of avian diseases. There is a number of options for minimising or substituting antimicrobials use, such as, vaccination, feed additives like enzymes, prebiotic, probiotic, acidifiers, plant extracts, essential oils and yeast (FAO 2010). Natural products are emerging as an attractive way to combat coccidiosis. Antioxidant rich essential oils have been evaluated to alleviate the damage caused by oxidative stress during coccidiosis. However, results are very divergent and often not as satisfactory as expected (Idris et al., 2017). Several challenges in the anticoccidial use of natural products such as anticoccidial efficacy, identification of active compounds, mechanism, safety, and cost-effectiveness of plant extracts and compounds need to be overcome prior to large scale applications (Muthamilselvan et al., 2016).

Probiotics have long been considered as alternatives to the antibiotics (Menconi et al., 2014). Probiotics are "live organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). They have been reported to improve health of the host animal by enhancing the beneficial microflora, improving the host resistance to pathogenic microorganisms, and increasing the level of immunomodulation of the host (Jin et al., 1998). Most common bacterial probiotics species in poultry belong to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* (Kabir, 2009). Some of the important criteria for a probiotic bacterial species are it must be a normal inhabitant of the gut, able to adhere to intestinal epithelium, to survive at low pH and exposure to bile salt, to compete with other microorganisms in the gastrointestinal tract, to increase immune response, and to maintain high viability rate in normal storage condition (Walker and Gilliland, 1993).

A number of studies have been conducted to evaluate the efficacy of probiotics to alleviate the effects of coccidiosis in poultry. Ritzi et al. (2016) reported that the combination of probiotics and coccidiosis vaccines (*Eimeria* oocysts) could enhance

performance and provide protective effect against a mixed *Eimeria* challenged. Similarly, Bozkurt et al. (2014) also observed that probiotics and prebiotics supplementation to chickens exposed to experimental coccidiosis alleviated the influence of the disease and lessen the depression in growth. In another study, Lee et al. (2007) also observed that *Pediococcus*-based probiotic effectively enhanced the resistance of birds but partially alleviated the negative growth effects associated with coccidiosis. On the other hand, Abu-Akkada and Awad, (2015) reported that multispecies probiotic mix (Lacto G[®]) supplementation to broilers prevented mortality, reduced oocyst output and the severity of *E. tenella* lesions, but the protective effects were not associated with an improved growth performance. Abdelrahman et al. (2014) also reported that broilers that received the several-species host-specific probiotic (PoultryStar) in their diet performed hugely same as to broilers that received salinomycin in terms of overall feed conversion ratio, oocyst shedding, mortality, and intestinal lesions. However, body weights of broilers in the probiotic group at d 42 were significantly lower than the salinomycin or unchallenged group. Similarly, Lee et al. (2007b) reported that MitoGrow (as a probiotic) administered to *E. tenella*-infected birds, did not improve weight gains or oocyst output.

These findings clearly demonstrated the positive effects of probiotics in enhancing resistance of birds towards coccidiosis, but the positive effects on growth performance of birds challenged with *Eimeria* infection were not apparent.

Problem statement

Several probiotics have been shown to protect broiler chickens against coccidiosis, but the protective effects were not always associated with an improved broilers growth performance. Therefore, it was imperative to find probiotics species which possess anti-coccidiosis activity and at the same time capable of improving broilers growth performance when challenged with *E. tenella*.

Objective

The main objective of the study was to isolate and screen beneficial bacteria with probiotic properties and anti-coccidial activity as feed additive for broiler chicken. The specific objectives were:

- i. To isolate and characterize beneficial *Lactobacillus* spp. from broiler chicken intestine with probiotic properties.
- ii. To evaluate the efficacy of isolated probiotics in the performance of broiler chickens challenged with *Eimeria tenella*.

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BIODATA OF STUDENT

The author, Wan Nur Fadhilah Binti Shamsudin was born in 1986 (27th April) in Klang, Selangor. She did her Diploma in Science in 2008 at Universiti Teknologi MARA (UiTM) Jengka, Pahang. She was then conferred her Bachelor in Science (Hons) degree (Biomolecular Science) in 2012 by Universiti Teknologi MARA (UiTM) Shah Alam. Upon graduation, she started her career as a research officer at the Agro-Biotechnology Institute, Malaysia (ABI), Serdang. At the same time, she enrolled as a Master of Science student in Applied Microbiology at the Institute of Bioscience, Universiti Putra Malaysia under the supervision of Prof. Dr. Ho Yin Wan (until she retired in 2015), followed by the late Assoc. Prof. Dr. Sieo Chin Chin (until October 2015) and then under Prof. Dr. Norhani Abdullah. She was involved in a research project funded by the Ministry of Science under ScienceFund in a project titled “Evaluation of Probiotic Formulations for The Control of Coccidiosis in Broiler Chickens”. The author was actively involved as facilitator and committee member in several workshops and seminars: (i) Organizing Committee for Facility Handover on Plant Tissue Culture Project in Kampung Lapasan, Tuaran, Sabah In June 2017; (ii) Facilitator for plant tissue culture in Tuaran, Sabah, under Science Technology and Innovation event in May, 2017 (iii) Committee Member for organizing the Workshop on Plant Tissue Culture Technology under MOSTI Social Innovation Fund in Kota Kinabalu, Sabah, ion December 2016; (iv) Committee Member for National Institutes of Biotechnology Malaysia (NIBM) Convention, at MGI, Bangi in October 2016; (v) Organizing Committee in the Workshop on Bacteriophage Propagation and Purification (BPP) 2014 at Laboratory of Vaccines and Immunotherapeutics (LIVES), Institute of Bioscience, UPM. During her study, she had published her scientific findings in several conferences locally.

LIST OF PUBLICATIONS

Proceedings of paper presented in conferences and seminar:

- Shamsudin.W.N.F.**, Ho, Y.W., Sieo, C.C., Wan, K.L., Azizi, A.A., Shanmugavelu.S., and Loo, S.S. Effects of *Lactobacillus* Isolated from Broiler Chickens on Growth Performance and Coccidiosis Infection Towards Broiler Chickens. International Conference on Life Sciences Revolution: Past, Present, Future and Beyond. Shah Alam 24-25th November 2015. Page: 149-151.
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- Shamsudin.W.N.F.**, Loo, S.S., Sieo, C.C., Kalavathy, R., Lim, S.M., Lai, K.S., Ho, Y.W. 2014. *In Vitro* Screening for *Lactobacillus* with Adherence Property from Broiler Chicken Intestines. National Postgraduate Seminar 2014. Page: 75.
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