



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF PREBIOTIC, PROBIOTIC AND SYNBIOTIC ON
PERFORMANCE AND CECAL MICROBIOME OF
LAYING HENS***

SHIRLEY TANG GEE HOON

IB 2016 11



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By

SHIRLEY TANG GEE HOON

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

July 2016

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DEDICATIONS

This thesis is dedicated to my lovely grandmother, mother, aunties, uncle, husband and my siblings for their love, endless support and encouragement



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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Chairman : Associate Professor Wan Zuhainis Saad, PhD
Institute : Bioscience

The rampant use of antibiotics as growth promoters in poultry feed has led to development of antibiotic resistant bacteria and accumulation of antibiotic residues in poultry eggs and meat. Probiotic bacterial strains, prebiotic oligosaccharides and their combination as synbiotics (probiotics + prebiotics) have been considered as alternatives to antibiotic growth promoters (AGPs) in poultry production. A recent study showed that prebiotic isomaltooligosaccharide (IMO) significantly improved the performance and intestinal health of broiler chickens. The effects and role of IMO however, have not been explored on laying hens. Therefore, the present study was initiated to evaluate the *in vivo* effects of prebiotic IMO (PRE) and probiotic PrimaLac[®] (PRO), administered singly, and in combination as a synbiotic (SYN) (IMO + PrimaLac[®]), on performance, biochemical and hematological responses, egg quality and chemical compositions of egg yolks, and diversity and functional potential of cecal microbiome of laying hens. The layer feeding trial showed that the supplementation of PRE, PRO or SYN significantly ($P < 0.05$) improved the body weight gain, feed intake, feed conversion ratio (FCR), egg production, egg weight, egg mass and egg size of laying hens at 20-36, 37-52 and/or 20-52 weeks of age. PRE, PRO or SYN supplementation also significantly ($P < 0.05$) lowered the levels of serum total cholesterol, low-density-lipoprotein (LDL) cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), heterophil (H) percentages and H/L ratio, and increased lymphocyte (L) percentages at 36 and 52 weeks of age. PRE, PRO or SYN supplementation resulted in significant ($P < 0.05$) decreases in the cholesterol (24 and 28 weeks of age) and saturated fatty acid (SFA; 28, 32 and 36 weeks of age), and increases in the unsaturated fatty acid (UFA; 28, 32 and 36 weeks of age) and polyunsaturated fatty acid (PUFA; 28 weeks of age), including linoleic and alpha-linolenic acids of eggs without affecting the egg quality (Haugh unit, relative weights of the albumen and yolk, specific gravity, shell thickness and yolk color) (20-36 weeks of age), and lipid, carotenoid and tocopherol contents of eggs (24, 28, 32 and 36 weeks of age). The quantification of the bacterial population using quantitative real-time PCR (qPCR) showed that PRE, PRO or SYN supplementation significantly ($P < 0.05$) increased the populations of cecal lactobacilli and

bifidobacteria at 36 and 52 weeks of age, but decreased the populations of total cecal Enterobacteriaceae and *Escherichia coli* at 36 weeks of age. The cecal volatile fatty acids (VFA; acetic, propionic and butyric acids) and non-VFA (lactic and succinic acids) were significantly ($P < 0.05$) increased whilst harmful microbial enzyme activities (β -glucosidase, β -glucuronidase and urease) significantly ($P < 0.05$) decreased after PRE, PRO or SYN supplementation at 36 and 52 weeks of age. The 16S rRNA deep-sequencing analysis revealed that the relative abundance of cecal lactobacilli was significantly ($P < 0.05$) increased in all supplemented hens (PRE, PRO or SYN) at 36 and 52 weeks of age. The relative abundance of cecal bifidobacteria was also increased significantly ($P < 0.05$) in PRE- or SYN-supplemented hens at 36 weeks of age, and in PRE-, PRO- or SYN-supplemented hens at 52 weeks of age. At 36 weeks of age, the relative abundance of cecal Enterobacteriaceae decreased significantly ($P < 0.05$) after PRE, PRO or SYN supplementation. The whole metagenome shotgun sequencing analysis indicated that the microbiome of PRE-, PRO- or SYN-fed hens were predicted to have higher percentages of genes involved in carbohydrate metabolism (gluconeogenesis/glycolysis, citrate cycle, butanoate and pyruvate metabolisms); xenobiotics biodegradation and metabolisms (dioxin, xylene and toluene degradation); lipid metabolism (arachidonic acid metabolism and fatty acid elongation); Adenosine Triphosphate (ATP)-binding cassette transporters (ABC transporters); signalling molecules and interaction; immune and digestive systems as compared to control hens at 36 weeks of age. In conclusion, the results of the present study showed that the supplementation of PRE, PRO or SYN could improve the hen performance, produce eggs with lower cholesterol and SFA, and promote the hen health by altering the microbial populations, diversity and functional potential of cecal microbiome without affecting the egg quality of laying hens.

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

**KESAN PREBIOTIK, PROBIOTIK DAN SINBIOTIK TERHADAP
PRESTASI DAN MIKROBIOM SEKUM AYAM PENELUR**

Oleh

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Penggunaan antibiotik sebagai penggalak pertumbuhan dalam makanan poltri secara berleluasa telah menyebabkan perkembangan bakteria rintangan antibiotik dan pengumpulan sisa antibiotik dalam telur dan daging poltri. Strain bakteria probiotik, oligosakarida prebiotik dan kombinasi mereka sebagai sinbiotik (probiotik + prebiotik) telah dipertimbangkan sebagai alternatif kepada antibiotik penggalak pertumbuhan dalam pengeluaran poltri. Baru-baru ini, satu kajian menunjukkan bahawa prebiotik isomaltooligosakarida (IMO) dapat mempertingkatkan prestasi dan kesihatan usus ayam pedaging dengan signifikannya. Walau bagaimanapun, kesan dan peranan IMO terhadap ayam penelur masih belum dikaji. Oleh demikian, kajian ini dijalankan untuk menilai kesan-kesan *in vivo* prebiotik IMO (PRE), probiotik PrimaLac[®] (PRO), yang diberikan secara individu, dan secara kombinasi sebagai sinbiotik (SYN) (IMO + PrimaLac[®]) terhadap prestasi, respon biokimia dan hematologi, kualiti telur dan komposisi kimia kuning telur, serta diversiti dan fungsian potensi mikrobiom sekum ayam penelur. Hasil kajian *in vivo* menunjukkan bahawa suplementasi PRE, PRO atau SYN menambah baik penambahan berat badan, pengambilan makanan, kadar penukaran makanan (FCR), produksi telur, berat telur, jisim telur dan saiz telur ayam penelur secara signifikan ($P < 0.05$) pada umur 20-36, 37-52 dan/atau 20-52 minggu. Suplementasi PRE, PRO atau SYN juga menurunkan paras jumlah serum kolesterol dan kolesterol LDL, ALP, ALT, peratusan heterofil (H) dan nisbah H/L, dan meningkatkan peratusan limfosit (L) dengan signifikannya ($P < 0.05$) pada umur 36 dan 52 minggu. Suplementasi PRE, PRO atau SYN telah mengurangkan kolesterol (pada umur 24 dan 28 minggu) dan asid lemak tepu (SFA; pada umur 28, 32 dan 36 minggu), dan meningkatkan asid lemak tak tepu (UFA; pada usia 28, 32 dan 36 minggu) dan asid lemak politaktepu (PUFA; pada umur 28 minggu), termasuk asid linoleik dan alfa linolenik dalam telur dengan signifikannya ($P < 0.05$) tanpa memberi kesan kepada kualiti telur (Unit Haugh, berat relatif albumen dan kuning telur, graviti spesifik, ketebalan kulit telur dan warna kuning telur) (dari umur 20-36 minggu), dan lemak, karotenoid dan kandungan tokoferol telur (pada umur 24, 28, 32 dan 36 minggu). Kuantifikasi populasi bakteria dengan menggunakan kuantitatif PCR (qPCR) menunjukkan bahawa suplementasi PRE, PRO atau SYN meningkatkan populasi sekal laktobasili

dan bifidobakteria dengan signifikannya ($P < 0.05$) pada umur 36 dan 52 minggu, tetapi, mengurangkan populasi jumlah sekel Enterobacteriaceae dan *Escherichia coli* pada umur 36 minggu. Asid lemak meruap (VFA; asid asetik, propionik dan butyrik) dan bukan VFA (asid laktik dan suksinik) dalam sekum ayam penelur meningkat secara signifikan ($P < 0.05$), dan aktiviti enzim mikrob berbahaya (“ β -glukosidase”, “ β -glukuronidase” dan “urease”) menurun secara signifikan ($P < 0.05$) selepas suplementasi dengan PRE, PRO atau SYN pada umur 36 dan 52 minggu. Analisis penjujukan 16S rRNA menunjukkan bahawa kelebihan relatif laktobasili telah ditingkatkan secara signifikan ($P < 0.05$) dalam sekum ayam penelur yang disuplementasi dengan PRE, PRO atau SYN pada umur 36 dan 52 minggu. Kelebihan relatif bifidobakteria juga ditingkatkan dengan signifikannya ($P < 0.05$) dalam sekum ayam penelur yang disuplementasi dengan diet PRE atau SYN pada umur 36 minggu, dan diet PRE, PRO atau SYN pada umur 52 minggu. Pada umur 36 minggu, kelebihan relatif sekel Enterobacteriaceae diturunkan dengan signifikannya ($P < 0.05$) selepas suplementasi PRE, PRO atau SYN. Analisis penjujukan metagenomik “shotgun” menunjukkan bahawa mikrobiom ayam penelur yang diberi diet PRE, PRO atau SYN dijangka mempunyai peratusan gen yang lebih tinggi dalam metabolisme karbohidrat (glukoneogenesis/glikolisis, kitaran sitrat, metabolisme butanoate dan piruvat); xenobiotik biodegradasi dan metabolisme (degradasi dioksin, xilena dan toluena); metabolisme lemak (metabolisme asid arakidonik dan pemanjangan asid lemak); pengangkut “ATP-binding cassette” (pengangkut ABC); pengisyaratan molekul dan interaksi; sistem imun dan pencernaan, jika berbanding dengan ayam penelur kawalan pada umur 36 minggu. Kesimpulannya, hasil-hasil kajian tersebut menunjukkan suplementasi PRE, PRO atau SYN dapat menambah baik prestasi, menghasilkan telur dengan paras kolesterol dan SFA yang rendah, serta meningkatkan kesihatan ayam penelur dengan memanipulasikan populasi, diversiti dan fungsian potensi mikrobiom sekum tanpa mempengaruhi kualiti telur ayam penelur.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my ex-supervisor, Professor Dr. Ho Yin Wan, for her attentive guidance, endless patience and encouragement throughout the course of my study. Moreover, I have obtained valuable insights through her advices that have helped me grow not only academically but also build my character, resilience and grit. I am grateful and honored for being given the opportunity to pursue my doctoral degree under her supervision. I wish her the very best of health and happiness in her retirement.

I wish to express my deep appreciation and most sincere gratitude to the late Associate Professor Dr. Sieo Chin Chin (who was chairperson of my supervisory committee till she passed away in October 2015), for her invaluable guidance, support and encouragement in completing this research work. Her warm, engaging and friendly approach has boosted my confidence throughout this study.

My heartfelt appreciations are extended to the chairperson of my supervisory committee, Associate Professor Dr. Wan Zuhainis Saad, for her kind assistance, support and advice that nurtured my passion for research.

I wish to extend my sincere thanks to my co-supervisor, Associate Professor Dr. Kalavathy Ramasamy, for her assistance and constant encouragement throughout the course of my work and in the preparation of the thesis. Special appreciation goes to Mr Wong Hee Kum, ex-Deputy Director of Strategic Livestock Research Centre, MARDI, for sharing his expertise in layer management and feed formulation.

I am indebted and thankful to Madam Haw Ah Kam, Mr Nagayah Muniandy, Mr. Khairul Kamar Bakri, Cik Nadia, staffs of the Probiotic and Prebiotic Technology Programme, Laboratory of Vaccine and Immunotherapeutics, Institute of Bioscience, for their technical support and kind assistance throughout my study.

I sincerely thank the Department of Higher Education, Ministry of Education, for the PhD funding and the Ministry of Science, Technology and Innovation (MOSTI) for the research project funding.

I appreciate the friendship, motivation and support from my labmates and friends, Wei Li, Su Ting, Fadhilah, Helen Tang, Ling Lit, Saminathan, Shaufi, Soo Ning, Ying Ying, Ying Jie and Mei Chee. I would like to thank them for their help, moral support, active co-operation and invaluable support. I have enjoyed the time that I spent with them throughout my studies.

I am truly blessed and fortunate to have my beloved husband Pin Jern, who has selflessly supported my decision to pursue my PhD. I thank him for always being there, at every step of all my endeavours, and for helping me overcome my fears and failures. I truthfully appreciate his courage, understanding and trust which have been the pillars in our relationship.

To my beloved grandmother, mother, father-in-law, mother-in-law, uncle, aunties, sisters, brother, brother-in-laws, sister-in-laws and cousins, your support, both morally and financially, have enabled me to complete my study with confidence and without having to fear and worry.



I certify that a Thesis Examination Committee has met on 14 July 2016 to conduct the final examination of Shirley Tang Gee Hoon on her thesis entitled "Effects of Prebiotic, Probiotic and Synbiotic on Performance and Cecal Microbiome of Laying Hens" 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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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACE	Abundance-based Coverage Estimators
AGPs	Antibiotic growth promoters
ALA	Alpha-linolenic acid
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BCFA	Branched chain fatty acid
bp	Basepair
BSM	Bifidus Selective Medium
BSH	Bile salt hydrolase
C	Coverage
Ca	Calcium
cm	Centimeter
cfu	Colony-forming unit
CH ₄	Methane
CK	Creatinine kinase
Cl	Chloride
Cq	Quantitative cycle
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
E	Amplification efficiency
EC	Enzyme Commission
EDTA	Ethylene diamine tetraacetate
EFAs	Essential fatty acids
EMP	Embden Meyerhof Parnas
EPA	Eicosapentaenoic acid
FAMES	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
FID	Flame ionization detector
FISH	Fluorescence <i>in situ</i> hybridization
fL	Femtoliter
FOS	Fructooligosaccharide
g	Gram
g	Gravity
GB	Gigabyte
GC	Gas chromatography
GGT	Gamma-glutamyl transpeptidase
GOS	Galactooligosaccharide
H	Heterophil

h	Hour
H/L	Heterophil/Lymphocyte ratio
H ₂ S	Hydrogen sulphide
Hb	Hemoglobin
HCN	Hydrocyanic acid
HDL	High-density lipoprotein
HMG CoA	Hydroxymethylglutaryl coenzyme A
HPLC	High performance liquid chromatography
HT-NGS	High-throughput next generation sequencing
HU	Haugh unit
IDBA-UD	De Bruijn Graph De Novo Assembler for Short Reads Sequencing data with Highly Uneven Sequencing Depth
IL	Interleukine
IMO	Isomaltooligosaccharide
IU	International units
K	Potassium
k	Kilo
K ₂ EDTA	Potassium enthylenediaminetetraacetate
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
L	Lymphocyte
L	Large
L	Liter
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
M	Medium
m	Meter
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MEGAN	MetaGenome Analyzer
mg	Milligram
min	Minute
MJ	Millijoules
ml	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimole
mol	Mole
MOS	Mannanligosaccharide
MRS	de Man, Rogosa and Sharpe
MTBE	Methyl-tert-butyl ether
MUFA	Monounsaturated fatty acid

N50	Average length of a set of sequences
Na	Sodium
NaCl	Sodium chloride
NaClO	Alkaline hypochlorite
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NDOs	Non-digestible oligosaccharides
NGS	Next generation sequencing
NH ₃	Ammonia
NH ₄ Cl	Ammonium chloride
(NH ₄) ₂ SO ₄	Ammonium sulphate
NK	Natural killer
nm	Nanometer
ng	Nanogram
nt	Nucleotide
NTC	No-template control
OTU	Operational taxonomic unit
P	Phosphorus
PANDAseq	PAired-eND Assembler for DNA sequences
PCR	Polymerase chain reaction
PCV	Packed cell volume
PP	6-phosphogluconate
PRE	Basal diet + 1% prebiotic IMO
PRO	Basal diet + 0.1% probiotic PrimaLac [®]
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinyl difluoride
PyNASt	Python Nearest Alignment Space Termination
q	Qscore
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
R ²	Correlation coefficient values
RAM	Random access memory
RBC	Red blood cell
RFO	Raffinose oligosaccharide
RNA	Ribonucleic acid
S	Small
s	Second
SCFA	Short chain fatty acid
SD	Standard deviations
SEM	Standard Error Mean
SFA	Saturated fatty acid
sIgA	Secretory Immunoglobulin A
SMS	Shotgun metagenomic sequencing

SOS	Soy-oligosaccharide
SRA	Sequence Read Archive
STAMP	Statistical Analysis of Metagenomic Profiles
STOC	Sucrose thermal oligosaccharides
SYN	Basal diet + 1% IMO + 0.1% PrimaLac [®]
TAE	Tris-acetate EDTA
TGF- β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
THF	Tetrahydrofuran
TLR	Toll-like receptor
TMS	Targeted metagenomic sequencing
TOS	Transgalactooligosaccharide
U/L	Units per litre
UFA	Unsaturated fatty acid
V	Volt
v/v	Volume per volume
VFA	Volatile fatty acid
VLDL	Very-low-density lipoprotein
WHO	World Health Organization
XL	Extra large
XOS	Xylooligosaccharide
μg	Microgram
μL	Microliter
μm	Micrometer
μM	Micromolar
μmol	Micromole

CHAPTER 1

INTRODUCTION

Since the late 1940s, antibiotics have been widely used for treatment of animals and as growth promoters for livestock (Aarestrup and Jensen, 2007). The efficiency of antibiotics in improving feed utilization, animal growth, and reducing morbidity and mortality due to clinical and subclinical diseases is well documented (Dibner and Richards, 2005; Niewold, 2007; Hao *et al.*, 2014). The imprudent use of antibiotics in animal production has resulted in the prevalence of antibiotic resistance bacteria and accumulation of antibiotic residues in animal products such as poultry meat and eggs, posing large threats to public health (Vieira *et al.*, 2011; Hao *et al.*, 2014). The prevalence of multi-drug resistant bacteria in poultry and other farm animals are on the rise throughout the world including Malaysia (Ahmed Geidem *et al.*, 2012; Mansouri-najand *et al.*, 2012; Szmolka and Nagy, 2013; Fan *et al.*, 2015). Consequently, many countries have started to restrict or ban the inclusion of antibiotics in poultry diets as routine means of growth promotion. In 2006, European Union banned the use of antibiotic growth promoters (AGPs) in animal feed. The withdrawal of antibiotics as growth promoters in poultry feed instigated the search for new alternatives to maintain animal welfare without affecting health and performance. A strategy envisaging the use of probiotics, prebiotics, or synbiotics, as feed additives in poultry nutrition has been proposed as substitutes for AGPs to enhance gastrointestinal health, poultry performance and produce safe poultry products.

Probiotics have been defined in various ways over the years but the Food Agriculture Organization (FAO)/World Health Organization (WHO) definition of probiotics remains applicable to scientific, industrial and regulatory communities. They coined probiotics as “live microorganisms which when administered in adequate amounts confer a beneficial effect on health of the host” (FAO/WHO, 2002). The majority of probiotics are lactic acid bacteria that belong to the genera *Lactobacillus* and *Bifidobacterium*. The supplementation of probiotics in poultry feed has been reported to improve growth performance (Jin *et al.*, 1998a; Jin *et al.*, 1998b; Kalavathy *et al.*, 2003; Awad *et al.*, 2009; Kalavathy *et al.*, 2009; Mountzouris *et al.*, 2010; Li *et al.*, 2014; Zhang and Kim, 2014; Calik and Ergün, 2015), nutrient retention (Li *et al.*, 2008; Mountzouris *et al.*, 2010; Shim *et al.*, 2012), cecal microbial balance (Teo and Tan, 2007; Mountzouris *et al.* 2007, 2010; Li *et al.*, 2014; Zhang and Kim, 2014; Calik and Ergün, 2015), egg yolk cholesterol contents (Haddadin *et al.*, 1996; Kalavathy *et al.*, 2009; Mikulski *et al.*, 2012) and immune response (Zuklifi *et al.*, 2000; Panda *et al.*, 2008; Molnár *et al.*, 2011; Zhang and Kim, 2014).

Prebiotics on the other hand are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of health-promoting bacteria in the colon and thus improve host health” (Gibson and Roberfroid, 1995). Unlike probiotics, prebiotics provide nourishment that enhances growth of the resident microflora in the gastrointestinal

tract. They are non-digestible oligosaccharides (NDOs) that are resistant to digestion in the upper gastrointestinal tract, and resistant to gastric acid and hydrolytic enzymes. Hence, they pass unaltered to the colon where the resident microflora, such as lactobacilli and bifidobacteria, can selectively ferment the NDOs to produce beneficial effects in the hosts. There are several types of NDOs, such as fructooligosaccharides (FOS; oligofructose and inulin), galactooligosaccharides (GOS), transgalactooligosaccharides (TOS), lactulose, glucooligosaccharides, glycol-oligosaccharides, isomaltooligosaccharides (IMO), lactitol, maltooligosaccharides, xylooligosaccharides (XOS), stachyose, raffinose and sucrose thermal oligosaccharide, which have been reported to possess prebiotic effects (Patterson and Burkholder, 2003; Desai *et al.*, 2004; Gaggia *et al.*, 2010; De Souza Oliveira *et al.*, 2011). Prebiotics have been reported to improve intestinal microflora, enhancing the health and well-being of the hosts (Gaggia *et al.*, 2010). Recently, prebiotics have been shown to confer health benefits in poultry by increasing the number of specific probiotic bacteria such as lactobacilli and bifidobacteria (Shang *et al.*, 2010; Kim *et al.*, 2011; Tako *et al.*, 2014), reducing pathogenic bacteria by mimicking their attachment sites on the intestinal mucosa (Iji and Tivey, 1998; Kim *et al.*, 2011), enhancing ileal digestibility (Huang *et al.*, 2005; Amirdahri *et al.*, 2012), increasing mineral absorption (Sohail *et al.*, 2011) and reducing serum cholesterol levels (Chen *et al.*, 2005a; Li *et al.*, 2007; Al-Saad *et al.*, 2014).

The term “synbiotics” refer to nutritional supplements combining probiotics and prebiotics in a synergistic form. Gibson and Roberfroid (1995) defined synbiotic as “a mixture of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, thus improving the host health”. Synbiotics potentiate the efficacy of probiotic preparations (Awad *et al.*, 2009). It enables the probiotics to survive for a longer period in the gastrointestinal tract of the host (Collins and Gibson, 1999). Most of the previous research on poultry involved the use of probiotics or prebiotics alone whilst the use of synbiotics has emerged in poultry only recently (Abdelqader *et al.*, 2013a, 2013b; Dibaji *et al.*, 2014).

A recent study evaluated the effects of a prebiotic IMO on the performance, cecal bacterial populations and cecal metabolic profiles of broiler chickens (Mookiah *et al.*, 2014). The results of the study demonstrated that the performance of broiler chickens (weight gain and feed efficiency), the populations of cecal beneficial bacteria and the cecal fermentative concentrations improved significantly after supplementation of IMO in broiler chickens. The role of IMO and its combination with a probiotic as a synbiotic however, has not been explored on laying hens.

Therefore, the present study was aimed to evaluate the effect of prebiotic IMO, and a commercial probiotic (PrimaLac[®]), administered singly, or in a combination as a synbiotic, on laying hens. This is the first study on the dietary effects of the prebiotic (IMO), and its synbiotic on laying hens. This study will also unravel the role of probiotics, prebiotics and synbiotics in modulating cecal microbial diversity. The specific objectives of the study were:

- i. to determine the efficacy of the prebiotic IMO (PRE), and probiotic PrimaLac[®] (PRO), administered singly, and their combination as a synbiotic (IMO + PrimaLac[®]) (SYN) on the performance, biochemical and hematological responses, and relative organ weights of the laying hens,
- ii. to determine the effects of the PRE, PRO and SYN on egg quality and chemical compositions of egg yolk (total lipids, cholesterol, fatty acid composition, tocopherols and carotenoids) of laying hens,
- iii. to evaluate cecal microbial populations, cecal metabolite profile and microbial enzyme activities of laying hens supplemented with PRE, PRO and SYN diets,
- iv. to evaluate cecal microbial diversity of hens fed PRE, PRO and SYN using 16S rRNA high-throughput-Next Generation Sequencing (HT-NGS),
- v. to investigate the functional potential of hen cecal microbial in response to PRE, PRO and SYN treatments using whole metagenome shotgun sequencing.

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