UNIVERSITI PUTRA MALAYSIA

PREBIOTIC EFFECTS OF PALM KERNEL EXPELLER OLIGOSACCHARIDES EXTRACT ON INTESTINAL MICROBIOTA AND IMMUNE RESPONSE IN Salmonella-CHALLENGED BROILER CHICKEN

SIAMAK REZAEI

IPTSM 2016 1

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By

SIAMAK REZAEI

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

November 2016

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DEDICATION

This thesis is dedicated to my parents for their loves, endless support and encouragement

Abstract of thesis presented to the Senates of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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November 2016

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Institute : Tropical Agriculture and Food Security

The increased of the development of microbial resistance due to the use of antibiotics in animal feeds leads to search for a replacement for antibiotics. Prebiotics, which are non-digestible food ingredients, that beneficially affect the host by selectively stimulating the growth and/or activity of beneficial bacterial species residing in the colon had been reported as potential substitutes for antibiotics. Oligosaccharides from palm kernel expeller (PKE) have been reported to exhibit prebiotic effect in chicken. The aim of this study was to examine the periodic effects of oligosaccharides extract from PKE (OligoPKE) on immune system in broiler chickens.

The first study examined the prebiotic effects of OligoOKE on growth performance, cecal microbiota and immune response in ninety 1-day-old Cobb500 broiler chicks. Dietary treatments were basal diet as control, basal diet plus 0.5% OligoPKE, and basal diet plus 1% OligoPKE. Chicken performance, caecal microbial quantification, all white blood cells type counted and immunoglobulins (IgA and IgM) were determined. Results showed that chickens fed OligoPKE supplemented diet had better FCR ($P<0.05$). Immunoglobulin and hematological data suggested that OligoPKE improved immune responses in broiler chickens, been more efficient during their younger age when their immune system was still not fully developed.

The second experiment was designed to farther examine the prebiotic effects of OligoPKE on cecal microbiota including *Salmonella* population and immune response in chicks challenged with *Salmonella enteritidis* and *Salmonella typhyimuriu*m. A total of ninety 1-day-old chicks were randomly allocated to six treatment groups of 15 birds each. From day 1, chicks in three treatment groups were fed corn-soy based starter diet (22% CP and 3000 kcal ME/kg) and birds in the remaining three treatment groups received the same diet supplemented with 1%

OligoPKE. On day 3, chicks in the first group within each dietary treatments were orally administered with 0.5 ml of *S. enteritidis* (1.0 x 10^9 CFU) and the second group with equal amount of *S. typhimurium* (1.0 x 10^9 CFU) while the third group was kept as control. OligoPKE supplementation reduced *Salmonella* population in both intact and challenged birds with stronger suppression effect in *S. typhimurium* group ($P<0.05$). Both challenged groups had higher level of plasma IgA, and OligoPKE supplementation elevated the plasma IgA response in *S. typhimurium* and jejunal IgA in *S. enteritidis* challenged birds. This result reaffirmed that OligoPKE acts as prebiotic, capable of enhancing beneficial cecal microbiota, suppressing pathogens and enhancing immune response in *Salmonella* challenged chicks.

The third experiment was designed to examine the prebiotic effects of OligoPKE on cecal microbiota including *Salmonella* population and immune response in chicks challenged with different level of *S. typhimurium*. A total of one hundred and twenty 1-day-old chicks were randomly allocated to eight treatment groups of 15 birds each. From day 1, chicks in four treatment groups were fed corn-soy based starter diet (22% CP and 3000 kcal ME/kg) and birds in the remaining four treatment groups received the same diet supplemented with 1% OligoPKE. On day 3, chicks in the first groups within each dietary treatment were orally administered with 0.5 ml of *S. typhimurium* (1.0 x 10^8 CFU/mL), and the second groups were orally administered with 0.5 ml of *S. typhimurium* (1.0 x 10^6 CFU/mL) and third 0.5 ml of *S.* typhimurium (1.0 x 10^4 CFU/mL) respectively. While the fourth groups of each dietary treatments were kept intact. Samples were taken at day 10. Results of this experiment showed that OligoPKE supplementation reduced *Salmonella* population with all levels of contaminations in cecum and jejunum. In addition, OligoPKE increased the immunoglobulin A response in chicken in all the challenged groups. Data of immune gene expression showed that oligosaccharides could improve the total immunity (IL-2, IL-8, IL-10, IFN- γ, TNF- α and Muccin) of chicken.

The last experiment was designed to examine the prebiotic effects of OligoPKE on cecal microbiota including *Salmonella* population and immune response in chicks challenged with *S. enteritidis* from three different sources, named ground, food and water. A total of eighty 1-day-old chicks were randomly allocated to eight treatment groups of 10 birds each. From day 1, chicks in four treatment groups were fed corn-soy based starter diet (22% CP and 3000 kcal ME/kg) and birds in the remaining four treatment groups received the same diet supplemented with 1% OligoPKE. On day 3, chicks in the first group within each dietary treatment received water contaminated with *S. enteritidis* (1.0 x 10^9 CFU/mL). While birds in second group were challenged with *S. enteritidis* (1.0 x 10^9 CFU/g) via contaminated feed. And the third group located in cages with contaminated floor with *S. enteritidis* (1.0 x 10^9 CFU/cm). While the fourth groups of each dietary treatments were kept intact. Results of this study showed that OligoPKE supplementation reduced *Salmonella* population in cecum and jejunum in both groups of contaminated water and feed with stronger effect in the contaminated water group ($P<0.05$). OligoPKE increased immunoglobulin A response in chicken in all challenged groups. Once again, the immune gene expression data showed that oligosaccharides could improve the total immunity (IL-2, IL-8, IL-10, IFN- γ, TNF- α and Muccin) of chicks.

In conclusion, OligoPKE had shown its capability as a prebiotic by reducing pathogens in cecum and jejunum in *Salmonella* infected birds, increasing the immune response by effecting immunoglobulin production and the expression of immune related genes.

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

KESAN PREBIOTIK OLEH OLIGOSAKARIDA DARIPADA ISIRONG KELAPA SAWIT TERHADAP MIKROB USUS DAN RESPON DAYA TAHAN PENYAKIT DI DALAM AYAM PEDAGING YANG DIJANGKITI *Salmonella*

Oleh

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Penggunaan antibiotik dalam penyediaan makanan haiwan telah sangat meluas terutama bagi membantu tumbesaran dan mencegah penyakit. Kesan penggunaan antibiotik dalam makanan haiwan ini telah mengakibatkan pertumbuhan gen yang mempunyai daya tahan tinggi terhadap mikrob, dan ini telah mencetuskan kajian untuk mendapatkan pengganti bagi antibiotik. Pribiotik yang mampu menggantikan antibiotik, ialah bahan makanan yang sukar dicernakan tetapi memberi manfaat pada haiwan dengan merangsang tumbesaran dan/atau aktiviti bakteria bermanfaat yang terdapat di dalam sekalnya. Isirong kelapa sawit merupakan produk hiliran dari industri kelap sawit yang sangat penting. Dengan komposis serat yang tinggi, ia sering digunakan sebagai bahan makanan dalam ternakan ruminan. Tujuan penyelidikan ini dijalankan adalah bagi mengkaji kesan berkala oleh ekstrak oligosakarida daripada isirong kelapa sawit (OligoPKE) terhadap sistem imunisasi ayam pedaging.

Kajian yang pertama melibatkan kesan berkala OligoPKE terhadap tumbesaran, mikrobiota sekal, dan respon daya tahan penyakit oleh ayam pedaging. Sembilan puluh (90) ekor ayam pedaging baka Cobb500 berumur 1 hari telah dibahagikan secara rambang kepada tiga kumpulan (diet) yang terdiri daripada enam sangkar (replikasi) untuk satu kumpulan, dimana lima ekor ayam diletakkan di dalam satu sangkar. Kumpulan diet terdiri daripada diet asas sebagai kawalan, diet asas ditambah 0.5% OligoPKE, dan diet asas ditambah 1% OligoPKE. Tumbesaran ayam dinilai pada peringkat permulaan (hari 1 ke 21), penamat (hari 22 ke 35) dan keseluruhan tempoh ujikaji. Digesta, kuantifikasi mikrob, semua jenis sel darah putih dan imunoglobulin (IgA dan IgM) telah ditentukan. Hasil kajian menunjukkan ayam yang diberi diet suplemen OligoPKE mempunyai FCR yang lebih bagus ($P<0.05$). Pada umur 21 hari dan 35 hari, konsentrasi IgA darah meningkat dengan

peningkatan kadar OligoPKE di dalam diet, dimana 1% suplemen OligoPKE merekodkan satu lipatan meningkat berbanding kumpulan kawalan. Bilangan heterofil dan besofil dalam ayam yang diberi diet suplemen OligoPKE lebih rendah berbanding kumpulan kawalan pada umur 21 hari. Dapatan imunoglobulin dan hematologi di atas menunjukkan OligoPKE mampu menambah baik respon daya tahan penyakit dalam ayam pedaging, terutama diperingkat umur yang lebih muda dimana sistem imuninasi masih belum terbentuk sempurna.

Kajian kedua dirangka untuk mengkaji kesan pribiotik oleh OligoPKE terhadap mikrobiota sekal termasuk populasi *Salmonella* dan respon daya tahan penyakit anak ayam yang terdedah kepada *Salmonella enteritidis* dan *Salmonella typhyimuriu*m. Sembilan puluh ekor anak ayam berumur 1 hari telah dibahagikan secara rambang kepada enam kumpulan rawatan yang terdiri daripada 15 ekor anak ayam sekumpulan. Tiga kumpulan rawatan diberi makan diet permulaan jagung-soya (22% CP dan 3000 kkal ME/kg) dan tiga kumpulan lain diberi diet sama yang disuplemen dengan 1% OligoPKE. Pada hari 3, daripada setiap kumpulan diet, anak ayam daripada kumpulan pertama diberi 0.5 ml *S. enteritidis* (1.0×10^9 CFU) secara oral, anak ayam daripada kumpulan kedua diberi 0.5 ml *S. typhimurium* (1.0×10^9 CFU) secara oral dan kumpulan ketiga dibiarkan sebagai kawalan. Hasil kajian menunjukkan suplemen OligoPKE meningkatkan populasi *Lactobacillus* dan *Bifidobacterium* serta mengurangkan populasi *Entrobacter* di dalam sekum. Jumlah populasi *Salmonella* di dalam anak ayam yang terdedah kepada *S. typhimurium* atau *S. enteritidis* meningkat berbanding kumpulan kawalan yang bebas *Salmonella*. Walaubagaimanapun, suplemen OligoPKE dapat mengurangkan populasi *Salmonella* di dalam kedua-dua keadaan anak ayam, dimana kesan yang lebih kuat dilihat pada kumpulan *S. typhimurium* ($P<0.05$). Kedua-dua kumpulan yang didedahkan kepada jangkitan mempunyai kadar plasma IgA yang lebih tinggi, dan suplemen OligoPKE meningkatkan plasma IgA apabila didedahkan kwpada *S. typhimurium* dan jejunal IgA apabila didedahkan kepada *S. enteritidis.* Sebagai konklusi, kajian ini menunjukkan OligoPKE dapat bertindak sebagai pribiotik, dan ia mampu memperbaik mikrobiota sekal bermanfaat, menahan patogen, dan meningkatkan respon daya tahan jangkitan *Salmonella* di dalam ayam.

Kajian ketiga dijalankan untuk mengkaji kesan pribiotik oleh OligoPKE terhadap mikrobiota sekal termasuk populasi *Salmonella* dan respon daya tahan penyakit dalam anak ayam yang didedahkan kepada kadar jangkitan yang berbeza oleh *S. typhimurium.* Satu ratus dua puluh ekor anak ayam berumur 1 hari telah dibahagikan secara rambang kepada lapan kumpulan rawatan yang terdiri daripada 15 ekor ayam sekumpulan. Empat kumpulan rawatan diberi makan diet permulaan jagung-soya (22% CP dan 3000 kkal ME/kg) dan empat kumpulan lain diberi diet sama yang disuplemen dengan 1% OligoPKE. Pada hari 3, daripada setiap kumpulan diet, anak ayam daripada kumpulan pertama diberi 0.5 ml *S. typhimurium* (1.0×10^8 CFU) secara oral, anak ayam daripada kumpulan kedua diberi 0.5 ml *S. typhimurium* (1.0×10^6 CFU) secara oral, anak ayam daripada kumpulan ketiga diberi 0.5 ml of *S.* typhimurium (1.0×10^4 CFU) secara oral dan kumpulan keempat dibiarkan sebagai kawalan. Sampel telah diambil pada hari ke 10. Pada pengambilan sampel pertama, suplemen OligoPKE kepada anak ayam didapati telah mengurangkan populasi Salmonella dalam sekum dan jejunum pada semua tahap kontaminasi. Pengurangan

populasi Salmonella berterusan pada pengambilan sampel kedua. OligoPKE telah meningkatkan respon IgA dalam ayam pada semua tahap kontaminasi, dan peningkatan respon direkodkan apabila usia ayam meningkat. OligoPKE juga boleh meningkatkan jumlah imuniti (IL-2, IL-8, IL-10, IFN- γ, TNF- α dan Muccin) oleh ayam, menerusi ujikaji ekspresi oleh gen imun.

Kajian terakhir dijalankan bagi mengkaji kesan pribiotik oleh OligoPKE terhadap mikrobiota sekal termasuk populasi *Salmonella* dan respon imun dalam anak ayam yang didedahkan kepada *S. enteritidis* dari sumber tanah, makanan, dan air. Lapan puluh ekor anak ayam berumur 1 hari telah dibahagikan kepada lapan kumpulan rawatan yang terdiri daripada 10 ekor ayam sekumpulan. Empat kumpulan rawatan diberi makan diet permulaan jagung-soya (22% CP dan 3000 kkal ME/kg) dan empat kumpulan lain diberi diet sama yang disuplemen dengan 1% OligoPKE. Pada hari 3, daripada setiap kumpulan diet, anak ayam daripada kumpulan pertama diberi air yang dicemari *S. enteritidis* (1.0×10^9 CFU/mL), anak ayam daripada kumpulan kedua diberi makanan yang dicemari dengan *S. enteritidis* (1.0×10^9 CFU/g), anak ayam daripada kumpulan ketiga ditempatkan di dalam sangkar berasingan di atas lantai yang dicemari dengan *S. enteritidis* (1.0×10^9 CFU/cm) dan kumpulan keempat dibiarkan sebagai kawalan. *Salmonella* tidak dapat dikesan di dalam sampel sekum dalam kumpulan kawalan dan kumpulan yang dicemari lantai/tanah. Suplemen OligoPKE telah mengurangkan populasi *Salmonella* dalam kedua-dua kumpulan air dan makanan tercemar, di mana kesan yang impak tahan yang lebih kuat daripada kumpulan air tercemar ($P<0.05$). Di dalam sampel jejunum, ayam lebih menunjukkan impak tahan yang lebih kuat terhadap air yang tercemar, walaupun pengurangan populasi *Salmonella* masih dapat dilihat dalam kumpulan makanan dan lantai/tanah tercemar. OligoPKE meningkatkan respon IgA di dalam ayam pada semua kumpulan kontaminasi. OligoPKE juga boleh meningkatkan jumlah imuniti (IL-2, IL-8, IL-10, IFN- γ, TNF- α dan Muccin) oleh ayam, menerusi ujikaji ekspresi oleh gen imun.

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I certify that a Thesis Examination Committee has met on 18 November 2016 to conduct the final examination of Siamak Rezaei on his thesis entitled "Prebiotic Effects of Palm Kernel Expeller Oligosaccharides Extract on Intestinal Microbiota and Immune Response in *Salmonella*-Challenged Broiler Chicken" 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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BW	body weight
BWG	body weight gain
CDC	centre of disease control
CFU	colony forming unit
cm	centimeter
CP	crude protein
DOA	date of age
E. coli	*Escherichia coli*
FCR	feed conversion ratio
FDA	food and drug administration
FI	feed intake
FOS	fructo-oligosaccharides
g	gram
g/L	gram per liter
GALT	gut associated lymphoid tissue
GAPDH	glyceraldehyde phosphate dehydrogenase
GIT	gastrointestinal tract
GOS	galacto-oligosaccharides
h	hour
HDL	high-density protein
IACUC	institutional animal care and use committee
IFN	interferon
IFN-α	Interferon-α
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-8	Interleukin-8
IMO	isomalto-oligosaccharides
L	liter
LDL	low-density protein
ME	metabolizing energy
mg	milligram
ml	millilitre
MOS	mannan-oligosaccharides

MUC	muccin
NDO	non-digestible oligosaccharides
NSPs	non-starch polysaccharides
ºC	degree centigrade
OligoPKE	oligosaccharides extracted from plam kernel expeller
PI	prebiotic index
PKE	Palm Kernel Expeller
PKM	Palm Kernel Meal
rpm	round per minute
S. enterica	*salmonella enterica*
S. enteritidis	*salmonella enteritidis*
S. gallinarum	*salmonella gallinarum*
S. Paratyphi	*salmonella paratyphi*
S. pullorum	*salmonella pullorum*
S. typhi	*salmonella typhi*
S. typhimurium	*salmonella typhimurium*
SFB	segmented filamentous bacteria
SOS	soy-oligosaccharides
TGOS	transgalacto-oligosaccharides
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-α
VFA	volatile fatty acids
VRI	veterinary research institute
XOS	xylo-oligosaccharides
µg	microgram
µg/ mL	microgram per millilitre
µg/g	microgram per gram
µL	microliter

CHAPTER 1

INTRODUCTION

Antibiotics have been widely used as supplements in animal feed to promote growth and prevent diseases in commercial farms in both, the developed and developing countries. The increase of the development of microbial resistance genes due to the continuous use of antibiotics in animal feeds (Houshmand et al., 2011) leads to search for substitutes for antibiotics. Probiotics are live micro-organisms that administered in adequate amounts confer a health benefit on the host, while prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of beneficial probiotic bacterial species residing in the intestine (Saad et al., 2013; Dhama et al., 2015). Probiotics and prebiotics have been shown to be potential substitutes for antibiotics.

Salmonella are gram-negative bacteria consisting of non-spore forming bacilli and are a member of the *Enterobacteriaceae* family (Dunkley et al., 2009). *Salmonella* microorganism, with 2,668 different serotypes, can cause diseases in most of animal species (human, animal, bird, reptile and even insects). *Salmonella enterica* and *Salmonella bongori* are the two main species causing *Salmonella* infections. While *Salmonella bongori* with 21 serotypes can be usually found in cold blood animal, *Salmonella enterica* which is divided into 6 subspecies according to chromosomal DNA hybridization and multilocus enzyme electrophoresis, cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals. *Salmonella pullorum* and *Salmonella gallinarum* and recently *Salmonella enteritidis* and *Salmonella typhimurium* were reported to cause infection in poultry (Akbarmehr et al., 2010). And *Salmonella enteritidis* has been reported to be one of the most pathogenic in chickens which can contaminate egg products by infected laying chickens resulting in serious food-borne illness and human poisoning (Fernandez et al., 2002b; Jawale and Lee, 2014).

Salmonella can cause intestinal infections in birds with high mortality rates especially in young birds below two weeks old (Dunkley et al., 2009). Egg shells can be contaminated with *Salmonella* as a result of passage through the intestinal tract and the ability of the pathogen to penetrate into the avian egg. Not only the egg shell is known as source of contamination, some *Salmonella* strains can be transferred to the next generation from the egg. *Salmonella* is invasive in laying hens by contaminating egg content by colonization in the intestinal tract and by trans-ovarian transmission (Humphrey, 1994). *S. enteritidis* can also colonize the vaginal epithelium of chickens and spread in the flock rapidly via contaminated faeces and contaminated eggs (Mizumoto et al., 2005).

Palm Kernel Expeller (PKE) is an important byproduct from the oil palm industry in many tropical and sub-tropical countries, including Malaysia. Being high in fiber, PKE is commonly used as feed ingredient in ruminant diets (Erickson et al., 2012); however, many attempts have been made to use PKE as energy and protein sources

in broiler diets, but with inconsistent results (Sundu et al., 2006; Saenphoom et al., 2013). Since between 58 to 78% of the PKE fiber are made up of insoluble hemicelluloses-mannan (Daud and Jarvis, 1992; Dusterhoft and Voragen, 1991), and recent studies demonstrated that the extract of PKE containing a mixture of mainly mannan- monosaccharides and oligosaccharides (Jahromi et al., 2016) which could modulate gut microflora, particularly reducing pathogenic bacteria, such as *E. coli*, in rats (Chen et al., 2015). Theoretically, these oligosaccharides can withstand the digestive enzymes and flow to the lower intestines intact to be used by beneficial bacteria. Most enteric pathogens, including *Salmonella*, must be able to attach to the mucosal surface of the gut wall of the host animals in order to stay and penetrate in the gastrointestinal tract. Thus, one way to prevent attachment of these pathogens to the gut wall is by providing competition for the attachment sites (Schneitz et al., 1993). Because the attachment is often mediated through binding of bacterial lectins to receptors containing D-mannose (Al, 1978), it may be possible to block the lectins with mannose and to inhibit bacterial attachment (Spring et al., 1999).

With the global demand for new prebiotic sources as an antibiotic replacement in order to avoid increasing of antibiotic resistant gene, the aim of this thesis was to examine the efficacy of oligosaccharides extract from PKE, as a prebiotic source, on intestinal microbiota population, and immune response in *Salmonella* infection in broiler chicks. To achieve this objective, four experiments were designed to examine oligoPKE prebiotic effects on two main non-typhoid *Salmonella* strains (*S. typhimurium* and *S. enteritidis*), with different level of contamination and different sources of contamination, in immune system.

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