UNIVERSITI PUTRA MALAYSIA

EFFECTS OF POSTBIOTIC AND INULIN SUPPLEMENTS ON GROWTH PERFORMANCE, GUT MORPHOLOGY, GENE EXPRESSION AND FECAL CHARACTERISTICS OF BROILER CHICKENS

KARWAN YASEEN KARREM

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By

KARWAN YASEEN KARREM

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

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DEDICATION

I would like to dedicate this work to those who taught, motivated and helped me throughout my study. This work is also dedicated to my dearest wife, Dlgash Nooraldin Kareem without her support and love, I could not have accomplished many of the things that I have accomplished.

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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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By

KARWAN YASEEN KAREEM

May 2016

Chairman : Professor Loh Teck Chwen, PhD
Faculty : Agriculture

Antibiotic growth promoters (AGP) are effective in modulating the gut microflora and promoting growth performance in birds. Nonetheless, prolonged use of AGP can cause antibiotic resistance and residual effects, which could imperil the health of birds and human consumers. Prebiotics, probiotics and their combinations are potent replacements for AGP. Nonetheless, commercial probiotics have consistently failed to meet the anticipated capability and their efficacy is uncertain. In addition, the use of probiotics could lead to the occurrence of antibiotics gene resistance that could be transferred between organisms. This justifies the need to search for suitable and safe alternative to probiotics. Postbiotic, a metabolite of probiotics exhibits probiotic effect without living cells. Despite the efficacies of postbiotics and prebiotics, the synergistic effects of prebiotic and postbiotic combinations have not been elucidated. This study examined the influence of postbiotic produced by strains of *Lactobacillus plantarum*, and inulin combinations on growth performance, gut morphology, meat quality, gene expression and fecal characteristics of broiler chickens. The study was partitioned into three experiments. The first experiment examined the inhibitory activity of postbiotic produced by strains of *Lactobacillus plantarum* using reconstituted media supplemented with inulin. Postbiotics were produced by six strains of *L. plantarum* (RG11, RG14, RI11, UL4, TL1 and RS5) using reconstituted media supplemented with different levels of inulin (0, 0.2, 0.4, 0.6, 0.8, and 1.0) yielding 36 combinations. The modified inhibitory activity (MAU) of the produced postbiotics were tested against indicator microorganism, *Pediococcus acidilactici* and pathogenic microorganisms; *Listeria monocytogenes*, *Salmonella enterica*, Vancomycin-Resistant Enterococci (VRE) and *Escherichia coli*. The combination of postbiotic and inulin had higher ($P< 0.05$) MAU than postbiotic alone against all indicator organisms except *P. acidilactici,* and *E. coli*. The RI11 + 0.8% Inulin, RG14 + 0.8% Inulin and RG14 + 0% Inulin had significantly ($P< 0.05$) higher MAU/mL against *P. acidilactici* than other treatments. The RI11 + 0.8% Inulin and RG14 + 0.4% Inulin had a significantly ($P< 0.05$) higher MAU/mL against VRE. The MAU/mL against *L. monocytogenes* was greater in RI11 + 1.0% Inulin, RI11 + 0.6% Inulin and RI11 + 0.8% Inulin. The combinations of RS5 + 1.0% Inulin, RS5 + 0.8% Inulin and RS5 + 0.6% Inulin had greater MAU/mL

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against *S. enterica*; whereas in *E. coli*, the inhibitory activity had higher activity that can only be found in RS5 + 0.8% Inulin. The combination of postbiotics and inulin had higher optical density and lower pH, which corresponds to increased inhibitory activity against indicator organisms. The results of this study showed that postbiotics and inulin combinations inhibit the proliferation of pathogenic bacteria. Four combinations of postbiotic and inulin with the highest MAU were used as a feed additive in the second experiment.

The second experiment examined the influence of postbiotics on growth performance, apparent ileal digestibility (AID), meat quality and gene expression in broiler chickens. A total of 280, one day-old male, Cobb broiler chicks were randomly assigned to 8 treatment groups. The treatments include; basal diet (negative control), basal diet+ neomycin and oxytetracycline (positive control), (T1) basal diet+0.3% postbiotic RI11, (T2) basal diet+0.3% postbiotic RG14, (T3) basal diet+0.3% postbiotic RI11+0.8% inulin, (T4) basal diet+0.3% postbiotic RI11+1.0% inulin, (T5) basal diet+0.3% postbiotic RG14+0.8% inulin, and (T6) basal diet+0.3% postbiotic RG14+1.0% inulin. Birds fed postbiotic and inulin combinations had greater ($P< 0.05$) final body weight, body weight gain and feed efficiency compared with the control birds. Similarly, supplementation of postbiotic and inulin combinations increased ($P< 0.05$) the population of faecal lactic acid bacteria and villi height of small intestine and lowered faecal *Enterobacteriaceae*. Birds fed postbiotics and inulin combinations had lower ($P< 0.05$) drip loss and higher ($P< 0.05$) lightness of *Pectoralis major* muscle compared with the control birds. Birds fed diets supplemented with postbiotic and inulin combinations had greater ($P<0.05$) percentage of tibia bone Ca and P, faecal acetic acid, propionic acid and total organic acid compared with the control birds. The liver of birds fed T4 and T6 had higher Insulin-like growth factor 1 (IGF-I) expression compared with other treatments while T6 had higher growth hormone receptor (GHR) mRNA expression compared with other treatments. The supplementation of postbiotics and inulin combination had beneficial effect on total body weight, feed efficiency, mucosal architecture, and expression of IGF-I and GHR mRNA in broiler chickens. The third experiment examined the effect of graded levels of RG14 and 1% inulin on growth performance, meat quality and gene expression in broiler chickens. A total of 216, one day old male Cobb chicks were randomly assigned to six dietary treatments. The treatment were basal diet (negative control), basal diet+ neomycin and oxytetracycline (positive control), (T1) basal diet+0.15% postbiotic RG14+1.0% inulin (T2) basal diet+0.3% postbiotic RG14+1.0% inulin (T3) basal diet+0.45% postbiotic RG14+1.0% inulin, (T4) basal diet+0.6% postbiotic RG14+1.0% inulin. Supplementation of 0.15 and 0.45 % RG14 with 1% inulin improved final body weight, weight gain and meat quality of broiler chickens compared with other treatments. The supplementation of postbiotic and inulin combinations increased the concentration of acetic acid, ileal cytokine expression, plasma immunoglobulin IgG and IgM, and population of total bacteria and beneficial bacteria and reduced the population of *Enterobacteria* and *E. coli* compared with the control diet. The combination of 0.15% RG14 with 1% inulin is potential replacement for antibiotic growth promoter (AGP) in the poultry industry.

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

KESAN MAKANAN TAMBAHAN POSBIOTIK DAN INSULIN KE ATAS PRESTASI TUMBESARAN, MORFOLOGI USUS, EKSPRESI GEN DAN CIRI-CIRI TINJA AYAM PEDAGING.

Oleh

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Penggalak Tumbesaran Antibiotik (AGP) diakui berkesan dalam memodulasi mikroflora usus dan menggalakkan prestasi tumbesaran ayam. Walau bagaimanapun, penggunaan AGP yang berpanjangan boleh menyebabkan rintangan antibiotik dan kesan sampingan, yang mampu membahayakan kesihatan ayam dan manusia yang memakannya. Prebiotik, probiotik dan kombinasinya merupakan pengganti AGP yang berpotensi. Namun, probiotik komersil seringkali gagal memenuhi keupayaan yang diharapkan dan keberkesanannya adalah tidak menentu. Tambahan pula, penggunaan probiotik boleh membawa kepada berlakunya rintangan gen antibiotik yang boleh berpindah sesama organisma. Ini menunjukkan bahawa perlunya untuk mencari alternatif kepada probiotik yang selamat dan sesuai. Posbiotik yang merupakan suatu metabolit bagi probiotik menunjukkan kesan probiotik tanpa kehadiran sel-sel hidup. Meskipun posbiotik dan prebiotik adalah berkesan, kesan sinergi di antara kombinasi prebiotik dan posbiotik belum pernah diperjelaskan. Kajian ini menguji pengaruh posbiotik yang dihasilkan oleh strain *Lactobacillus plantarum*, dan kombinasi inulin ke atas prestasi tumbesaran, morfologi usus, kualiti daging, ekspresi gen dan ciri-ciri tinja pada ayam pedaging. Kajian ini dibahagikan kepada tiga eksperimen. Eksperimen pertama mengkaji aktiviti perencatan posbiotik yang dihasilkan oleh strain *Lactobacillus plantarum* menggunakan media yang dibentuk semula dan ditambah dengan inulin. Posbiotik telah dihasilkan dari enam strain *L. plantarum* (RG11, RG14, RI11, UL4, TL1 and RS5) menggunakan media yang dibentuk semula dengan aras inulin yang berbeza (0, 0.2, 0.4, 0.6, 0.8, dan 1.0) menghasilkan sejumlah 36 kombinasi. Aktiviti Perencatan Terubah suai (MAU) bagi posbiotik yang dihasilkan telah diuji dengan mikroorganisma indikator, *Pediococcus acidilactici* dan mikroorganisma patogen; *Listeria monocytogenes*, *Salmonella enterica*, Vancomycin-Resistant Enterococci (VRE) dan *Escherichia coli*. Kombinasi di antara posbiotik dan inulin menunjukkan MAU yang lebih tinggi berbanding posbiotik melawan kesemua organisma indikator secara tunggal, kecuali *P. acidilactici,* dan *E. coli*. Kombinasi RI11 + 0.8% Inulin, RG14 + 0.8% Inulin dan RG14 + 0% Inulin mempunyai ($P< 0.05$) MAU/mL yang ketara lebih tinggi melawan *P. acidilactici* berbanding rawatan-rawatan lain. RI11 + 0.8% Inulin RG14 + 0.4% Inulin menunjukkan ($P< 0.05$)

MAU/mL yang ketara lebih tinggi melawan VRE. MAU/mL melawan *L. monocytogenes* adalah lebih tinggi dalam RI11 + 1.0% Inulin, RI11 + 0.6% Inulin dan RI11 + 0.8% Inulin. Kombinasi RS5 + 1.0% Inulin, RS5 + 0.8% Inulin dan RS5 + 0.6% Inulin mempunyai MAU/mL yang lebih tinggi secara ketara melawan *S. enterica*; manakala dalam *E. coli*, aktiviti perencatan menunjukkan aktiviti yang lebih tinggi yang hanya boleh ditemukan dalam RS5 + 0.8% Inulin. Kombinasi di antara posbiotik dan inulin mempunyai ketumpatan optik yang lebih tinggi dan pH yang lebih rendah, yang merujuk kepada peningkatan aktiviti perencatan melawan organisma indikator. Keputusan kajian ini menunjukkan bahawa penambahan posbiotik dan inulin merencatkan pembiakan bakteria patogenik. Empat kombinasi posbiotik dan inulin dengan MAU tertinggi telah digunakan sebagai additif makanan dalam eksperimen kedua. Eksperimen kedua menguji pengaruh posbiotik ke atas prestasi tumbesaran, kebolehcernaan ketara ileum(AID), kualiti daging dan ekspresi gen dalam ayam pedaging. Sejumlah 280 anak ayam pedaging Cobb yang berumur sehari, telah dibahagikan kepada 8 kumpulan rawatan secara rawak. Rawatan-rawatannya termasuklah; diet asas(kawalan negatif), diet asas+ neomisin dan oksitetrasiklin (kawalan positif), (T1) diet asas+0.3% posbiotik RI11, (T2) diet asas+0.3% posbiotik RG14, (T3) diet asas+0.3% posbiotik RI11+0.8% inulin, (T4) diet asas+0.3% posbiotik RI11+1.0% inulin, (T5) diet asas+0.3% potbiotik RG14+0.8% inulin, dan (T6) dietasas+0.3% posbiotik RG14+1.0% inulin. Makanan ayam dengan kombinasi posbiotik dan inulin menunjukkan ($P< 0.05$) berat badan akhir, penambahan berat badan dan keberkesanan makanan yang lebih tinggi berbanding ayam kawalan. Demikian juga, penambahan posbiotik inulin telah meningkatkan ($P< 0.05$) populasi asid laktik yang dihasilkan bakteria dalam tinja dan ketinggian vilus dalam usus kecil dan menurunkan *Enterobacteriaceae* dalam tinja. Ayam yang diberi makan kombinasi posbiotik dan inulin mempunyai kehilangan titis yang lebih rendah ($P< 0.05$) dan keringanan otot *Pectoralis major* yang lebih tinggi ($P< 0.05$) berbanding ayam kawalan. Ayam yang memakan diet yang ditambahkan dengan kombinasi posbiotik dan inulin mempunyai perstusan Ca dan P dalam tulang tibia, asid asetik dalam tinja, asid propionik dan jumlah asid organik yang lebih tinggi ($P<0.05$) berbanding ayam kawalan. Hati bagi ayam yang telah diberi makan T4 dan T6 mempunyai ekspresi faktor tumbesaran seiras-insulin 1 (IGF-I) berbanding rawatan-rawatan lain manakala T6 mempunyai ekspresi reseptor hormon tumbesaran (GHR) mRNA yang lebih tinggi berbanding rawatan-rawatan lain. Penambahan kombinasi posbiotik dan insulin mempunyai mempunyai kesan berfaedah ke atas berat bahan keseluruhan, keberkesanan makanan, seni bina mukosa, dan ekspresi IGF-I dan GHR mRNA dalam ayam pedaging. Eksperimen ketiga mengkaji kesan aras-aras RG14 dan 1% inulin berperingkat ke atas prestasi tumbesara, kualiti makanan dan ekspresi gen dalam ayam pedaging. Sejumlah 216, ayam jantan baka Cobb yang berusia sehari telah dibahagikan secara rawak kepada enam rawatan pemakanan. Rawatan tersebut adalah diet asal (kawalan negatif), diet asas+ neomisin dan oksitetrasiklin (kawalan positif), (T1) diet asas+0.15% postiotik RG14+1.0% inulin (T2) diet asas+0.3% posbiotik RG14+1.0% inulin (T3) diet asas+0.45% posbiotik RG14+1.0% inulin, (T4) diet asas+0.6% posbiotic RG14+1.0% inulin. Penambahan 0.15 dan 0.45 % RG14 dengan 1% inulin telah meningkatkan berat badan akhir, penambahan berat badan dan kualiti daging ayam pedaging berbanding rawatan-rawatan lain. Penambahan kombinasi posbiotik dan inulin telah meningkatkan kepekatan asid asetik, ekspresi sitokin ileum, immunoglobulin plasma IgG dan IgM, dan populasi jumlah bakteria dan bakteria berfaedah dan menurunkan populasi Enterobakteria dan bakteria *E. coli* berbanding diet kawalan. Kombinasi 0.15% RG14 dengan 1% inulin telah dikenal pasti sebagai

pengganti bagi Penggalak Tumbesaran Antibiotik (AGP) yang berpotensi dalam industri poltri.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µL	Microliter
µm	Micrometer
a*	Yelloness
AGP	Antibiotic growth promoter
AID	Apparent ileal digestibility
ATP	Adenosine triphosphate
b*	Redness
BHA	Butylated hydroxyanisole
BMC	Bone mineral density
BW	Body weight
BWG	Body weight gain
cDNA	Complementary-DNA
CFU	Colony forming units
Cm	Centimeter
CP	Crude protein
CPO	Crude palm oil
CRD	Complete randomized design
CT	Cycle threshold
DC	Dendritic cell
DM	Dry matter
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
EE	Ether extract
ENT	Enterobacteriaceae
FCR	Feed conversion ratio
FI	Feed intake
FOS	Fructooligosaccharide
G	Gram
GARS	Generally recognized as safe
GHR	Growth hormone receptor
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLM	General linear model
GOS	Galactooligosaccharide
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HDL-C	High density lipoprotein cholesterol
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IGF-I	Insulin-like growth factor 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMO	Isomaltooligosaccharide
kg	Kilogram
L	Liter

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L*	Lightness
LAB	Lactic acid bacteria
LDL-C	Low-density lipoprotein cholesterol
M	Molar
MAU	Modified arbitrary unit
mL	Milliliter
Mm	Millimeter
mM	Millimolar
MOS	Mannanoligosaccharide
mRNA	Messenger ribonuclic acid
MRS	de-Mann Rogosa Sharp
N CL	Sodium chloride
OD	Optical density
OF	Oligofructose
PKa	The dissociation constant
PSE	Pale, soft, exudative
RNA	Ribonuclic acid
Rpm	Rounds per minute
SAS	Statistical analysis system
SCFA	Short chain fatty acid
SOS	Soyaoligosaccharide
TCHOL	Total cholesterol
TG	Triglyceride
Th1	T-helper 1
TiO_2	Titanum dioxide
TLR	Tolllike receptor
v/v	Volume versus volume
VFA	Volatile fatty acid
VLDL-C	Very low-density lipoprotein cholesterol
VRE	Vancomycin-Resistant Enterococci
w/v	Weight versus volume
WHC	Water holding capacity
XOS	Xylooligosaccharide

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CHAPTRE ONE

GENERAL INTRODUCTION

Poultry production is an active and dynamic industry that is central to the wellbeing of many people and an indispensable part of the economy in many countries (FAO, 2014). The main targets of poultry production are high growth rate, optimum performance and feed efficiency at least cost. Meeting these targets is premised solely on the genetic potential of the birds, environmental conditions, quality of the diets, and outbreak of diseases. Aside the aforementioned-factors, gut health has recently been the subject of intense studies in poultry production (Rinttilä and Apajalahti, 2013).

The gut plays a vital role in mediating the uptake and use of nutrients by the animals and a major site of potential exposure to environmental pathogens (Yegani and Korver, 2008). Thus, a healthy and well-functioning gut is germane for optimum performance and health of birds. When the gut function and health are impaired, digestion and absorption of nutrients are affected and the health and performance of birds will be compromised (Sugiharto, 2014).

Besides being responsible for the absorption of nutrients from the lumen, intestinal mucosa of broiler chicken plays an important role in providing an effective barrier between the hostile luminal content and the host internal tissues. In this respect, intestinal mucosa is an important determinant of gut health and performance of chicken (Rinttilä and Apajalahti, 2013).

Among the factors responsible for the gut health and performance of chicken, commensal microbiota in the gut seem to have pivotal roles as they help to direct the development of gut structure and morphology, modulate the immune responses, offer protection from luminal pathogens and aid the digestion and utilization of nutrients (Sugiharto, 2014). In order to support the intestinal mucosal barrier functions, the dynamic balance between the mucus layer, epithelial cells, microbiota and immune cells in the intestine is of importance (Schenk and Mueller, 2008).

Various factors associated with diet and infectious disease agents affect this dynamic balance, and subsequently affecting the health status and production performance of the chicken (Yegani and Korver, 2008). Decreased incidence of disease may be related to changes in immune regulation through cytokine secretion. Thus, there is evidence of correlation between the composition of the colonizing microbiota and variations in immunity (O'Hara and Shanahan, 2006). The dependence of nutritional and growth hormones on hepatic IGF-I production has been demonstrated (Moriyama, 1995; Shamblott *et al.,* 1995). Moreover, amongst the genes influencing growth, IGF-I has been demonstrated as an indicator of growth rate in chicken (Beccavin *et al.,* 2001; Jones and Clemmons, 1995). The overall nutritional status of the animal modulates the ability of hepatic tissue to respond to growth hormones (Beckman, 2011).

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Antibiotics growth promoters (AGP) have been widely used in the poultry industry for decades to maintain the balance of ecosystem in the gut and to promote growth performance of birds (Huyghebaert *et al*., 2011). Nonetheless, the usage of antibiotics as feed additives for long periods in poultry diets can lead to antibiotic resistance (Shazali *et al*., 2014) and high residue levels in animal products.

Genes encoding for this resistance have the potential to be transferred to other formerly susceptible bacteria, therefore posing a threat to animal and human health (Montagne *et al*., 2003). The use of AGP in poultry production has been banned or restricted in many countries due to public concern (Ohimain and Ofongo, 2012). The need to optimize gut health and growth performance in birds to produce a safe and quality products to consumers has created an impetus to find alternatives for the usage of antibiotics in poultry production (Kleter and Marvin, 2009; Sugiharto, 2014). Some of these additives are prebiotics, probiotics, symbiotics and postbiotics (metabolic products by probiotic) (Alloui *et al*., 2013; Loh *et al*., 2014).

Prebiotics such as inulin (Rebolé *et al*., 2010) are effective in controlling pathogens such as *Escherichia coli* and *Salmonella* and in stimulating the growth of *Bifidobacteria* and *Lactobacilli*, thus promoting health and performance of animals (Bogusławska-Tryk *et al*., 2012; Jung *et al*., 2008). Probiotics are beneficial bacteria capable of colonizing the host digestive system, increasing the natural flora and preventing colonization of pathogenic organisms (Loh *et al*., 2014). In spite of beneficial effects of probiotics, the major problem with their application is that some probiotics have antibiotic resistance genes, especially those encoded by plasmids, which can be transferred between organisms (Marteau and Shanahan, 2003). This justifies the need to search for suitable and safe alternative to probiotics.

As a substitute to probiotics, metabolite products synthesized from probiotic known as postbiotics could be used. Postbiotics exhibit probiotic effects without living cells (Loh *et al*., 2010; Thanh *et al*., 2009; Thu *et al*., 2011). The efficacy of metabolites produced from *L. plantarum* to inhibit the proliferation of pathogenic bacteria such as *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli* and Vancomysin Resistant *Enterococci* has been documented (Loh *et al*., 2010). Furthermore, improvements in growth performance, faecal lactic acid bacteria and villus height were observed when metabolite combinations were added to the feed of broilers (Thanh *et al*., 2009), laying hens (Choe *et al*., 2012) and pigs (Thu *et al*., 2011).

Synbiotics, the combination of prebiotics and probiotics may possibly produce greater beneficial effects on gut health than individual application of probiotics and prebiotics (Abdel-Fattah and Fararh, 2009; Mookiah *et al*., 2014; Hamasalim, 2016). Synbiotics exerts synergistic and/or additive effects in the improvement of gut health and growth performance in livestock (Nekoubin and Sudagar, 2012; Al-Baadani *et al*., 2016; Hamasalim, 2016).

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Despite the beneficial roles of synbiotics, there is a paucity of information on the synergistic effects of the combination of postbiotics and prebiotics on gut health, growth performance, meat quality, gene expression and faecal characteristics of broiler chickens. Thus, the current study was initiated to examine such effects with the following hypothesis and objectives.

Hypothesis statements

1. The combinations of inulin and postbiotics produced by *Lactobacillus plantarum* would exhibit inhibitory activity against pathogenic bacteria.
2. The combinations of postbiotics and inulin would alter gut morphology, promote nutrient digestibility, growth performance and meat quality in broiler chickens.
3. Different levels of postbiotics and inulin would alter faecal bacteria profile, gene expression and cytokine expression in broiler chickens.

Objectives

The main objective of this research was to examine the effects of combinations of postbiotics and inulin on gut health, growth performance, gene expression and faecal characteristics of broiler chickens.

The specific objectives of this study were:

1. To determine the modified inhibitory activity of postbiotics produced by *Lactobacillus plantarum* RG11, RG14, RI11, UL4, TL1 and RS5 using reconstituted media supplemented with different levels of inulin.
2. To examine the impact of dietary postbiotics and inulin combinations on gut morphology, nutrient digestibility, faecal bacteria, growth performance, meat quality and hepatic IGF-I and GHR mRNA expressions in broiler chickens.
3. To determine the effect of feeding different levels of postbiotic RG14 and 1% inulin on growth performance, gut morphology, faecal bacteria, and cytokine expression in broiler chickens.

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