CELLULAR AND MOLECULAR EFFECTS OF DIET SUPPLEMENTED WITH POTENTIAL PREBIOTIC CRUDE EXTRACTS FROM SELECTED LOCAL PLANT RESOURCES IN BROILER

By

WONG LING LIT

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

This thesis is dedicated to my beloved late supervisor,
Associate Professor Dr. Sieo Chin Chin
(11th Nov 1971 - 5th Oct 2015)

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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Chair : Prof. Abdul Rahman bin Omar, PhD
Institute : Bioscience

Poultry industries have grown rapidly over the years due to the high demand from consumers. The quality and safety of the meat have become a major concern in which consumers prefer meat produced from natural resources. In the past, antibiotics were used as a growth promoter to increase body weight as well as feed efficiency of the birds. However, this resulted in the emergence of bacterial resistance to antibiotics, and residues of antibiotics in the meat may also affect humans' health. Thus, other alternatives that could replace antibiotics are being extensively studied. Prebiotics, which are complex oligosaccharides, have been reported to selectively enhance beneficial bacteria populations in the gut, leading to inhibition of adhesion of pathogenic bacteria. Consequently, a good gastrointestinal system enhances the growth rate, feed efficiency and also immune status of the birds.

Plant extracts have been reported as one of the sources of prebiotics and it can be hypothesized that certain plant extracts that is obtained locally may represent a rich source of prebiotics. However, the potential of various local plant extracts as a new source of prebiotic to effectively substitute the usage of antibiotics in broilers has not been extensively studied. Hence, the general objective of the study is to screen prebiotic from local plant extracts and investigate its effects on the broiler performance, nutrients absorption, immunity with the combination of commercial probiotic strains, as well as the changes of their gut microbiota.

This study showed that among the six selected local plant candidates including coconut shred, rice bran, corn cob, oil palm frond, pineapple peel, and banana peel extracted using autohydrolysis method, corn cob extract contained the highest total carbohydrate concentration. Corn cob also resulted in the significant highest relative growth of *Lactobacillus reuteri* C1, *Lactobacillus. reuteri* I16, and

Lactobacillus salivarius I24. Therefore, corn cob is the best candidate for the study because it fulfilled one of the prebiotic properties, “promoting the growth of beneficial intestinal microorganisms” and substrate availability. Autohydrolysis extraction of corn cob at 80 °C for 20 minutes has been found to give the best yield of possible prebiotics. Corn cob extracts totally inhibited the growth of *Campylobacter jejuni* co-cultured with *Pediococcus pentosaceus* PP6, in addition to the reduction of all non-beneficial bacteria. The corn cob extract was able to selectively enhance the growth and activity of beneficial bacteria better compared to the commercialised XOS.

NMR revealed a mixture of monosaccharides, disaccharides and oligosaccharides, 3-carbon oligosaccharides are present in the crude corn cob extract. There are 3 ^{1}H signals in the proton anomeric region at 5.40, 5.18, and 4.58 ppm, with 6 ^{13}C signals in the anomeric region at 106.77, 104.61, 101.07, 99.16, 95.25, 95.20 ppm. This result was further supported by LC-MS/MS that trimaltotriose based sugar compounds were identified based on the mass and the LC-MS/MS spectrum.

In overall, *in vivo* broilers fed with 0.1% probiotic and 1.0% corn cob extract (SYN10) resulted in significant ($P < 0.05$) better feed conversion ratio (FCR), body weight gain (BWG), lower serum triglyceride and higher antibody of IgA and IgM compared to BD. SYN10 also significantly ($P < 0.05$) enhanced the population of *Lactobacillus* and *Bifidobacterium*, in conjunction with the reduction of *Escherichia coli* and *Clostridium perfringens* population in caeca and ilea during the finisher period compared to BD. On the other hand, 0.1% probiotic and 0.5% corn cob (SYN05) significantly ($P < 0.05$) lowered the LDL level during the finisher period compared to BD. SYN05 supplemented diet resulted in a significantly ($P < 0.05$) higher bacteria richness and diversity reflected compared to BD during the finisher period. Besides, SYN05 also significantly ($P < 0.05$) elevated the beneficial bacteria and suppressed the growth of pathogenic bacteria in caeca. In addition, SYN05 significantly ($P < 0.05$) elevated the metabolism of cofactors of vitamins and nucleotide metabolism compared to BD.

From the overall results of this study, it can be concluded that the diets supplemented with corn cob extracts give positive and promising results on all aspects of the broilers’ growth and health performance comparing to the BD at varying degrees. The information of this study is important for the development of a potential prebiotic from local resources to be used to produce quality meat in the country, and the corn cob extract with probiotic can be further studied as a potential alternative to AGPs in poultry production.

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

KESAN SELULAR DAN MOLEKUL DARIPADA DIET YANG DITAMBAHKAN EKSTRAK MENTAH DENGAN POTENSI PREBIOTIK DARIPADA SUMBER TANAMAN TEMPATAN TERPILIH KE ATAS AYAM PEDAGING

Oleh

WONG LING LIT

June 2021

Pengerusi : Prof. Abdul Rahman bin Omar, PhD
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Industri ternakan ayam telah berkembang dengan mendadak semenjak beberapa tahun ini akibat kenaikan permintaan daripada pengguna. Kualiti dan keselamatan daging telah menjadi perhatian utama di mana pengguna lebih cenderung terhadap daging yang dihasilkan daripada sumber semula jadi. Pada masa lalu, antibiotik digunakan sebagai penggalak pertumbuhan untuk menambahkan berat badan dan kecekapan pemakanan ayam. Namun, pengunaan antibiotik telah menyebabkan kelahiran bakteria yang lali dengan antibiotik selain residu antibiotik dalam daging boleh menjejaskan kesihatan manusia. Oleh itu, alternatif lain untuk mengganti penggunaan antibiotik sedang giat dikaji. Prebiotik, yang merupakan oligosakarida komplek, telah dilaporkan dapat meningkatkan populasi bakteria bermanfaat tertentu di dalam usus, seterusnya mengurangkan bakteria patogenik. Kesannya, sistem gastrousus yang sihat dapat meningkatkan kadar tumbesaran, kecekapan pemakanan dan sistem imunisasi ayam.

Ekstrak tanaman telah dilaporkan sebagai salah satu sumber prebiotic dan ia boleh dicadangkan bahawa terdapat sesetengah ekstrak tanaman yang diperoleh dalam negara yang sebenarnya kaya kandungan prebiotik. Namun, potensi pelbagai ekstrak tanaman tempatan sebagai sumber baru prebiotik untuk mengganti pengunaan antibiotik dalam ayam daging masih tidak dikaji secara meluas. Oleh itu, objektif umum kajian ini adalah untuk memilih ekstrak tanaman tempatan yang mengandungi oligosakarida komplek yang mempunyai potensi prebiotik dan mengkaji kesan prebiotik ke atas prestasi ayam daging, penyerapan nutrien, imunisasi dengan kombinasi strain probiotik komersial, serta perubahan mikrobiota usus ayam daging.

Hasil kajian menunjukkan daripada enam calon terpilih tanaman tempatan termasuklah hampas kelapa, dedak padi, tongkol jagung, pelepah kelapa sawit, kulit nenas, dan kulit pisang yang diekstrak menggunakan kaedah autohidrolisis, ekstrak tongkol jagung mengandungi kandungan keseluruhan karbohidrat yang tertinggi. Tongkol jagung turut memberi keputusan tumbesaran relatif *Lactobacillus. reuteri* C1, *Lactobacillus reuteri* I16, and *Lactobacillus salivarius* I24 paling tinggi yang ketara. Oleh itu, jagung merupakan calon yang terbaik untuk kajian bagi memenuhi salah satu sifat prebiotik iaitu "menggalakkan tumbesaran mikroorganisma usus yang bermanfaat" and juga ketersediaan substrat. Ekstrak tongkol jagung daripada autohidrolisis pada suhu 80°C untuk tempoh 20 minit telah memberi hasil oligosakarida terbaik. Ekstrak tongkol jagung telah sepenuhnya menghalang tumbesaran *Campylobacter jejuni* kultur bersama dengan *Pediococcus pentosaceus* PP6, selain penurunan semua bakteria tidak bermanfaat. Ekstrak tongkol jagung berupaya untuk meningkatkan tumbesaran dan aktiviti sesetengah bakteria bermanfaat dengan lebih baik jika dibandingkan dengan XOS yang dikomersialkan. NMR menunjukkan campuran monosakarida, disakarida and oligosakarida, di mana 3-karbon oligosakarida wujud dalam ekstrak mentah tongkol jagung. Terdapat isyarat 3 ^{1}H dalam kawasan proton anomer pada 5.40, 5.18, and 4.58 ppm, dengan isyarat 6 ^{13}C dalam kawasan anomer pada 106.77, 104.61, 101.07, 99.16, 95.25, 95.20 ppm. Hasil kajian di atas turut disokong oleh LC-MS/MS, di mana campuran gula berasaskan trimaltriose telah dikenalpasti, berdasarkan jisim dan spektrum LC-MS/MS.

Secara keseluruhannya, ayam daging dalam percubaan *in vivo* yang diberi makan dengan 1.0 % ekstrak tongkol jagung dan 1.0 % probiotik (SYN10) dengan ketaranya ($P < 0.05$) menghasilkan nisbah penukaran makanan (FCR), kenaikan berat badan (BWG) yang lebih baik, trigliserida serum yang lebih rendah dan antibodi IgA dan IgM yang lebih tinggi jika dibandingkan dengan BD. SYN10 turut dengan ketaranya meningkatkan populasi *Lactobacillus* dan *Bifidobacterium*, selaras dengan penurunan populasi *Escherichia coli* dan *Clostridium perfringens* dalam caeca and ilea semasa tempoh pengakhiran jika dibandingkan dengan BD. Sementara itu, 0.1% probiotik + 0.5% tongkol jagung (SYN05) dengan ketaranya ($P < 0.05$) merendahkan paras LDL semasa tempoh pengakhiran jika dibandingkan dengan BD. Pemakanan yang ditambah dengan SYN05 menghasilkan kesuburan dan kepelbagaian bakteria yang lebih tinggi dengan ketara ($P < 0.05$) jika dibandingkan dengan BD. Selain itu, SYN05 juga meningkatkan pertumbuhan bakteria bermanfaat dengan ketara ($P < 0.05$) and menghalang pertumbuhan bakteria patogenik di caeca. Tambahan lagi, SYN05 turut meningkatkan dengan ketaranya ($P < 0.05$) metabolisme kofaktor vitamin dan metabolisme nukleotida berbanding dengan BD.

Daripada keseluruhan hasil kajian ini, kesimpulannya, rawatan permakanan dengan tambahan ekstrak tongkol jagung menunjukkan kesan yang positif dan penuh harapan dari ke semua segi prestasi pertumbuhan dan kesihatan ayam daging berbanding dengan BD. Informasi daripada kajian ini amatlah penting untuk perkembangan sumber tempatan yang mempunyai potensi sebagai prebiotik untuk digunakan dalam pengeluaran daging yang berkualiti dalam

negara, dan ekstrak tongkol jagung dengan probiotik boleh dikaji dengan lebih mendalam sebagai potensi alternatif AGPs dalam pengeluaran ayam daging.

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× g	Relative centrifugal force
AGP	Antibiotic Growth Promoter
ANOVA	Analysis of Variance
ARC	Animal Research Centre
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
bp	Base pair
BW	Body weight
BWG	Body weight gain
cfu	Colony Forming Units
CID	Collision-induced Dissociation
CJ	*Campylobacter jejuni* (*C. jejuni*)
CP	*Clostridium perfringens* (*C. perfringens*)
Cq	Quantification cycle
CSS	Cumulative Sum Scaling
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic Acid
dNTP	deoxynucleotide Triphosphate
EC	*Escherichia coli* (*E. coli*)
EFm	*Enterococcus faecium*
EFs	*Enterococcus faecalis*
ESR	Erythrocyte Sedimentation Rate
EU	European Union
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio

FDR	False Discovery Rate
FI	Feed Intake
FOS	Fructooligosaccharides
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GOS	Galactooligosaccharides
H : L	Heterophil : Lymphocyte Ratio
Hb	Hemoglobin
HDL	High Density Lipoprotein
HMBC	Heteronuclear Multiple Bond Coherence
HPLC	High-performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
HT-NGS	High-throughput Next Generation Sequencing
IACUC	Institutional Animal Care and Use Committee
IDT	Integrated DNA Technologies
IEC	Intestinal Epithelial Cell
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMO	Isomaltoligosaccharide
KEGG	Kyoto Encyclopedia of Genes and Genomes
L.	*Lactobacillus*
LC	Liquid Chromatography
LDL	Low-density Lipoprotein
LEC	Ligand Exchange Chromatography

M	Molar
MARDI	Malaysian Agriculture Research and Development Institute
MS	Mass Spectrometry
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MOS	Mannanoligosaccharides
mRNA	Messenger RNA
MRS	de Man, Rogosa and Sharpe
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NDO	Non-digestible Oligosaccharides
NEB	New England Biolab
NGP	Natural Growth Promoter
NGS	Next-generation sequencing
NMR	Nuclear Magnetic Resonance
NRC	National Research Council
NTC	Non-template control
OD	Optical Density
OTU	Operational Taxonomic Unit
PCoA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
pH	Potential of Hydrogen
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
ppm	parts per million

qPCR	Quantitative Polymerase Chain Reaction
R^2	Correlation Coefficient Value
RBC	Red Blood Cell
RFU	Relative Fluorescence Unit
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Retention Time
RWO	Relative Weight of Organ
SA-HRP	Streptavidin-conjugated Horseradish Peroxidase
SBO	Soybean Oligosaccharides
SCFA	Short Chain Fatty Acid
SE	*Salmonella enterica*
spp	Several Species
SPSS	Statistical Package for Social Science
ST	*Salmonella thyphimurium*
TEA	Triethylamine
TOF-MS	Time-of-Flight Mass Spectrometer
TTP	2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt
UHPLC-MS/MS	Ultra-high Performance Liquid Chromatography Tandem Mass Spectrometry
UK	United Kingdom
US	United States
WBC	White Blood Cell
WHO	World Health Organization
XOS	xylooligosaccharides

CHAPTER 1

INTRODUCTION

Chicken meat is one of the most consumed protein sources compared to other animal proteins (Iyayi, 2008; Zaefarian et al., 2021). In order to achieve the high demand for chicken meat, intensive farming of broilers is required. However, there are several challenges in broilers' farming that need to be overcome such as enhancing weight gain and decreasing the occurrence of infectious diseases. Thus, antibiotics are widely used at sub-therapeutic levels to improve feed conversion and prevent infectious diseases in broilers. Nevertheless, the emerging of cross-resistance and multi-antibiotic resistance of pathogenic bacteria have raised concern and public awareness towards poultry products (Gustafson and Bowen, 1997). In response to this emergence of antibiotic resistance, the authorities of several regions of the world control and restrict the use of antibiotics in livestock including poultry and establishing programs for routine surveillance and monitoring of antibiotics application. They propose that antimicrobials belong to the same class as those used for humans, unless if risk analyses have been performed (WHO, 2000). For example, several European countries have restricted or banned antibiotics as growth promoters by limiting the use of antibiotics for broilers that are not associated with human treatment (Casewell et al., 2003). Looking at how the demand for chicken meat as a high-value animal protein source is expected to surge along with the growing world population and at the same time, the restricted usage of antibiotics in addressing the challenges in intensive broilers' farming, there is an urgent need to develop an effective alternative approach that would preclude drastic changes to existing poultry production practices to meet the increasing chicken meat's demand.

In the process of finding the alternative approach for the usage of antibiotics, the role of antibiotics in improving the feed conversion and preventing infectious diseases in broilers needs to be investigated. The gastrointestinal of the broilers does not only play an important function as the nutrient intake part but also as the primary protective barrier from pathogens. The mechanisms by which antibiotics enhance animal growth and feed efficiency are by altering the composition and activities of gut microflora of broilers, resulting in body weight gain. It has been suggested that inhibition of microbial activity by antibiotics reduced the competition with the host for nutrients (Niewold, 2007). These findings prove that there is direct relation of gastrointestinal microbiota with the health of broilers and thus, improving the feed efficiency. Prebiotics and probiotics are two of several approaches that can potentially to reduce enteric disease in poultry and subsequent contamination of poultry products by interfering with the gastrointestinal microbiota.

Probiotic is first defined by Fuller, (1989) as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial

balance" (Fuller, 1989, p. 366). Later, according to the adopted definition by FAO/WHO, (2002), probiotics are: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Bielecka, 2006). Inclusion of probiotics will lead to the competition of nutrients and adhere site on the intestinal epithelia with the pathogenic bacteria (competitive exclusion). Besides, probiotics can ferment carbon sources into lactic acid that lowers the pH condition of the gastrointestinal. Several antimicrobial substances include organic acids, hydrogen peroxide and bacteriocins. These compounds may reduce not only the number of viable cells, but may also affect the bacterial metabolism or toxin production. Thus, the gastrointestinal become unfavourable for pathogenic bacteria. There are several probiotics species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* (Bai et al., 2013; Sharifi et al., 2012, Bai et al.,2017) which have a beneficial effect on broiler performance (Awad et al., 2008; Mountzouris et al., 2007; Willis et al., 2007; Park and Kim 2014). The beneficial effects of those probiotics include enhancing the body weight gain, decreasing the feed intake, lowering the feed conversion ratio (FCR), modulation of intestinal microflora and pathogen inhibition, intestinal histological changes, immunomodulation, certain haemato-biochemical parameters, improving sensory characteristics of dressed broiler meat and promoting microbiological meat quality of broilers. The most extensively used genera are *Lactobacillus* and *Bifidobacterium*. However, an efficient probiotic must be viable and attached to the intestinal epithelia to carry out its function inside the host gastrointestinal. In addition, the shelf life of probiotic is very short and sensitive to temperature and moisture. When the probiotic reached the gastrointestinal, it has to compete not only with the pathogenic bacteria, but also with the native probiotic. This lead to the strategy of using nutrients/ingredients to optimize the existing beneficial gut microbiota of chickens instead of introducing a new strain of probiotic.

Non-digestible oligosaccharides (NDO) are intermediate between simple sugars and polysaccharides in the carbohydrate family. According to the updated version of the prebiotic definition, there are 3 criteria: First, by resistance to gastric acidity, hydrolysis by mammalian digestive enzymes and gastrointestinal absorption; Second, fermentation by intestinal microflora; and third, selective stimulation of the growth and/or activity (ies) of one or a limited number of intestinal bacteria beneficially associated with health and well-being (Roberfroid et al., 2010). Therefore, only functional oligosaccharides that fulfilled all of the criteria can be claimed as prebiotic. Unlike probiotics, prebiotic act as feed ingredients to the specific beneficial bacteria that lead to a better surviving chance, then competitively exclude other bacteria in the broiler gastrointestinal. The best known functional oligosaccharides include fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO), soybean oligosaccharides (SBO), xylooligosaccharides (XOS). Oligosaccharides naturally exist in plants, fruits, milk, honey and also other edible foods. The types of oligosaccharides extracted are depended on the source. Inulin and FOS are commonly found in fruits; IMO are naturally found in honey and fermented soy sauce (Ibrahim, 2018); XOS are most abundant in fibrous parts of plants (Chung and Day, 2004; Kabel et al., 2002; Xu et al., 2003; Yang et al., 2005; Boonchuay et al., 2021). The beneficial effects of prebiotic supplementation are similar to the probiotic supplement on broilers (Bouhnik et

al., 2004; Iji et al., 2001; Shashidhara and Devegowda, 2003; Yang et al., 2016; Zhang et al., 2003; Basir, 2020). There are some challenges to extract oligosaccharides from plant sources when they need to be safe for animals' consumption. Apparently, studies showed that oligosaccharides yield best using different organic solvent ratios with water. However, water is the only solvent that is safe to be utilised in animal feed. This is because the residues of organic solvent will have toxicological or adverse health effects even in a minute amount. Currently, most of the available prebiotic are manufactured overseas. A recent paper reported that oligosaccharides could be found in our local fruits (Mohd Adzim Khalili et al., 2014).

The synergic effects of mixing probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, and another one selectively stimulating the growth and/or activating the metabolism of health-promoting bacteria, and thus improving host welfare (Gibson and Roberfroid, 1995). It has been suggested that synbiotic are much more efficient when used in combinations than singly (Alloui et al., 2013; Dizaji et al., 2012). However, not every synbiotic showed two-fold synergy effects as reported by Abdel-raheem et al. (2012) and Mookiah et al. (2014). This might be due to the differences in the type of prebiotics and the strains of probiotics.

Therefore, this study was performed to investigate the potential prebiotic effects of plant extracts obtained locally on the broiler performance, nutrients absorption, immunity with the combination of commercial probiotic strains, as well as the changes of their gut microbiota. The specific objectives of the study were, first, to screen for prebiotics from local plant resources; second, to optimize the extraction condition of selected local plant resources for the yield of prebiotcs; third, to identify the prebiotics present in the selected crude extract via Nuclear Magnetic Resonance (NMR), High-performance liquid chromatography (HPLC), and Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC LC/MS) analysis; fourth, to study the effects of the selected plant extract on the growth performance, haematological and serum lipid profiles, immunological response, jejuna and ilea villus height and crypt depth, and relative weight of organ (RWO) of broilers and lastly, to investigate the effects of the combination of selected crude extracts and Primalac® probiotic with different dosage on broilers' caecal and ileal microbial community using real-time quantitative PCR (qPCR) and high-throughput next-generation sequencing (HT-NGS).

CHAPTER 2

LITERATURE REVIEW

2.1 Poultry Industry in Global and Malaysia

There is a rapid growth in poultry farming worldwide following the great demand for chicken meat and other poultry products. The demand for poultry products is attributed to the quality of nutrition, affordable prices, and the appetizing tastes of the products (Wahyono and Utami, 2018). The global preferences and consumption of poultry products, mainly chicken meat, is greatly acknowledged by consumers globally compared with other meat. The increased consumption of poultry meat across the world is associated with rapid growth in the poultry sector (Vukasović, 2014). The main key factors that influence the global growth and consumption of poultry meat are lower prices of the poultry complements and higher income. Mottet and Tempio (2017) mentioned that in the agricultural sub-sector, the poultry sector is the fastest growing industry following the demand for products like eggs and meat. The demand for meat in the poultry sector is associated with rising incomes, urbanization, and the rapidly growing population worldwide. In rural and urban areas, poultry farmers in medium and low-income levels engage in poultry farming because of the growing market that provides income and enhances market participation among such populations (Mottet and Tempio, 2017).

Similarly, the global poultry industry is growing as a result of the rapid growth in the economy. This is revealed by meat consumption levels between 2011 and 2015. The demand for poultry meat in 2011 was estimated to increase with average meat consumption of 14.5 kg per capita. The total consumption of poultry meat globally increased in 2015 to about 41.3 kg per capita, and the increase is estimated to reach about 45.3 kg per capita in the consumption of poultry meat by 2030 (Wahyono and Utami, 2018). The increase in consumption indicates high demand and high income in the poultry industry. The economic growth of the poultry industry following the demand and income in poultry meat, poultry production systems such as backyards, broilers, and layers have developed at the global level. The establishment of these production systems has helped achieve increased capital input, higher poultry productivity levels, and enhanced produced feed across poultry industries in the world (Mottet and Tempio, 2017). Further, Mottet and Tempio (2017) cited the information presented by Global Livestock Assessment Model and disclosed that poultry meat production across the globe is approximately 100 million tons. Using the information presented through the modeling approach, the global production of poultry meat is high from the broiler systems which accounts for nearly 92% compared to the production through layer estimated to yield 6% of the poultry meat (Mottet and Tempio, 2017).

The poultry industry across countries in Africa, Asia, and South America has experienced rapid growth in broiler meat production. The demand for poultry meat in Asia has been increasing rapidly with broiler meat production being the key systems in these countries (Wahyono and Utami, 2018). The consumption per capita (PCC) in Malaysia stood at 51.2 kilogrammes annually in 2016. Thus, led to the significant growth in Malaysia's poultry industry. Majid and Hassan (2014) asserted that broiler farming is the key production system that has promoted poultry production across Malaysia. Through broiler systems, poultry production has increased in Malaysia following technological advances in chicken feeds, animal husbandry, and nutrition. Also, contract farming with broilers is widely practiced in Malaysia and is dominating a larger percentage (75%) of the broiler production nationally (Majid and Hassan, 2014). The growth in poultry demand is associated with the demand and supply of chicken meat in Malaysia. The demand is attributed to the lower cost of chicken meat than the prices of other meat, the religious acceptance of chicken meat consumption, and the increased income from the products (Jayaraman et al., 2013). Following a sufficient domestic supply of poultry products like meat and eggs, Malaysia's Third National Agricultural Policy was established between 1998 and 2010. This policy was developed to improve the growth in the poultry industry and to ensure the efficiency of the domestic supply of poultry products among the small-scaled farm industry (Majid and Hassan, 2014).

Jayaraman et al. (2013) cited that in 2010, the poultry industry in Malaysia experienced a huge supply of broiler chicks from stock farms. It is estimated that in Peninsular Malaysia, a total of 25 stock farm companies produce and supply over 500 million broiler chicks that are one-day-old. The main breeds of these chicks include the Ross and Conns which accounts for more than 90% of use in Malaysia. As cited by Majid and Hassan (2014), the broiler production in Malaysia during the year 2010 accounts for over 50% for the total livestock production and the consumption of broiler meat increased from 28 kg in 2000 to nearly 34 kg in 2010. It is estimated that broiler production will increase by 2.14% every year (Majid and Hassan, 2014). The broiler-producing farms in Malaysia produce about 523 million birds, and 43 million of the birds are transported to Singapore. Approximately 30% of the broilers are also exported via modern processing plants and sold in various supermarkets (Jayaraman et al., 2013). The remaining 70% of the broilers are sold to the wet markets (markets selling fresh meats) as live birds. One of the well-known poultry industries in Malaysia is the Leong Hup Holdings Berhad which was established in 1979 and since then, it has been producing more than 100 million one-day-old broiler chickens per annum. The industry gets about 23% of the market supply for broiler chicks and has become the leading distributor of about 3 million broiler chicks in a month (Jayaraman et al., 2013). Several broiler-producing industries are located in Kedah, Pulau Pinang, and Perak which are the leading producers of broilers in Peninsular Malaysia. These states have commercial companies for broiler production and they account for 60% of the total production of broilers in Malaysia (Majid and Hassan, 2014). Further, the broiler production sectors have parent breeder farms, processing plants, hatchery, and feed mills which enable them to produce many broiler chickens. It is estimated that the broiler farmers can manage to produce between 10,000 and 110,000 broiler chickens on average (Majid and Hassan, 2014).

In relation to the high production and selling of broiler chickens in Malaysia, the economic growth is high in the poultry industries. Jayaraman et al. (2013) indicated that the price for chicken meat is increasing every year. From the statistics between 2010 and 2011, there is an increase in chicken meat cost, which rose from RM7 for a kilo in 2010 up to RM7.80 per kilo in 2011. An increase in broiler chicken meat costs is due to the elevated prices of day-old broiler chickens, the global rise in maize price and the increased price of chicken feeds (Jayaraman et al., 2013). Using the economic analysis performed by Abdurofi et al. (2017), the analysis of the broiler poultry industry in Peninsular Malaysia shows that the consumption of broiler chickens accounts for more than 90% of the total consumption compared to the consumption of approximately 3% and 1% of ducks and other poultry. Based on this high demand, the broiler industry in Peninsular Malaysia is operating at sustainable production and has maintained its profitability which has helped in making chicken meat available at affordable prices. In addition, Jayaraman et al. (2013) mentioned that in Asian countries, Malaysia as the leading exporter of poultry meat mainly from turkey, duck, and chicken had gained high demand from other nearest Asian countries. Following the high production and export of poultry meat to other countries, Malaysia has gained rapid economic growth. Malaysia has achieved about RM2.7 billion exports of poultry chicken which have significantly resulted in improving the economic growth of the company. Abdurofi et al. (2017) explained that following the rapid rate in the growth of the broiler industry in Peninsular Malaysia, the country had attracted several investors into the broiler industry. Also, the reduction in the cost of broiler production has contributed to improved productivity from broiler stock farmers and enhanced exports. Therefore, the increased exports of poultry production to other states have facilitated a boost in the overall foreign exchange in the broiler business economy in Malaysia. Jayaraman et al. (2013) disclosed that price fluctuations in the Malaysian broiler industry are important to poultry agencies involved in the management of broiler production farms. Therefore, reducing the chicken meat prices is essential in ensuring successful management and in meeting the demands of the consumers.

2.2 Challenges Facing in the Poultry Industry

2.2.1 Antibiotic Growth Promoters (AGPs) in Poultry Production

The application of Antibiotic Growth Promoters (AGPs) in the poultry industry was first acknowledged in the 1940s following the administration of streptomycin in chickens. The administration of this antibiotic as a growth promoter led to enhanced growth and improved efficiency in the ability of chickens to feed (Brown et al., 2016). The main aim of AGPs in the poultry industry is to achieve increased production in terms of weight gain in chickens. According to Salaheen et al. (2017), AGPs is used in poultry farming to promote the growth promotion in chickens. AGPs are widely used in the poultry industry to enhance weight gain in poultry. These AGPs are administrated in poultry feeds to facilitate the growth

of chickens and in reducing the activity of bacteria in the poultry gut. The use of these AGPs has been found to increase the mean body weight among chickens and reduce the growth in population for *Lactobacillus* species which are the main producers of bile salt hydrolase that negatively impact the fat digestion in poultry (Dibamehr,2021). The AGPs are used in poultry production as a therapeutic and non-therapeutic method to promote growth in poultry. These antibiotics are used to reduce and control human infections associated with food animal production (Salaheen et al., 2017). Economou and Gousia (2015) explained that oligosaccharides avilamycin, flavophospholipid, bacitracin, avoparcin, and virginiamycin are used in poultry farming to control infections caused by *Clostridium perfringens* and to improve conversion efficiency in poultry feeds. Park et al. (2016) explained that AGPs in poultry feeds to prevent the spread of bacteria since the livestock feeds are their main reservoirs. Thus, using these antibiotics in poultry feeds reduces the spread of bacteria during slaughtering and processing that may eventually lead to human infections. As cited in Park et al. (2016), the use of AGPs for feed efficiency and to enhance growth and minimize the mortality of chickens in the poultry industry has become a common practice with a lack of thorough testing. The mechanism of actions associated with the use of antibiotics as growth promoters is strongly related to a decrease in the pathogenic bacteria in the intestinal sites of chickens.

2.2.1.1 Mode of Action of AGPs

AGPs are added to the poultry feeds as food additives and are administered to chickens in low quantities. As cited in Gadde et al. (2018), subtherapeutic and low amounts of AGPs are added to the feeds and are involved in the metabolic process. Subtherapeutic doses of AGPs are used to exert growth-promoting effects to the poultry by diminishing the number and decreasing the diversity of bacterial flora within the gut. The mode of action of AGPs is explained following the interaction with the gastrointestinal tract (GIT). According to Brown et al. (2016), the main site of AGPs action is the GIT. GIT physiology involves the interactions of host factors and dietary nutrients and the pathogenic and commensal bacteria. The gut microbiota is colonized by microbial communities which are involved in maintaining a balance of the intestinal health and diseases throughout the animal body (Brown et al., 2016). The maintenance of the balance of bacterial flora in the gut increases the bioavailability of the required nutrients to the host and reduces metabolite production in the gut microbiota which are deleterious to the growth of animals (Gadde et al., 2018). Also, the ingested AGPs induce changes in the gut microbiota hence reducing the competition for nutrients. The AGPs also prevent the colonization of harmful pathogens in the microbiota and enhance the growth of microorganisms that are involved in extracting more energy from the diet (Brown et al*.,* 2016). The role of bacterial in extracting energy from the diets provides an efficient approach for nutrient uptake which is essential for enhanced chickens growth. Similarly, the administration of AGPS in therapeutic concentrations leads to immunomodulation, where the AGPs moisten intestinal mucosa to reduce inflammation. The immunomodulation allows for the dedication of more nutritional resources to the anabolic process which is a key step for growth (Brown et al, 2016).

2.2.1.2 Sides Effects of Antibiotics in Poultry

The usage of antibiotics in intensive farming had led to two major concerns which are the antibiotic residues in animal-derived products and, eventually, antibiotic resistance. Once antibiotics were ingested/ injected into the chicken, the antibiotic residues will accumulate in the body including muscle tissues, blood, internal organs and eggs. Before the chickens were sent out to the market, they must go through a withdrawal period. The level of antibiotic residue detected in the meat must not exceed the legal maximum residual level (MRL). The Malaysian government has set the MRL of 100μg/KG for antibiotics either is parent drug,singly or in combination (Malaysian Foof Regulation 1985,2015). A study was conducted in 2018 to determine the occurrence of antibiotics (tetracyclines, sulphonamides and quinolones) residues in chicken meat from small and medium scale slaughterhouse in Peninsular Malaysia. Results from the study showed that 0.9% of the samples contained antibiotic residues above the MRL (Marzura et al., 2018).

Consumers should have aware of the fact that antibiotics residues may lead to the emergence of antibiotic resistance which could possibly transferred to men and resulted in treatment failure of clinically significant infections. With the significant use of AGPs for growth promotion in poultry production, the issues associated with the development of antibiotic resistance in the gut microbiota have been established. Gadde et al. (2018) pointed out that there are controversies following the utilization of AGPs for animals intended for meat production. The excessive use of these growth promoters in meat production in poultry for sometime is associated with the majority of bacteria developing resistance to antibiotics. The use of AGPs like virginiamycin has shown significant benefits in controlling infections, improving feed efficiency, and preventing acid lactosis in poultry. However, the overuse of these AGPs in poultry has contributed to the development of bacteria resistant to such antibiotics (Economou and Gousia, 2015). Similarly, the use of AGPs leads to the indirect spread of *Salmonella* by allowing unhygienic conditions in the environment where the animals are kept. For broiler chicken production, the confined housing is vulnerable to unhygienic conditions following the use of AGPs. This allows the pathogenic bacteria to spread through the restricted places and cause the spread of *Salmonella* which is the main cause of human infections when these broiler chickens are consumed (Economou and Gousia, 2015). Therefore, the use of AGPs in poultry feeds has been found to increase the emergence of antibiotic-resistant strains of bacteria that may cause harm to humans. Moreover, these growth promoters may cause disturbances in the dysbiosis of the microbiota which is strongly associated with the development of antibiotic-resistant diseases in humans (Costa et al., 2017). A study by Hao et al. (2014) established that the use of AGPs like enrofloxacin induces fluoroquinolone resistance in *Campylobacter* and *Salmonella* from poultry. The development of such resistant bacteria contributes to failures in antibiotic treatment among humans.

In a review, Park et al. (2016) cited that the use of AGPs in the poultry industry leads to the emergence and transmission of antibiotic-resistant bacteria to human beings. The transmission is associated with the consumption of animal products and through the handling of the animals. The use of AGPs causes the emergence of vancomycin-resistant enterococci that can be transmitted to healthy farm workers either by handling the animals or during the slaughtering and processing of poultry products. In addition, Maron et al. (2013) indicated that when the antimicrobials are given to poultry animals for growth promotion and to prevent diseases, they are administrated for an extended period. This leads to increased selective pressure for the growth of antibiotic-resistant bacteria. The administration of antimicrobials following the use of AGPs in poultry farming occurs through medicated feeds and drinking water using a herd-flock system, and this can result in the enhanced selection of antibacterial-resistant bacteria. Following the alarming risks associated with the increased resistance levels of the bacteria to the antibiotics, the European Union banned their use in the poultry industry and such banning policies have been implemented across various countries. Maron et al. (2013) explained that the European Union decided to ban the AGPs use in the poultry industry for animal feeds following the incidences of antibiotic resistance and the risk of human infections. As a result of the risks of antimicrobial-resistant pathogens and consumer concerns regarding the residues of antibiotics in increasing human infections, AGPs use in poultry was banned leading to the adoption of other methods in the poultry industry (Diaz-Sanchez et al., 2015). The banning of AGPs aims at ensuring other antibiotic resistance by some bacteria is prevented through the adoption of alternative methods. The European Union banned AGPs as food additives based on the reports that these growth promoters enhance the overgrowth of harmful pathogens in the flora like the increased development of *Clostridium difficile*, reduced production of short-chain fatty acids which are valuable compounds needed to achieve a normal flora, and increased vulnerability of infections to both animals and humans (Maron et al., 2013).

2.2.1.3 Alternative to AGPs

Maintaining a healthy gut environment in poultry is a prerequisite for successful broiler performance. Antibiotics as growth promoters have been widely used since the 1940s to ensure a healthy gut environment and improve performance in broiler systems (Murugesan et al., 2015). However, the AGP compounds in poultry diets have been banned and the poultry industry has been put under pressure to seek other viable alternatives that can be used in poultry production for improved performance, to safeguard the health of the poultry, and to sustain profit margins (Murugesan et al., 2015). Banning AGPs use in poultry feeds and the establishment of AGPs alternatives are associated with concerns of harmful infections that occur due to the consumption of AGPs in feed additives, resulting in an imbalance in the gut microbiota. This contributes to the severe loss of productivity, sudden changes in dietary intake in poultry, the emergence of intestinal diseases like coccidiosis, and suppression of immune systems (Sethiya, 2016). Similarly, the primary concerns of transmission and the

overgrowth of resistant bacteria in humans through food consumption of poultry products that have used AGPs in their diets led to the establishment of alternatives in the poultry industry (Park et al., 2016).

Park et al. (2016) cited that after the termination of use of AGPs, the impact of banning the growth promoters was investigated where animal productivity and health were assessed. From the investigation, the phasing out AGPs use contributed to the performance issues in animals and increased animal infections such as the emergence of necrotic enteritis. There were also incidences of bacterial proliferation in the gut microbiota, increased contamination of food products and feces in water content, and malabsorption in the gut (Park et al., 2016). Following the ban on antibiotic use, the search for potential AGPs alternatives has, therefore, reached momentum (Park et al., 2016). Redondo et al., (2014) indicated that establishing alternatives to AGP originated from the public health programs where probiotics and prebiotics were used as nutritional interventions to minimize the incidences of chronic health diseases in humans. The formulation of these alternatives is mainly focused on improving gut health in the animal industry to achieve improved welfare and promote animal productivity. Among the alternatives are natural commercial additives which have been investigated as the alternative strategies that are residue-free and are found to be safe in the poultry industry. These commercial additives are Natural Growth Promoters (NGPs), which are currently used in preserving and maintaining the balance of gut microbiota in animals like poultry (Sethiya, 2016).

Natural products as growth promoters have been established as the appropriate alternatives to AGPs in maintaining the safety of animal feeds and products. The most common NGPs used in poultry feeds are probiotics, prebiotics, synbiotics, nucleotides, organic acid, clay minerals, phytobiotics, and exogenous enzymes (Jaiswal et al., 2017). Using NGPs as alternatives in poultry diets has been tested and evaluated as the best feed additives in improving gut microbiota functionality. Natural products have beneficial roles in ensuring that the health of poultry is improved and protected from infectious diseases (Sethiya, 2016). The NGPs improve the morphology of intestinal microbiota and the absorption of nutrients. When added to the poultry diets, the functionality of gut microbiota and nutrient absorption is improved for enhanced growth and overall performance of poultry animals (Sethiya, 2016).

Using NGPs like probiotics and prebiotics improves the colonization resistance against bacteria and enhances the immune responses in poultry (Jaiswal et al., 2017). These NGPs in poultry diets also provide adequate energy needed to improve important bacteria in the gut. The promoters work to ensure microbial balance in the gut and suppress harmful pathogens in the microbial gut of chickens (Jaiswal et al., 2017). The NGPs act by reducing the risks of severe infections and reduces the amount of metabolites that can depress the growth of poultry. Natural products also have a key role in controlling microbiota shifts, inhibit cytokine production and excretion, and ensure improved inflammatory responses in microbiota shifts (Sethiya, 2016). In addition, dietary supplementation using plant extracts has been reported to promote the growth

of broilers. As cited in Salaheen et al. (2017), plant phenolic extracts are used as alternative AGPs in poultry diets and have a beneficial role in improving health among broilers. These extracts have anti-inflammatory, antimicrobial, anti-oxidant, and anti-carcinogenic properties which are essential in enhancing the growth performance and health of broilers. Besides, Murugesan et al. (2015) mentioned that plant extracts in poultry diets stimulate enzymatic activity and digestive secretion; therefore, providing important actions in the digestive tract and modulate the function of gut microbiota. The plant extracts as NGPs modulate the functions of the gut in broilers and support digestive activities for improved growth in broilers. Additionally, botanical extracts in poultry feeds are essential in stimulating and proliferating bacterial growth, particularly the growth of important bacteria like *Bifidobacteria* and *Lactobacilli* in the gut (Sethiya, 2016). The extracts serve as immunostimulants and protect the gut from microbial damages. They enhance the productivity and enzymatic activity and increase hydrophobicity to hinder the virulence activity in poultry intestines. These beneficial roles of botanic extracts have growth-promoting effects on feed additives (Sethiya, 2016).

2.3 Probiotic (Lactic Acid Bacteria)

2.3.1 Definition

Probiotics have beneficial properties. According to Shi et al. (2016), probiotics exhibit significant antihypertensive and antihypercholesterolemic effects, lactose intolerance effects, and intestinal microbial balance. The properties have essential health benefits that promote healthy living. In addition, probiotics or lactic acid bacteria have beneficial properties such as the competition for nutrients, adherence to epithelium walls, antagonistic or inhibitory activity, and stimulating effects on the immune system. In relation to hypocholesterolemic property, probiotics have a mechanism of action. They work through cholesterol assimilation, allow the binding of cholesterol into the cellular surface, cause co-precipitation of cholesterol, and caused conjugation of bile acids via the secretion of bile salt hydrolase (Shi et al., 2016). Probiotics also interfere with the micelle development for absorption in the intestinal tract (Shi et al., 2016). The antimicrobial effect of probiotics is associated with the ability of the lactic acid bacteria to secrete antimicrobial substances. According to Reis et al. (2016), some lactic acid bacteria produce antimicrobial compounds which have psychological effects of reducing and inhibiting the growth of intestinal pathogens. Probiotics produce lactic acids in high concentration which when combined with bile salts, inhibit the growth of pathogens, mainly the Gram-negative bacteria in the intestines.

When probiotics are used in animal feeds, they have beneficial properties in improving digestion and nutrient absorption. When incorporated into animal feeds or administered through water, the probiotics act in the digestive tract of animals by antibiosis, where they hinder the colonization of potential pathogens. They also act by competing with the potential bacteria in the intestines for

nutrients and space in the host; hence inhibiting pathogen colonization (Hemaiswarya et al., 2013). In addition, probiotics act by inhibiting microbial metabolism and stimulate vitamin production for improved nutrition in animals. Probiotics also cause the detoxification of dietary compounds and the breakdown of indigestible substances into small compounds. These properties are essential in preventing infections from harmful pathogens and for better growth and development of animals (Hemaiswarya et al., 2013).

2.3.2 Isolated Sources and Types of Probiotics

The main types of probiotics are *Lactobacillus* species. These probiotic strains are isolated from different sources, including fermented foods and drinks, non-dairy fermented food products, non-intestinal sources, fruit juices, and vegetables. The unconventional isolate sources are the main sources of isolating different strains or species of probiotics, mainly the *Lactobacillus* strains (Sornplang and Piyadeatsoontorn, 2016). Animal products and gut are the main sources used to isolate probiotics. According to Fontana et al. (2013), dairy and dairy-related products are the best probiotic isolation sources. Fermented milk has a complex composition of lactic acid bacteria; hence is considered a useful source of probiotics. The fermented milk produces lactobacillus strains like *Lactobacillus delbrueckii*. A recent study conducted by Sornplang and Piyadeatsoontorn (2016) established that the probiotic isolate sources are direct fed microbial supplementation. The main sources of lactic acid bacteria, especially those found in poultry include the GIT system. Also, probiotics are isolated from the feces of animal species like chickens and ruminants. Indigenous broiler chickens, broiler chickens, laying hens, and traditional fermented dairy products are the main sources for isolating the strains of probiotics or lactic acid bacteria (Sornplang and Piyadeatsoontorn, 2016). Besides, Latha, et al. (2016) explained that indigenous broiler chickens are the main sources for isolating strains like *Streptomyces* species. These species have psychological features of resisting growth inhibitors and mainly use phenylalanine and cellulose as the sole source of carbon and nitrogen needed for growth. As such, these strains are mainly found in broiler practices where they are used as feed additives to promote enhanced broiler production (Latha et al., 2016). This makes broiler chickens the main isolate source of lactic acid bacteria. Lactic acid bacteria isolates also involve plants and animal feeds. A study by Damayanti et al., (2017) found that lactic acid bacteria like *Lactobacillu*s species are isolated from fermented cassava and silage feeds. The probiotic isolates from cassava and silage feeds are the main source because they have antifungal activities which make the strains find a favorable place for their growth and survival.

The types of probiotics are bacteria, and yeasts and molds. The main common bacteria species are *Lactobacillus*, *Bifidobacterium, Streptococcus, Leuconostoc mesenteroides, Bacillus, Enterococcus, Pediococcus, and Propionibacterium* (Amara and Shibl, 2015). Similarly, Jaiswal et al. (2017) asserted that *Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus lactis, Lactobacillus acidophilus, Enterococcus faecalis, Streptococcus*

thermophilus, and *Enterococcus faecium* are the main types of bacteria which are used as probiotics. These probiotic strains play important roles in poultry production. For instance, *Lactobacillus acidophilus* plays an important role in the gut of chicks where they compete with other bacteria like *Escherichia coli (E. coli).* The competition for nutrients with *E. coli* is to inhibit their growth in the gut of chicks. Also, *Lactobacillus acidophilus* is essential in maintaining balance in the intestinal microflora, enhancing immune response, and reducing fecal enzyme activity (Sethiya, 2016). *Bifidobacterium bifidum* also balances intestinal microflora and in preventing the proliferation and growth of rotavirus that may cause diarrhea to poultry (Sethiya, 2016). The most common types of yeast and molds that are used as probiotics are *Saccharomyces bourlardii, Aspergillusoryzue, Candida pintolopesii, and Saccharomyces cerevisiae* (Amara and Shibl, 2015). These strains are useful in poultry production because they significantly contribute to health improvements in chickens. The yeast, *Saccharomyces bourlardii,* is an important probiotic strain in the poultry diet since it is used in treating chickens infected with *Eimeria*. The yeast helps prevent the recurrence of infections caused by *Clostridium difficile* and prevents the reoccurrence of *calibacillosis* in chickens (Sethiya, 2016). Bacterial strains of *Lactobacillus* and *Bifidobacterium* species are considered the best and safe probiotics. These strains are known to cause no infections or food-borne illnesses to animals and humans. They are the predominant groups of probiotics that are helpful in the GIT microbiota (Shi et al., 2016). These lactic acid bacteria are useful in the intestinal microflora of poultry where they prevent the colonization of harmful pathogens that can cause infections and affect intestinal balance (Jaiswal et al., 2017). Also, among the yeasts identified as probiotics strains, *Saccharomyces boulardii* is the predominant probiotic bacteria with health benefits. These strains are regarded to be safe strains because they have the ability to adhere to GIT mucosa and can adapt to competitive nature with potential pathogens (Fontana et al., 2013). These probiotic strains have the ability to survive well in the GIT conditions, can survive well in the intestinal tract in the presence of gastric acid and bile and are non-toxic, non-pathogenic, and cannot cause any side effects to the host (Fontana et al., 2013).

2.3.3 Beneficial Effects of Probiotic on Boilers

Probiotics are included in broiler nutrition for growth performance. According to Allahdo et al. (2018), the newly hatched broiler chicks have an immature GIT system. When the broiler chicks start to ingest feed, the GIT develops functional and microbial gut flora. At this time, the chicks are highly vulnerable to pathogens. Allahdo et al. (2018) further indicated that under the modern broiler production system, the exposure of chickens to stress is high during the production time. As a consequence, probiotics are being used as an alternative to antibiotics to improve intestinal microflora in broiler chickens. Podolian (2016) mentioned that probiotics in the broiler feeds are essential for growth performance by increasing pectoral muscle and femoral muscle weight in chickens. The overall weight of the broiler chickens consuming probiotics as feed additive increases and the survival rate also increases. Probiotics maintain the balance of the intestinal microbial population and stimulate the growth and proliferation of useful pathogens to maintain animal health for improved growth

performance (Allahdo et al., 2018). Adding lactic acid bacteria like *Lactobacillus* species to the feeds of broiler chickens leads to body weight gain and the improvement of feed conservation ratio. Feeding broiler chickens with a diet containing *Lactobacillus acidophilus* and *Lactobacillus faecium* significantly promotes increased body weight and improvement on body weight gain. Similarly, Olnood et al. (2015) explained that the addition of *Lactobacillus* species to the diets of broiler chickens significantly improves growth performance by increasing the total number of anaerobic bacteria in the ileum and *Lactobacilli* in the caeca that enhances increased weight in the small intestines of broilers. Adding probiotics to the poultry feeds improves the feeding efficiency which is essential for growth performance. Allahdo et al. (2018) disclosed that feeding the broiler chickens with probiotic supplements increases the digestion of feeds in the chickens, promoting the feed intake and improving growth performances.

Probiotics in broiler feeds are also useful in maintaining a healthy balance of gut microbiota. The addition of probiotics to the poultry diet maintains a healthy microbial balance in the gut through the process of competitive exclusion (Sugiharto, 2016). This process involves useful pathogens to exclude the potential microbes by competing with them for nutrients and space or attachment sites in the intestines. The beneficial bacteria also act through antagonism, where they produce lactic acids in the host intestines to inhibit the proliferation and growth of pathogenic bacteria (Sugiharto, 2016). Similarly, a healthy balance of gut microbiota is achieved through the action of beneficial bacteria by stimulating the immune system. Poultry feeds with probiotics enhanced the cellular and humoral immunity against invading *Eimeriatenella* that can cause infections in chickens. These probiotics provide a defense mechanism in the GIT where they prevent chicken infections by *Salmonella* and *Eimeria* species. The mechanism of action of probiotics through the stimulation of the immune system thus assists in reducing the risk of diseases in chickens since they act by preventing pathogenic bacteria from proliferating in the intestinal tract (Wang et al., 2017).

Probiotics in broiler feed are also associated with immunomodulation. The addition of probiotic strains to broiler feeds stimulates immune response through immunomodulatory action. In this immunomodulatory activity, the probiotics induce cytokine production that modulates the adaptive and innate immune responses (Sugiharto, 2016). For instance, adding probiotic strains like *Lactobacillus acidophilus* to broiler feeds induces the production of T-helper-1 cytokines while the addition of *Lactobacillus salivarius* stimulate anti-inflammatory responses which are essential in improving the productivity and performance of broiler chickens (Sugiharto, 2016). In an experiment that was conducted by Bai et al. (2013), the results indicated that probiotics, particularly *Lactobacillus fermentum* and *Saccharomyces cerevisiae,* significantly impact the intestinal immune response in broilers. Using these probiotic-supplemented diets in broiler feeds stimulates the T-cell immune system without affecting the growth performance in broiler chickens. Probiotics in broilers also stimulate immunomodulation by enhancing macrophage activity through the production of antibodies. According to Fathi et al. (2017), the probiotic strains phagocytose the

pathogenic microorganism in the gut and enhance the production of antibodies, mainly the Immunoglobulin. The production of these antibodies takes place at the gut mucosal surfaces, where they assist in immune responses.
The key factors influencing the probiotic efficiency in broilers are the antimicrobial and antagonistic activity of the probiotic strains in the gut microbiota. According to Reis et al. (2016), lactic acid bacteria are susceptible to antibodies and they produce an antimicrobial impact in the gut. They produce antimicrobial substances that act on the pathogenic bacteria by inhibiting their proliferation and growth in the intestinal surfaces. Besides, the lactic acid bacteria produce higher concentrations of lactic acid and bile salts in the gut which hinder the growth of pathogenic bacteria. In addition, the efficiency of probiotics in broilers is their ability to compete with the pathogenic bacteria in the gut through antagonism. A study by Shokryazdan et al. (2017) showed that the probiotic strains act through pathogenic interference where they alter the growth and colonization of pathogens. Probiotics act by competitive exclusion through nutrient and attachment site competition with pathogens in the intestinal walls to interfere with their proliferation and growth. Such completion helps provide microbial balance in the intestinal epithelial walls in GIT system that promotes the development and prevention of infectious illness in broilers.

2.4 Prebiotics

2.4.1 Definitions

Prebiotic is also known as non-digestible oligosaccharides (NDO), are compounds that modulate the composition and activity of the microbiota through pathogenic metabolization. This confers valuable psychological effect in the host body (Bindels et al., 2015). The prebiotic definition was almost unchanged when it was first introduced in 1995 by Glenn Gibson and Marcek Roberfroid as “a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon, and thus improves host health” (Gibson and Roberfroid,1995). Thus, to fit the criteria as prebiotic, the compound should be: a) resistant to gastrointestinal digestion, b) can be selectively fermented by intestinal microbiota, and c)also selectively stimulated the growth/activity of the intestinal bacteria and improves host’s health (Gibson.2010) The prebiotic is able to escape from digestion due to the structure of the compound. First is the degree of polymerization of prebiotic. The higher degree of polymerization caused the prebiotic even harder to breakdown and enhanced the growth and fermentation of greater microbiota diversity in gastrointestinal (Erola et al*.,* 2019). Another factor is the beta-bond of the prebiotic structure. As most mammalians’ pancreatic only produced alpha amylase that can only break down alpha bond (Pajic et al.,2018). Thus, those prebiotics that are linked by beta bonds will be indigestible and reached the colon for further fermentation by gastrointestinal microbioda.

2.4.2 Types of Prebiotics

The main types of prebiotics are dietary fibers that are mainly found in vegetables and fruits. The most common dietary fibers are oligosaccharides such as mannanoligosaccharides, fructooligosaccharides, xylooligosaccharides, lactose, soya oligosaccharides, galactooligosaccharides, and isomaltooligosaccharides (Sugiharto, 2016). Other types of prebiotics are soluble dietary fiber pectins, mainly the pecticoligosaccharides, which are found in agricultural by-products like fruits such as cranberries, citrus peel, and sugar beet pulp (Holck et al., 2014). The commercially available prebiotics that are mostly used includes transgalactooligosaccharides, fructooligosaccharides, oligochitosan, mannanoligosaccharides, inulin, and galactooligosaccharides, and lactulose. They are established prebiotics and are readily available in the market, where they are recommended as dietary supplements for health benefits (Samal and Behura, 2015).

2.4.3 Sources of Prebiotics

2.4.3.1 Fruit and Fruit Waste

The extractions of fruits have been regarded as the source of prebiotics. Manuel (2014) asserted that extracting pectin from fruit and fruit peels of apple, lemon, and oranges is a prebiotic source. Fruit extracts are conducted through enzymatic hydrolysis of polysaccharides to find the potential prebiotic carbohydrates. Also, banana and pineapple peels are used as isolate sources of prebiotics. Pereira et al. (2018) explained that bananas are the sources of xylooligosaccharides and fructooligosaccharides. Ripe banana peel and pulp are extracted to produce more significant amounts of fructooligosaccharides and galactooligosaccharides. The extraction of banana peels to get oligosaccharides is achieved through the enzymatic activity of invertase which is the primary banana enzyme. Pineapple peels also have oligosaccharides like banana peels. Parra-Matadamas et al. (2015) asserted that peels are good sources of non-digestible carbohydrates like fiber and oligosaccharides. The extraction of pineapple peels during the processing of fruit juice is a good source of obtaining dietary fibers which are the primary components of prebiotics. The pineapple by-products or pineapple peels are extracted through the action of lactic acid bacteria that help in isolating fibers and oligosaccharides.

2.4.3.2 Plant and Plant Waste

Plants and plant wastes are also sources of prebiotics. The coconut plant and its residues are among the sources of prebiotics. According to Mohd Nor et al. (2017), coconut residues are extracted for crude polysaccharides which can be further digested under enzymatic activity to provide oligosaccharides used as prebiotics. The coconut waste products like coconut kernel and coconut milk are extracted to obtain non-digestible carbohydrates. Also, rice bran and corn cob have prebiotics. Rice bran yogurts can be used to obtain prebiotics through

acidification. The rice bran has a higher percentage (50%) of carbohydrates mainly oligosaccharides which are the key components of prebiotics (Demirci et al., 2017). Rice bran consists of hemicellulosic oligosaccharides which are indigestible carbohydrates due to glycosidic linkages. Through acidic hydrolysis, these hemicellulosic compounds are broken down to galactose, glucose, and mannose oligosaccharides through enzymatic activity to obtain prebiotics (Kurdi and Hansawasdi, 2015). Besides, corn cobs have oligosaccharides which are the main types of prebiotics. Corn cobs' residues are extracted to obtain xylooligosaccharides which are examples of non-digestive oligosaccharides (NDO) important in the gut microbiota. The corn cob residues are extracted to obtain carbohydrate products, mainly the xylooligosaccharides (Pu et al., 2016). The corn cobs are digested by xylanase through ultra-assisted enzymatic hydrolysis to obtain xylooligosaccharides. The xylanase helps in eliminating lignin and xylan that is hydrolyzed to obtain the main component of sugar oligomers or xylooligosaccharides (Sun et al., 2015).

2.4.3.3 Properties and Mode of Action of Prebiotics

Anti-toxic effect is one of the properties of prebiotics. Prebiotics such as oligofructose has antimicrobial and antioxidant properties which are essential in controlling the toxic effects of cycloalkenes, esters, and cycloalkanes in the gut (Mundi et al., 2017). The prebiotics reduces cytotoxicity in the gut cells and do not react with the body or exert harmful effects but prevent acidic and enzymatic activity in the gut microbiota (Mundi et al., 2017). Also, prebiotics has antihypertensive and hypocholesterolaemic properties. Prebiotics are known to reduce the level of lipids in the blood through the production of short-chained fatty acids (Miremadi et al., 2016). This prebiotic is not digested in the small intestines and they enter the colon where they are fermented by lactic acids and acted upon by the lactic acid bacteria to yield lactate. The lactate undergoes further fermentation in the colon, where it produces compounds like acetate and propionate (Miremadi et al., 2016). The antihypertensive effect of prebiotics is also associated with the ability to reduce the absorption of cholesterol, which leads to the regulation of low-density lipoproteins receptors. According to Markowiak and Śliżewska (2017), the consumption of inulin assists in reducing the levels of triacylglycerol in the blood which enhances the reduction of very low-density lipoproteins in the blood. It also facilitates lipid metabolism by reducing the levels of cholesterol and triacylglycerol and thus, reduces blood pressure. Also, prebiotics reduces the pressure used in arteries to pump blood. The reduction of the absorption of cholesterol in the gut minimizes the rigidity of arteries hence reducing blood pressure (Miremadi et al., 2016).

Prebiotics are available in natural products which when ingested, are not digested in the gut. The main function of this prebiotic is to promote health and nutritional value. The prebiotics are indigestible by host enzymes and are passed into the colon through an unaltered process. In the colon, the prebiotics undergoes fermentation by saccharolytic bacteria of *Bifidibacterium* genus (Markowiak and Śliżewska, 2017). The fermentation affects the composition of microbiota in the intestines and affects metabolic activity. This occurs because

the modulation of lipid metabolism takes place which enhances calcium absorption and bowel function. The fermentation also assists in producing energy for useful bacteria in the gut microbiota. Through antagonism and competition for nutrients and attachment to epithelial surfaces in the intestines, the microbiota serves as the pathogenic barrier (Markowiak and Śliżewska, 2017). Carbohydrate metabolism takes place where short-chain fatty acids like propionate, acetate, and butyrate are formed and act as the source of energy to the host. In addition, carbohydrate fermentation produces compounds that inhibit the proliferation and growth of pathogens in the GIT (Slavin, 2013). This causes a pH reduction in the intestines. Based on the favorable effect on the growth of beneficial bacteria in the intestines, prebiotics act by inhibiting the growth and development of potential pathogens. The acidification in the intestines also occurs due to the fermentation of lactulose that enhances pathogen translocation from the bowel (Markowiak and Śliżewska, 2017).

2.4.3.4 Beneficial Effects of Prebiotic on Boilers

The growth performance in broiler chickens is improved with the dietary consumption of prebiotics. According to Sarangi et al. (2016), the addition of prebiotics to broiler feeds beneficially affects the chickens by improving their performance and growth. Prebiotics enhances the growth of beneficial pathogens in the gut like *Lactobacilli* and *Bifidobacteria*. In addition, dietary supplementation with oligochitosan or fructooligosaccharides has growth-promoting effects. Fructooligosaccharides induce beneficial effects to the broiler chickens by selectively improving the microbial growth of *Lactobacilli* and *Bifidobacteria* through inhibition of the growth of pathogens that compete with the useful bacteria for nutrients and space in the intestinal epithelial walls (Arsi et al., 2015). When consumed by poultry, prebiotics assists in restricting the growth of harmful microbial pathogens, regulating metabolism in the poultry, and promoting feed conservation ratio that is essential for growth performance (Wang et al., 2015). Additionally, prebiotics effectively influences body weight gain in broilers. Prebiotics increased the overall body weight by improving the daily weight gain in the ileum, duodenum, and jejunum (Wang et al., 2015).

Prebiotics also influence the balance of the ecosystem in gut microbiota. When added to chicken feeds, the prebiotics assists in maintaining a well-balanced ecosystem in the gut which improves the structure of intestinal mucosa (Pourabedin and Zhao, 2015). Prebiotics preserve the balance of the bacterial population in the gut by ensuring a proper balance between the beneficial bacteria and potentially pathogenic microbes. Prebiotics enhance the function of lactic acid bacteria in the gut which promotes the production of short-chain fatty acids. The production of fatty acids thus promotes the balance of bacteria in the gut of chickens by maintaining homeostasis and normal functions in the intestinal gut (Sugiharto, 2016).

In relation to the immunomodulation activity of prebiotics in broilers, their addition to broiler feeds enhances immune response. According to Pourabedin and Zhao

(2015), prebiotics in poultry production act by interacting with the immune systems through modulating the intestinal system. Prebiotics provide an immune-enhancing effect which leads to the rapid clearance of potential pathogens in the gut. The prebiotics interacts with the gut immune cells through preferential colonization of beneficial microorganisms and bacterial products in the immune system; hence supporting immune response (Sugiharto, 2016). Also, mannanoligosaccharides have immunomodulatory activity in broilers where they change gene expression and promote the production on immunoglobulin A (IgA). The modulation process occurs where the mannanoligosaccharides modulate the cell surface of the mannose receptor that identifies the microbial glycans and glycoproteins in the chickens (Pourabedin and Zhao, 2015). The modulation is facilitated by the binding lectins that activate the inflammatory responses by initiating a cascade expression of cytokines (Pourabedin and Zhao, 2015).

2.5 Extractions Methods of Prebiotics

2.5.1 Solvent Extraction

Various methods have been applied to extract prebiotics. Organic solvent extractions have shown significant benefits in obtaining prebiotics from carbohydrate-rich plants. The solvent extraction method with organic solvent involves the application of organic compounds like esters, benzene, chloroform, acetone, and methanol reagents (Bai et al., 2015). The use of these solvents helps in separating glucose under reduced pH and is examined through high-performance liquid chromatography (Bai et al., 2015). This is evident in an experiment conducted by Lu et al. (2017) that focused on extracting prebiotics from the Lotus seed using liquid chromatography. In this experiment, a high concentration of ethanol was used as the organic solvent. The study showed that adding ethanol to the extracts of lotus seed helps in separating prebiotics. Similarly, an experiment by Jahromi et al. (2015) showed that the extraction of prebiotics through organic solvent extraction with alcohol is effective. The results of the experiment showed that prebiotics like manna-oligosaccharides are effectively extracted when alcohol is used with neutral detergent solutions.

Pure water extraction can also be used to extract prebiotics. In a study conducted by Klinchongkon et al. (2017), subcritical water treatment with maximum temperature ranging from 100°C to 250°C is essential for oligosaccharide extraction. The study involved extracting prebiotics from a passion fruit peel. Water treatment of the fruit peels facilitates the extraction of pectin oligosaccharides. This indicates the effectiveness of the water extraction method in producing a prebiotic which is an essential dietary fiber. Water extract helps in extracting oligosaccharides through ultrasound-assisted extraction in the water medium. A study by Machado et al. (2015) examined the viability of the water extract method through the ultrasound-assisted medium. The study aimed at determining the extraction of prebiotic. From the results obtained, the water

extract method was effective in extracting different prebiotic sugars like fructooligosaccharides and raffinose was the main prebiotic extracted.

2.5.2 Autohydrolysis Extraction

2.5.2.1 Temperature Effects on Oligosaccharide and Sugar Yield

Hot water extraction is one of the autohydrolysis extraction methods for oligosaccharides. Hot water use helps in breaking down the bond linkage between the compounds. High temperature is the key factor in autohydrolysis. In hot water extraction, high temperature yields a higher extraction (Li et al., 2013). Autohydrolysis causes the depolymerization of compounds in high temperatures which enhances the extraction of oligosaccharides. Moura et al. (2015) cited that autohydrolysis can be used to obtain oligosaccharides from polysaccharides. Physical hydrolysis is used in this method, where the process occurs under high temperatures between 130 °C and 230 °C. Increasing the temperature when heating raw material results in depolymerization of hemicelluloses to generate hydronium ions from water autoionization and ionization of acetic and uronic acids. Depolymerization thus yields soluble oligosaccharides due to higher intensity and increased temperatures (Moura et al., 2015).

2.5.2.2 Duration Effects on Oligosaccharide and Sugar Yield

Under very high temperatures of high intensity, the extracted oligosaccharides are further hydrolyzed and monosaccharides are formed (Moura et al., 2015). The formed monosaccharide sugars are also decomposed and the formation of monomers increased where hydroxymethylfurfural and furfural are generated as dehydration products (Cardenas-Toro et al., 2014). The sugar yields are decomposed and partial dissolution of acid-soluble lignin takes place. Also, solubilization takes place where proteins from the raw material and amino acids react with sugars to form melanoidins (Cardenas-Toro et al., 2014).

2.5.3 Identification of Oligosaccharide

2.5.3.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is among the current techniques used to identify or determine oligosaccharides and their structures. The application of NMR spectroscopy in carbohydrates helps in identifying the structure and conformation of oligosaccharides (Lundqvist, 2015). The NMR spectroscopy determines the conformational properties of oligosaccharides through the identification of three-dimensional structures. Using the NMR

spectroscopy determines the conformation of mannose oligosaccharides and mannose recognizing lectins (Shahzad-ul-Hussan et al., 2017). The method helps in identifying the chemical shifts of undisclosed oligosaccharides. The determination of unidentified oligosaccharides indicates the usefulness of NMR spectroscopy for untargeted glycomics and identifying the chemical structure of unknown oligosaccharides (Klukowski et al., 2018).

2.5.3.2 Liquid Chromatography (LC) with Mass Spectrometry (MS)

Liquid chromatography with mass spectrometry assists in the identification of oligosaccharides. This liquid chromatography separates, identifies, and quantifies all the unknown components in a mixture (Pang et al., 2016). When used to identify oligosaccharides, liquid chromatography with mass spectrometry is an effective technique because of its high sensitivity and capability to provide sufficient information about the structure of oligosaccharides. Besides, liquid chromatography-mass spectrometry is accurate and has enough ability to quantify various oligosaccharides in a single run (Liu et al., 2014). Liquid chromatography-mass spectrometry technique is established as a built-in logical procedure where it assists in determining oligosaccharides using chromatogram. The technique works by determining glucose oligosaccharides (Hsu et al., 2018). A mass spectrometry having resonance excision and low-energy collision-induced dissociation with liquid chromatography is effective in determining oligosaccharides. Since carbohydrates have an increased number of isomers, identifying the structures of carbohydrates using mass spectrometer alone is difficult because of low ionization efficiency. Thus, using liquid chromatography with mass spectrometry is effective for structural analysis of glucose oligosaccharides due to high detection sensitivity (Hsu et al., 2018).

2.5.4 Challenges in Water Extraction of Oligosaccharide from Plant Cell Wall

Water extraction influences the intermolecular interactions of ions. In oligosaccharides, water extraction in high temperatures leads to depolymerization of oligosaccharides like hemicellulose, increasing the formation of hydroniums (Moura et al., 2015). Other challenges include the degradation and occurrence of chemical reactions during the extraction process. Plaza and Turner (2015) asserted that there are risks of chemical reactions when using pure hot water extraction, leading to erroneous results. In addition, the clogging of tubing may occur. This is associated with the precipitation of the extracted analytes when they are cooled to temperatures that the analytes cannot be soluble.

2.6 Beneficial Effects of Combination of Probiotic and Oligosaccharide (Synbiotic) on Broilers

Synbiotics involve using both or combining lactic acid bacteria and prebiotics. In poultry like chickens, synbiotics have significant roles in improving the survival and activity of beneficial microorganisms in the GIT (Scholz-Ahrens et al., 2016). The prebiotic and probiotic foods remain in the digestive system of chickens and act by minimizing the counts of potential pathogens while increasing the population of beneficial bacteria in the gut. Also, the consumption of symbiotic food enhances growth performance through the body weight gain of chickens (Abdel-Hafeez et al., 2017). Therefore, combining probiotics and oligosaccharides in broiler feeds may influence the growth and bacterial composition in the gut. According to Suthama et al. (2018), combining probiotics and oligosaccharides in chicken diets promotes the morphology of the intestinal gut and the absorption of nutrients which is important for improved growth performances in broilers. Including oligosaccharides in broiler feeds enhance protein digestibility, promoting intestinal development and providing health benefits in supporting nutrient digestibility for improved growth and poultry performance (Suthama et al., 2018). In addition, Sugiharto (2016) indicated that the combination of probiotics with oligosaccharides facilitates the survival of health-promoting microorganisms in the GIT of broilers. The combination also enhances the microbial ecosystem in the intestines of chickens and promotes the immune functions that improve the performance of broilers.

2.7 Study of Broiler Gut Microbiota

2.7.1 Conventional Microbiological Method

The conventional microbiological method involves using culturing techniques to examine the gut microbiota to study the microorganism. According to Guinane and Cotter (2013), conventional culturing techniques help reveal changes in the GIT microbial flora and metabolic activities of bacteria in the gut. The conventional method determines the composition of bacteria in the gut microbiota and in determining gene changes. The conventional culturing method involves using differential media to identify and study specific bacterial populations related to metabolic requirements (Rinttilä and Apajalahti, 2013). This method is applicable in recovering microbial isolates under aerobic and anaerobic conditions. Besides, the conventional culturing method provides a clear understanding of gut microbiota by investigating metabolic activities and the composition of a specific population of bacteria in the gut (Rinttilä and Apajalahti, 2013).

A real-time quantitative polymerase chain reaction (qPCR) method is among the best approaches used in determining and quantifying individual species. The qPCR method is applicable when identifying and quantifying the ecology of species. The method provides rich information about the genetic process in species (Feckler et al., 2017). The qPCR method is a sensitive method for gene quantification and is widely used in biomedical and biological fields. When using this method, the key principle is the amplification process where double-stranded sequences are amplified following the reactions of reagents (Rao et al., 2013).

When studying the gut microbiota, the qPCR method has shown significant contributions in identifying the psychological functioning and health in the gut. According to Kwok et al. (2014), the gut microbiota consists of a complex bacterial community involved in the digestion and metabolism of substances in the gut. Therefore, the qPCR method has shown significant benefits in studying the microbial composition and diversity of microbes in the gut. This method is an effective approach that provides accurate quantitative information on the gut microbiota. In addition, the qPCR method is used in studying the composition of GIT, its association with the diet, and assessing its function based on health and disease. The method is used in clinical assessment for the efficacy of probiotics in the gut microbiota (Davis, 2014).

2.7.2 High-throughput Next-generation Sequencing of 16S rRNA Gene

The 16S rRNA gene is also useful in studying gut microbiota. The 16S rRNA gene is used in studying the gut microbiota diversity and bacteria in it. The method provides new insights into the common bacteria and the balance of the ecological environment in the gut (Kwok et al., 2014). The next-generation sequencing of 16S rRNA is used in analyzing the composition of gut microbiota through DNA sequencing and analysis of primers. The method is more effective than the culturing method since it provides a fast and high throughput analysis of the intestinal microbiota using the DNA primers (Rintala et al*.,* 2017). The high throughput analysis of the gut microbiota provides significant information about the gut profile and the microbiome which is essential in understanding the composition in the intestines (Osman et al., 2018). The DNA sequencing through next-generation sequencing of 16S rRNA gene provides characterization of the gut microbiome that gives new insight about the phylogeny and taxonomy of the bacteria. The 16S rRNA gene is used as the gene marker and it has hypervariable regions that are used in identifying the bacteria in the gut (Osman et al., 2018).

2.8 Conclusion

The poultry industry is widely practiced globally and among the leading practices in Malaysia. Poultry farming with broiler chickens is practiced across Malaysia. Initially, the poultry industry in Malaysia practiced the use of AGPs to improve productivity. However, the use of antibiotics was banned following the incidences of infections and diseases that can be transmitted to humans. As such, new alternatives with natural products as growth promoters have been examined and have been found to be effective than AGPs. The NGPs have being used in the poultry industry to promote the growth and performance of chickens. Probiotics, prebiotics, and synbiotics are among the AGPs alternatives used in the poultry industry. The addition of these NGPs to broiler feeds promotes the growth performance, immune response, and body weight gain in chickens.

CHAPTER 3

SCREENING OF DIFFERENT LOCAL PLANT RESOURCES FOR POTENTIAL OLIGOSACCHARIDES TAT COULD ENHANCE GROWTH OF FRIENDLY GUT BACTERIA

3.1 Introduction

The emergence of antibiotic-resistant bacteria has led to the urge of replacing the antibiotic growth promoters (AGPs) with natural substances (Casewell et al., 2003; Donoghue, 2003; Preidis and Versalovic, 2009). Throughout these years, various type of replacements was tested in the lab, including enzyme, probiotic and prebiotic. However, the cost to reproduce all these potential AGPs' replacements on an industrial scale is too high. One of the promising AGPs' replacements, prebiotics are defined as the low molecular weight of carbohydrates made up of 2-10 sugar units, which also known as non-digestible oligosaccharides (NDO) (de Moura et al., 2015). Interestingly, NDO is naturally available in plants and fruits. Malaysia is well known for the industrial agriculture business and therefore, the existence of a huge amount of agricultural waste is undeniable. Rice bran, pineapple peel, oil palm frond, coconut shred, and corn cob are some examples of waste that are massively produced in Malaysia (Jamil, 2004). Despite these plant waste being considered as having no value and being discharged after their primary use, there are several studies that reported the existence of NDO in agricultural waste (Amulya et al., 2016; Singh et al., 2015; Carvalho et al., 2013). These studies suggested that there is a potential to reproduce the AGPs' replacements on an industrial scale with lower cost by utilizing these unwanted agricultural wastes. The extraction of oligosaccharides from agricultural waste can be performed with chemical, enzymatic or physical agents. However, this method requires specific enzymes and highly controlled reaction conditions, which usually will result in a lower yield, contributing to the synthesis being more expensive and difficult to reproduce on a large scale (Barreteau et al., 2006). On the other hand, mild autohydrolysis extraction method is an environmentally friendly technology for oligosaccharides production, especially from plants.

In the aspect of cost efficiency and reproducibility on an industrial scale, physical agents such as sonification and hydrolysis are the best choice for oligosaccharide production (Parajo et al., 1999; de Moura et al., 2015; Swennen et al., 2016). For extraction of low-molecular weight carbohydrates such as oligosaccharides from plant material, the optimal solvent to be used is water (Knudsen and Li, 1991). As the final product of the extract in this study will be fed to broilers in large amounts, simple hot water extraction (autohydrolysis) is carried out for optimization purposes.

In order to evaluate the potential content of the carbohydrates after the extraction process, the phenol-sulfuric acid method is a simple and rapid colorimetric method that can be used to determine the total carbohydrates in a sample. This method is able to detect virtually all classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides (Dubois et al. 1956; Chow and Landhäusser 2004; Masuko et al. 2005). In this method, xylose should be used to construct the standard curve for the assay, and the absorption is measured at 480 nm. For products that are high in hexose sugars, glucose is commonly used to create the standard curve, and the absorption is measured at 490 nm. The color for this reaction is stable for several hours, and the accuracy of the method is reported to be within ±2% under proper conditions. In contrast, a modified Dinitrosalicylic acid (DNS) method can also be applied to estimate the total reducing sugar in the plant crude extract. A reducing sugar is defined as any sugar that, in a solution, has a free aldehyde or a ketone group which will react with the reagent 3,5-dinitrosalicylic acid (DNS), and reduced into 3 amino 5 nitro salicylic acid under an alkaline solution. The 3 amino 5 nitro salicylic acid strongly absorbs light at 540 nm and allows the reducing sugars to be quantified . This reaction will result in color changed and the intensity can be determined by a spectrophotometer at 540 nm. Hence, several agricultural wastes, including coconut shreds, rice bran, corn cob, oil palm frond, pineapple peel, and banana peel, were selected to discover the potential of enhancing prebiotic bacteria.

Thus, the objective of the study was to screen for prebiotics from local plant resources. First, plant wastes were collected and extracted using sonification and autohydrolysis method, follow by determining the plant waste candidates that yield the highest total carbohydrates including monosaccharides, oligosaccharides and polysaccharides. Next, the prebiotic properties of the crude extracts of the plant wastes on *Lactobacillus* strains were studied. Lastly, optimization of the extraction methods of the selected plant waste candidate that give the highest yield of total carbohydrate.

3.2 Materials and Methods

3.2.1 Water Extraction of Plant Wastes for Potential Oligosaccharides via Sonification

Fresh coconut shred, rice bran, corn cob, oil palm frond, pineapple peel, and banana peel were rinsed with tap water and dried at oven (50 °C - 60 °C) until they were completely dry. The dried samples were then ground and sieved (2.0 mm) into powder form using a sieve grinding machine. The dried powder was sealed and stored in desiccators to prevent moisture. 1:10 of sample-to-solvent solution were weighed and measured in the conical flask. The mixtures were mixed well and undergo sonification in the water bath at 27 °C for 30 minutes. The mixtures were shaken for 6 hours at room temperature (25 °C) and filtered using a muslin cloth. The extracted solvents were further evaporated using a rotary evaporator at 55 °C. The concentrated solvents were freeze-dried into

powder form and stored in a desiccator before further analysis. The yield of each extraction was weighed to obtain the mass (g) per volume (mL).

3.2.2 Phenol-Sulfuric Acid Method for Total Carbohydrate

Five percent of phenol was prepared by dissolving 5g of solid phenol (C_6H_5OH) in 100 mL deionized distilled water using magnetic stirring. Glucose and xylose stock solutions (100 mg of glucose and xylose) were dissolved each in 1 mL deionized distilled water. 40, 80, 120, 160, 100 µL of the stock solution were pipetted into test tubes labelled 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL, respectively and topped up to final volume 2 mL with deionized distilled water. 0 mg/mL is purely deionized distilled water which was also used as blank. 1 mL of 5% phenol were added into each test tube followed by 5 mL of concentrated sulfuric acid (H_2SO_4). The mixtures were vortex vigorously for 10 minutes and incubated in 25 °C water bath for 20 minutes. The absorbance of glucose was read at 490 nm, whereas for xylose at 480 nm. The standard curves of absorbance values against the concentrations (mg/mL) for glucose and xylose were plotted. For sample preparation, 100 mg of every freeze-dried plant crude extract was dissolved in 1 mL deionized distilled water and was further diluted to 10-fold dilution. 2 mL of the diluted extract solution was pipetted into a test tube. The procedure carried out for glucose and xylose was repeated in a similar fashion with all the freeze-dried plant crude extract samples and their absorbance were read at both 480 nm and 490 nm wavelength. Their actual concentration (mg/mL) of total carbohydrate were determined using the slope equation for both glucose and xylose (Appendix I) and multiplied by 10. All measurements were performed in triplicate parallels and performed twice.

3.2.3 3,5-dinitrosalicylic Acid (DNS) Method for Total Reducing Sugar Test

The DNS reagent was prepared by slowly dissolved 2.18 g of DNS (MW=228.1 g/mol) in 80 mL of 0.5 M NaOH through heating and stirring at 70 ºC. 30 g of sodium potassium tartrate (MW=282.2 g/mol) was added and stirred until dissolved completely. The reagent was then topped up to 100 mL and cooled down at room temperature. The DNS reagent is very sensitive to light and thus, can only be used within 3-4 months if it is wrapped with aluminum foil and stored in a dark area. Glucose and xylose stock solutions: 100 mg of glucose were dissolved each in 1mL deionized distilled water. 40, 80, 120, 160, 100 µL of the stock solution were pipetted into test tubes labelled 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL, respectively, and topped up to final volume 2mL with deionized distilled water. 2 mL of DNS reagent was added into each sample and heated at 90 °C water bath for 15 minutes to develop red-brown color. The mixtures were vortex for 1 minute before the absorbance of glucose reading was taken at 540 nm wavelength using a spectrophotometer.

For sample preparation, 100 mg of each freeze-dried plant crude extract was dissolved in 1 mL deionized distilled water and was further diluted to 10-fold dilution. 2 mL of the diluted plant extract solution was pipetted into test tube. The procedure carried out for glucose was repeated in a similar fashion with all the freeze-dried plant crude extract samples and their absorbance were read at 540 nm wavelength. Their actual concentration (mg/mL) of total sugars was determined using the slope equation for glucose and xylose and multiplied by 10. Deionized distilled water and DNS mixture were set as blank. Each sugar and phenol combination was replicated 3 times.

3.2.4 Bacteria Strains, Culture Medium and Growth Conditions

Total of four *Lactobacillus* strains (Table 3.1) and 3 pathogenic strains including *Salmonella thyphimurium* NCTC13348 (ST), *Salmonella enterica* serovar Enteritidis 692/06 (SE), *Escherichia coli* ATCC25922 (EC) were selected for the single cultured relative growth test on plant extracts substrates. 16S DNA sequencing was conducted on all of the bacteria strains for confirmation. All bacteria strained were revived from the glycerol stock and inoculated on agar plate accordingly. *Lactobacillus* strains were inoculated in MRS broth at an anaerobic condition in anaerobic jars (Oxoid, UK) with Gas Generating Kits (Oxoid, UK), whereas pathogenic bacteria were inoculated in nutrient broth (NB) at the aerobic condition. All bacteria strains were cultured in an incubator at 37 °C. Stock cultures of all bacteria were prepared with a final concentration of 40% glycerol and stored in -20 °C.

Table 3.1: *Lactobacillus* strains and locations of isolation from the gastrointestinal tract of broilers.

Sequence analysis of 16S rRNA and 16S-23S rRNA gene by Lee *et al.* (2008)	Location of isolation from chicken gut
L. reuteri C 1	Cecum
L. gallinarum I 16	Ileum
L. salivarius I 24	Ileum
L. brevis I 218	Ileum

3.2.5 Modified Basal Growth Media

For the screening of the plant extract effects on the bacteria strains, all modified substrate-free basal media were prepared by mixing manually (Table 3.2) where glucose was excluded. All of the mediums were sterilized at 121 °C for 15 minutes.

Table 3.2: Compositions of modified de Man, Rogosa and Sharpe (mMRS), Nutrient broth (mNB) broth.

Components (g/L)	mMRS[1]	mNB[2]
Peptone (Merck, Germany)	10.00	15.00
Yeast extract (Oxoid, UK)	5.00	3.00
$NaCH_3COO$ x $3H_2O$ (Riedel-de Haen, Germany)	5.00	
NaCl (Merck, Germany)		6.00
K_2HPO_4 x $3H_2O$ (Merck, Germany)	2.00	
$(NH_4)_3C_6H_5O_7$ x $2H_2O$ (BDH, England)	2.00	
$MgSO_4$ x $7H_2O$ (Merck, Germany)	0.20	
$MnSO_4$ x $4H_2O$ (Merck, Germany)	0.05	
Tween 80 (Fluka, Switzerland).	1.00 mL	
pH	6.20	7.50

[1] mMRS broth was referred to Saarela et al., 2003.
[2]mNB broth was referred to Merck manufacture ingredients.

3.2.6 Crude Extract Substrates

The plant crude extracts that have the highest total sugar concentration (mg/mL) were selected as the substrate for bacteria. Stock solutions of these plant crude extracts were prepared in de-ionized water and filter sterilized with 0.2 µm filters (Minisart filters, Sartorius AG, Germany). The sterile plant crude extract solutions were added to autoclaved modified basal medium to obtain a final substrate concentration of 1% (w/v). Glucose (Sigma-Aldrich) (1%, w/v), which is a monosaccharide and a favorable carbohydrate source for all microorganisms strains and easily hydrolyzed in the upper gastrointestinal tract, was used as a control.

3.2.7 Determination of Relative Growth of Beneficial and Non-Beneficial Bacteria Strains on Plant Crude Extracts

In order to study the effects of plant crude extracts on the growth of *Lactobacillus* and pathogenic bacteria strains in utilization of the plant crude extracts, carbon-free basal broth medium were supplemented with 1% (w/v) of the test plant crude extracts or glucose (control) as the sole carbon source was inoculated with 1% (v/v) inoculum of *Lactobacillus* (6-h-old, log phase) or pathogenic bacteria (3-h-old, log phase) both giving a final cell concentration of 10^5 colony forming units per milliliter (cfu/mL). *Lactobacillus* strains were incubated in an anaerobic jar (Oxoid, UK) supplied with an anaerobic Gas Generator Pack (Mitsubishi, Thermo Fisher, US), whereas pathogenic bacteria strains were incubated in aerobic conditions. All bacteria strains were incubated at 37 °C. After 24 h of incubation, the cultures were vortexed for 30 s to disperse the bacterial cells, and the growth of each strain was determined by subtracting the optical density (OD 620 nm) values of the blank medium from each test media using a spectrophotometer

(Biomate 3, Thermo, US). Three replicates were made for each *Lactobacillus* and non-beneficial bacteria strain in each crude extract and control.

3.2.8 Determination of Relative Growth of Beneficial and Non-Beneficial Strains

The growth response of each bacteria stains on each plant crude extracts relative to its growth response on glucose was calculated using the method of (Kneifel et al., 2000). The formulation of the calculation was performed as shown below, where growth on glucose is set as 100%:

$$\text{Relative growth of bacteria strains on plant extract} = \frac{\text{OD620 value of a strain on a test plant extract}}{\text{OD620 value of the same strain grown on glucose}} \times 100\%$$

By this method, the results would reflect the growth of a specific bacteria strain on a particular plant crude extracts as the substrate to its growth on a glucose substrate that was fixed at 100%.

3.2.9 Optimization of Selected Plant Extract

The selected plant crude extract that has the highest mass yield (g/mL) and content of total carbohydrate was selected for the optimization of higher mass yield and total carbohydrate content with lower total reducing sugar. As the final product will be fed to broilers, organic solvents which is hazardous to broilers will not be tested in the optimization study. Mild hydrolysis was selected due to environmental and cost-friendly. Hence, two parameters were considered in the optimization: temperature (°C) and duration of extraction (minutes). The selected plant wastes were extracted at 45 °C, 50 °C , 60 °C, 70 °C , 80 °C, 90 °C, 100 °C, 110 °C, 121 °C and 135 °C by using the laboratory high-pressure steam sterilizer (SX-500, Digital Biology®, Tokyo) for 15 minutes. After hydrolysis, the mixtures were mixed well and undergo sonification in the water bath (27 °C) for 30 minutes. The mixtures were shaken for 6 hours at room temperature and filtered using a muslin cloth. The extracted solvents were further evaporated using a rotary evaporator at 55 °C to remove excess water. The concentrated solvent was freeze-dried into powder form and stored in a desiccator before further analysis. The total carbohydrate and total reducing sugars were measured by the phenol-sulphuric and DNS methods.

3.2.10 Antimicrobial Activity of Lactobacillus Strains on Pathogenic Bacteria

In order to determine the effect of corn cob extract on *Lactobacillus* bacteria utilization and antagonized properties toward pathogenic bacteria, an

antagonist/pathogen bacterial culture system was conducted. In a typical culture system, each of the *Lactobacillus* bacteria strains with each of the pathogenic bacteria strains was co-cultured to determine the effect of plant crude extract on the interaction between the two bacteria strains. Mono-culture of each *Lactobacillus* and pathogenic bacteria was also conducted to determine the viability of single bacteria growth on crude plant extract without any interference of another bacteria strains.

3.2.10.1 Bacteria Strains, Culture Medium and Growth Conditions

A total of 4 *Lactobacillus* strains (*Lactobacillus salivarious* I24, *Lactobacillus brevis* I25, *Lactobacillus gallinarium* I26, *Pediococcus pentosaceus* PP6) were tested using the following pathogenic bacteria as indicator strains: *Escherichia coli* (EC), *Salmonella thyphimurium* (ST), *Salmonella enterica* (SE), *Enterococcus faecium* (EFm), *Enterococcus faecalis* (EFs), *Clostridium perfringens* (CP), *Campylobacter jejuni* (CJ). The bacteria used in this study were maintained on the corresponding basal media and oxygen requirements (Table 3.3). Bacteria strains that maintained anaerobically were incubated in anaerobic jars with Gas Generating Kits whereas bacteria strains that maintained aerobically were incubated in an incubator shaker. All bacteria strains were incubated at 37 °C for 24 hours.

Table 3.3: List of bacteria strains with the basal media and oxygen requirement for growth.

Bacteria strains	Basal media	Oxygen requirement
***Lactobacillus* strains**		
Lactobacillus salivarious I24	MRS	Anaerobic
Lactobacillus brevis I25	MRS	Anaerobic
Lactobacillus gallinarium I26	MRS	Anaerobic
Pediococcus pentosaceus PP6	MRS	Anaerobic
Pathogenic strains		
Escherichia coli	NB	Aerobic
Salmonella thyphimurium	NB	Aerobic
Salmonella enterica	NB	Aerobic
Enterococcus faecium	BHI	Anaerobic
Enterococcus faecalis	BHI	Anaerobic
Clostridium perfringens	BHI	Anaerobic
Campylobacter jejuni	BHI	Anaerobic

3.2.10.2 Mixture Modified Basal Media of Antagonist/Pathogen Bacterial Culture System

Modified substrate-free MRS and NB were prepared as shown in previous Table 3.2. The modified substrate-free Brain Heart Infusion (BHI) broth contained the following components (g/L): Peptone (Merck, Germany), 10.00; NaCl (Merck, Germany), 5.00; Na_2HPO_4 (Merck, Germany), 2.50. The pH of the medium was adjusted to 9.00. All modified basal media were sterilized at 121 °C for 15 minutes. The basal media of co-culture is a mixture of mMRS and mNB/mBHI with a ratio of 1:1 according to the inoculated bacteria.

3.2.10.3 Determination of Viability of Mono- and Co-Culture of Lactobacillus and Pathogenic Strains

After 24 hours of anaerobic incubation of mono- and co-cultured of bacteria strains, the cultures were vortexed for 5 s to disperse clumps of cells, then 1 ml was taken, and 10-fold serial dilutions (10^{-1} – 10^{-9}) were made using peptone (1% w/v) and water diluents. Aliquots of 100 µL from the serial dilutions were plated on MRS agar, NB agar, BHI agar, accordingly and incubated anaerobically in anaerobic jars with Gas Generating Kits at 37 °C for 48 h, after which colonies formed on the plates were counted. Growth was determined as cfu/mL.

3.2.11 Statistical Analysis

Statistical analysis of data was performed using the IBM SPSS statistical for Windows, version 22.0 (Armonk, NY: IBM Corp). For the growth (OD 620 nm) study, significant differences in the effect of the different substrates, *Lactobacillus* and pathogenic strains and their interaction (substrate x strains) were tested using two-way analysis of variance (ANOVA). Significant differences between means were analysed using post hoc tests at $P < 0.05$.

3.3 Results

3.3.1 Total Carbohydrate and Total Reducing Sugar Content of Plant Crude Extracts

The total sugar and reducing sugar concentration of plant waste extracted through sonification in the water bath at 27 C for 30 minutes (Section 3.2.1) were tabulated in Table 3.4. Based on the results shown in Table 3.4, corn cob extract, pineapple peel extract and banana peel extract had significantly ($P < 0.05$) higher total carbohydrate and total reducing sugars than coconut shred extract, rice bran extract and oil palm frond. Corn cob extract showed the highest total

carbohydrate content, 48.28 ± 12.89 mg/mL and 50.88 ± 15.04 mg/mL, with the lower total sugar content of 30.59 ± 10.06 mg/mL, compared to pineapple peel extract and banana peel extract. Conversely, coconut shred had the lowest total carbohydrate and total sugar concentration, which is 5.05 ± 1.83 mg/mL, 4.98 ± 1.04 mg/mL, 2.63 ± 0.46 mg/mL, respectively. The four highest total carbohydrate and total reducing sugars content were selected for the next study on the relative growth effect of *Lactobacillus* and pathogenic bacteria.

Table 3.4: Total sugar concentration and reducing sugar of plant waste via aqueous extractions method.

Plant waste	Total carbohydrate concentration 480 nm (mg/mL)	Total carbohydrate concentration 490 nm (mg/mL)	Total reducing sugar concentration (mg/mL)
Coconut shred	5.05 ± 1.83[a]	4.98 ± 1.04[a]	2.63 ± 0.46[a]
Rice bran	8.76 ± 1.31[a]	8.83 ± 2.27[a]	5.88 ± 0.74[a]
Corn cob	48.28 ± 12.89[c]	50.88 ± 15.04[c]	30.59 ± 10.06[c]
Oil palm frond	18.89 ± 6.61[b]	14.20 ± 5.25[b]	12.57 ± 3.19[b]
Pineapple peel	44.96 ± 12.22[c]	50.48 ± 11.84[c]	44.89 ± 10.21[c]
Banana peel	45.32 ± 11.02[c]	50.43 ± 12.23[c]	40.01 ± 12.21[c]

[a-c] The total carbohydrate concentration and toal sugar concentration among the plant waste with different superscripts are significantly different ($P < 0.05$).

3.3.2 Screening of Selected Plant Extracts on The Relative Growth of Beneficial and Non-Beneficial Bacteria Strains

From the results of the screening study above, the four selected plant crude extracts (oil palm frond, corn cob, banana peel and pineapple peel) supported the growth of *Lactobacillus* and pathogenic bacteria at varying degrees. It was evident that the *Lactobacillus* strains exhibited plant crude extracts preference. Oil palm frond crude extract resulted in a significant low ($P < 0.05$) relative growth of all *Lactobacillus* strains compared to other plant crude extracts, in conjunction with the relative growth of pathogenic strains. On the contrary, pineapple peel extract was more favorable among the pathogenic strains, especially ST and EC ($P < 0.05$), but not among *Lactobacillus* strains. The relative growth of I16, I24 and CI were significantly highest ($P < 0.05$) in corn cob crude extract, whereas I128 resulted in the highest relative growth in the banana plant extract. In addition, corn cob crude extract is less favorable among pathogenic strains compared to other plant crude extracts.

In conclusion, corn cob crude extract exhibited prebiotic properties by enhancing most of the growth of the tested *Lactobacillus* strains but not pathogenic strains. In addition, the yield of total carbohydrate is the highest among the four-selected plant crude extract. Thus, corn cob was chosen for the next optimization study.

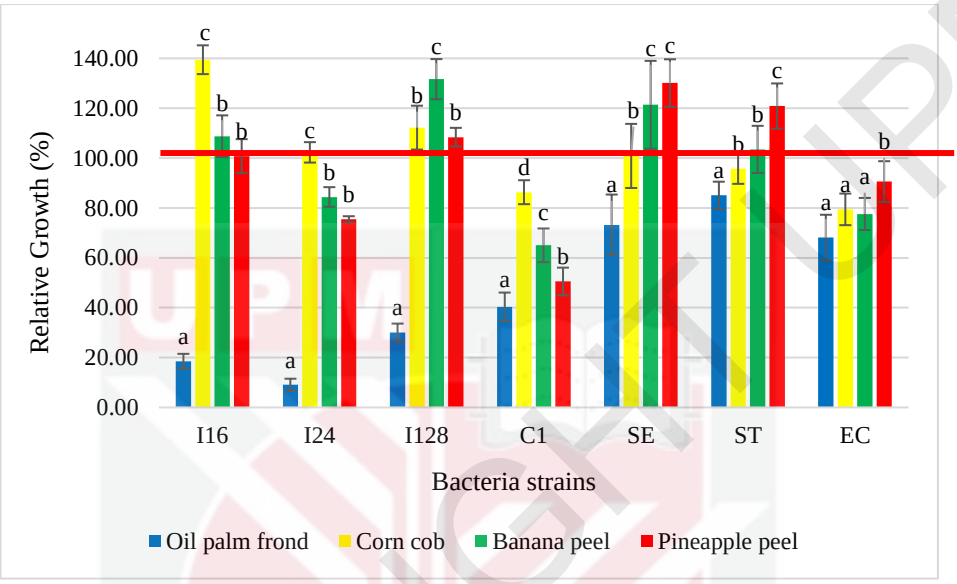


Figure 3.1: The relative growth of *Lactobacillus* and pathogenic bacteria on 1% plant extract compared to glucose.
The relative growth of bacteria strains on glucose are fixed at 100% (red line). *L. acidophilus* = I16, *L. salivarious* = I24, *L. brevis* = I218, *L. reuteri* = C1, *Salmonella enterica* = SE, *Salmonella typhimurium* = S and *Escherichia coli* = EC.
[a-c] The relative growth of specific bacteria strains in comparison to plant crude extract substrate with different superscripts are significantly different ($P < 0.05$).

3.3.3 Optimization in Yield, Total Carbohydrate and Total Reducing Sugar

The total carbohydrate and total reducing sugar (mg/mL) yield increases from 45 °C - 135 °C. Extraction at 45 °C and 50 °C resulted in significant ($P < 0.05$) lowest yield of total carbohydrate (mg/mL). As the extraction temperature increase, the yield of total reducing sugar (mg/mL) also increase by 10.63 ± 19.30 to 16.47 ± 1.93 mg/mL. Extraction at 80 °C was selected for the next time period optimization, due to the numerically highest total carbohydrate yield and significantly ($P < 0.05$) lowest total reducing sugars compared to 90 °C, 100 °C, 110 °C, 121 °C and 135 °C.

The extraction at 80°C for 20 minutes resulted significant ($P < 0.05$) increase of about 18.98-23.19 mg/mL of total carbohydrate compared to 15 minutes. However, no significant increase of total carbohydrate yield after 20 minutes of extraction duration. Furthermore, no significant total reducing sugar (mg/mL) were recorded. Thus, extraction of corn cob at 80°C for 20 minutes were selected for effect on *Lactobacillus* antigonistic properties against pathogenic bacteria compared to the commercial xylooligosaccharide (XOS).

Table 3.5: Total sugar concentration and reducing sugar of corn cob extracted at different temperatures (°C) via aqueous extraction method.

Temperature (°C)	Total carbohydrate 480 nm (mg/mL)	Total carbohydrate 490 nm (mg/mL)	Total reducing sugar (mg/mL)
45	30.12 ± 13.87^{a}	28.32 ±12.93^{a}	10.63 ± 19.30^{a}
50	31.11 ± 14.01^{a}	28.21 ± 14.15^{a}	10.33 ± 18.44^{a}
60	42.73 ± 10.51^{ab}	38.32 ± 11.83^{b}	13.43 ± 12.89^{a}
70	57.35 ± 10.87^{b}	53.10 ± 10.04^{c}	15.99 ± 15.38^{a}
80	**86.93 ± 11.37^{c}**	**80.32 ± 19.88^{d}**	**16.47 ± 1.93^{a}**
90	83.24 ± 10.52^{c}	79.43 ± 20.52^{d}	35.48 ± 13.59^{b}
100	80.67 ± 10.91^{c}	73.97 ± 19.90^{cd}	38.22 ± 12.96^{b}
110	81.63 ± 21.89^{bc}	80.10 ± 22.03^{d}	38.93 ± 10.37^{b}
121	81.32 ± 22.05b^{bc}	75.12 ± 21.00^{cd}	55.83 ± 18.42^{c}
135	83.55 ± 21.02^{bc}	73.94 ± 25.31^{cd}	55.93 ± 14.95^{c}

[a-d] The total carbohydrate concentration and toal sugar concentration of corn cob extracted at different temperature (°C) with different superscripts are significantly different ($P < 0.05$).

Table 3.6: Total carbohydrate and reducing sugar of corn cob extracted at different duration (minutes) at 80 °C via mild autohydrolysis extraction method.

Duration (minutes)	Total carbohydrate 480 nm (mg/mL)	Total carbohydrate 490 nm (mg/mL)	Total reducing sugar (mg/mL)
15	86.93 ± 11.37^{a}	80.32 ± 19.88^{a}	16.47 ± 1.93^{ab}
20	**105.91 ± 20.54^{b}**	**103.51 ± 23.91^{b}**	16.43 ± 1.10^{ab}
40	99.94 ± 4.82^{b}	99.00 ± 5.45^{b}	13.03 ± 2.28^{a}

Table 3.6: Continued

Duration (minutes)	Total carbohydrate 480 nm (mg/mL)	Total carbohydrate 490 nm (mg/mL)	Total reducing sugar (mg/mL)
60	98.44 ± 3.82^{b}	93.64 ± 2.35^{b}	14.51 ± 2.27^{a}
80	95.81 ± 2.95^{b}	92.72 ± 4.19^{b}	12.98 ± 1.27^{a}
100	100.88 ± 3.34^{b}	96.84 ± 2.21^{b}	14.83 ± 1.45^{a}

[a-b] The total carbohydrate concentration and toal sugar concentration of corn cob extracted at different duration (minutes) at 80°C with different superscripts are significantly different ($P < 0.05$).

Over analysis indicated that all *Lactobacillus* strains supplemented with corn cob crude extract resulted in better suppression or inhibition of pathogenic bacteria compared to the commercial XOS. Total inhibition of *S. typhimurium* NCTC 13348 were observed in I24, I26 and PP6, whereas I25 was able to suppress 0.54 cfu/mL of the growth. The growth of *E. coli* 01:K1, *S. thyphimurium* ATCC 81205, and *S. enterica* were suppressed by I24, I26 and PP6.

Among all of the *Lactobacillus* strains supplemented in corn cob extract, only PP6 capable of suppressing the growth of *E. feacium*. Both I26 and PP6 suppressed the growth of *E. feacalis* and *C. perfringen*. Total inhibition of *C. jejuni* by PP6 were recorded. In addition, total inhibition of *S. Typhimurium* by I24 and I26 were also recorded.

On the other hand, supplementation of commercial XOS in the antagonist/pathogenic culture system resulted in suppression only by I25 on *E. coli* 01:K1, *S. typhimurium* ATCC 81205, *S. Enterica, C. perfringens,* and *C. jejuni*, where no inhibition of any pathogenic bacteria was recorded.

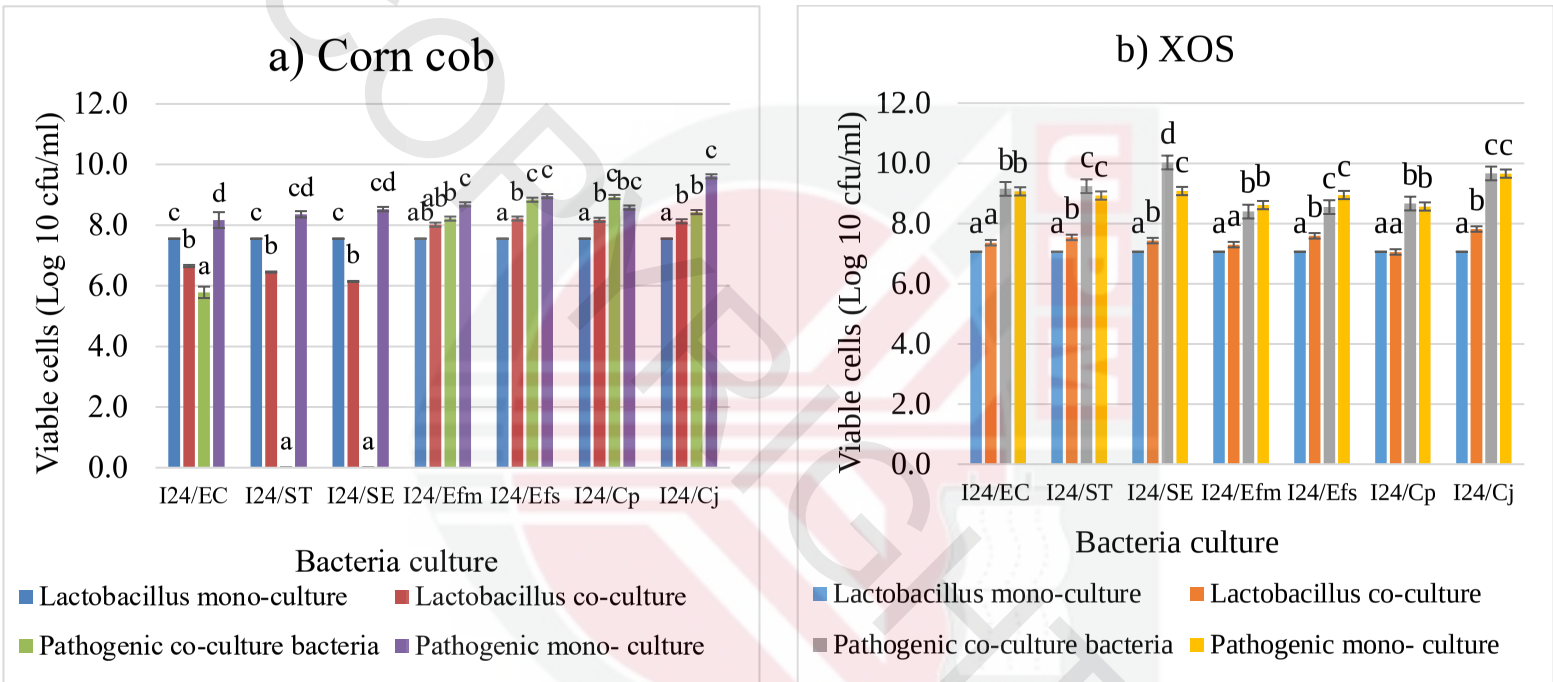


Figure 3.2: The viability (log 10 cfu/ml) of mono- and co-culture of *Lactobacillus salivarious* I24 and 7 pathogenic strains.
Escherichia coli (EC), Salmonella thyphimurium (ST), Salmonella enterica (SE), Enterococcus faecium (EFm), Enterococcus feacalis (EFs), Clostridium perfringens (CP), Campylobacter jejuni (CJ).
(a) corn cob extract and (b) xylooligosaccharides (XOS).
All bacteria culture were inoculated at standardized 6 log cycles.
[a-d] The viability (log cfu/ml) of specific Lactobacillus / pathogenic strains in mono- or co-culture ($P < 0.05$).

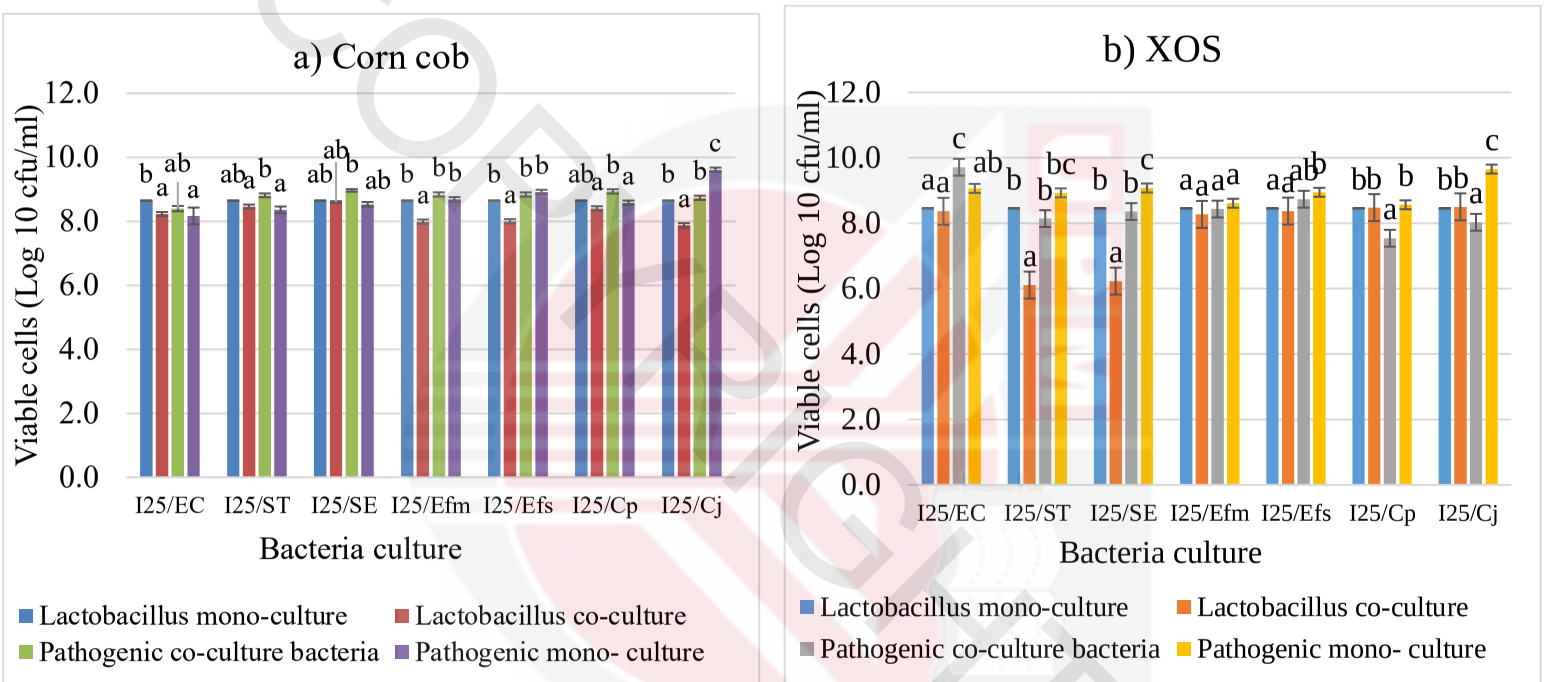


Figure 3.3: The viability (log 10 cfu/ml) of mono- and co-culture of *Lactobacillus brevis* I25, and 7 pathogenic strains.
Escherichia coli (EC), Salmonella thyphimurium (ST), Salmonella enterica (SE), Enterococcus faecium (EFm), Enterococcus feacalis (EFs), Clostridium perfringens (CP), Campylobacter jejuni (CJ).
(a) corn cob extract and (b) xylooligosaccharides (XOS).
All bacteria culture were inoculated at standardized 6 log cycles.
[a-c] The viability (log cfu/ml) of specific Lactobacillus / pathogenic strains in mono- or co-culture ($P < 0.05$).

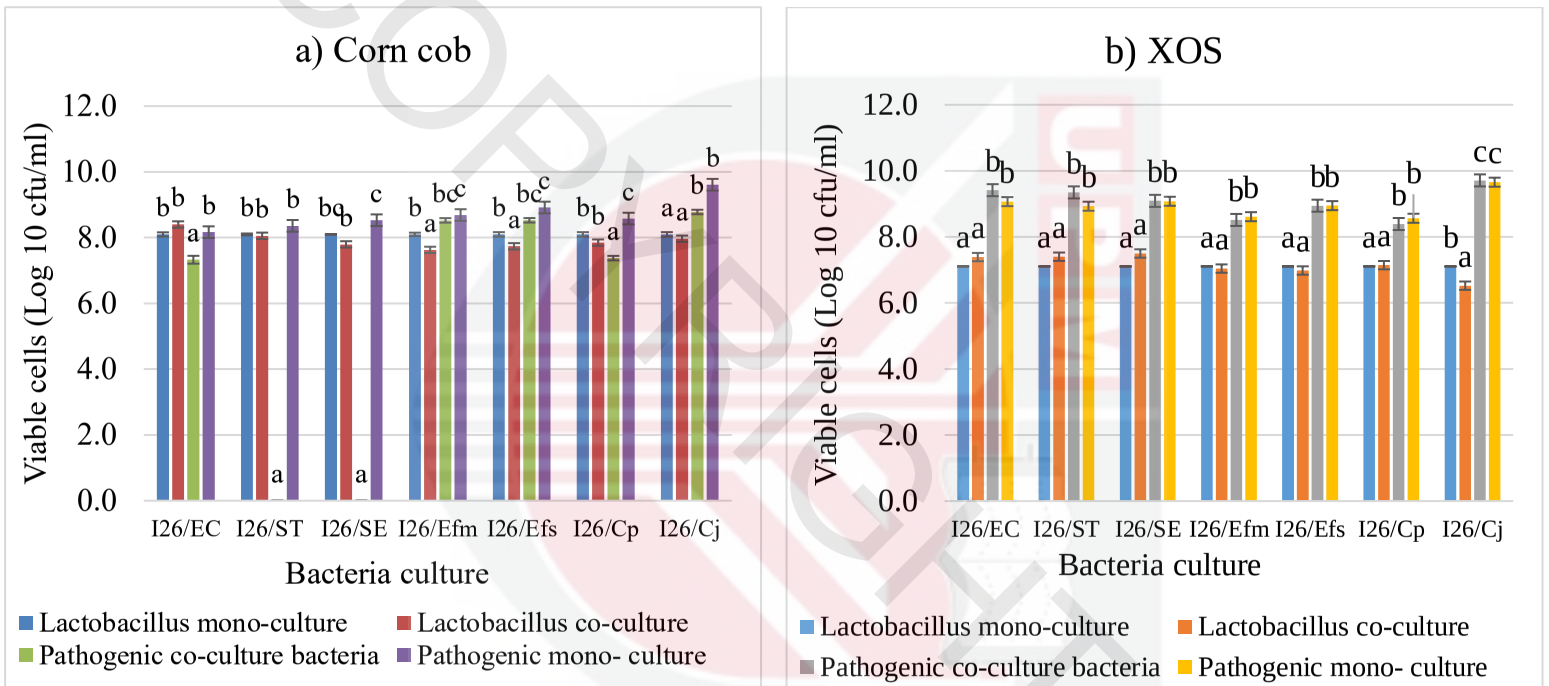


Figure 3.4: The viability (log 10 cfu/ml) of mono- and co-culture of *Lactobacillus gallinarum* I26 and 7 pathogenic strains.
Escherichia coli (EC), Salmonella thyphimurium (ST), Salmonella enterica (SE), Enterococcus faecium (EFm), Enterococcus feacalis (EFs), Clostridium perfringens (CP), Campylobacter jejuni (CJ).
(a) corn cob extract and (b) xylooligosaccharides (XOS).
All bacteria culture were inoculated at standardized 6 log cycles.
[a-c] The viability (log cfu/ml) of specific Lactobacillus / pathogenic strains in mono- or co-culture ($P < 0.05$).

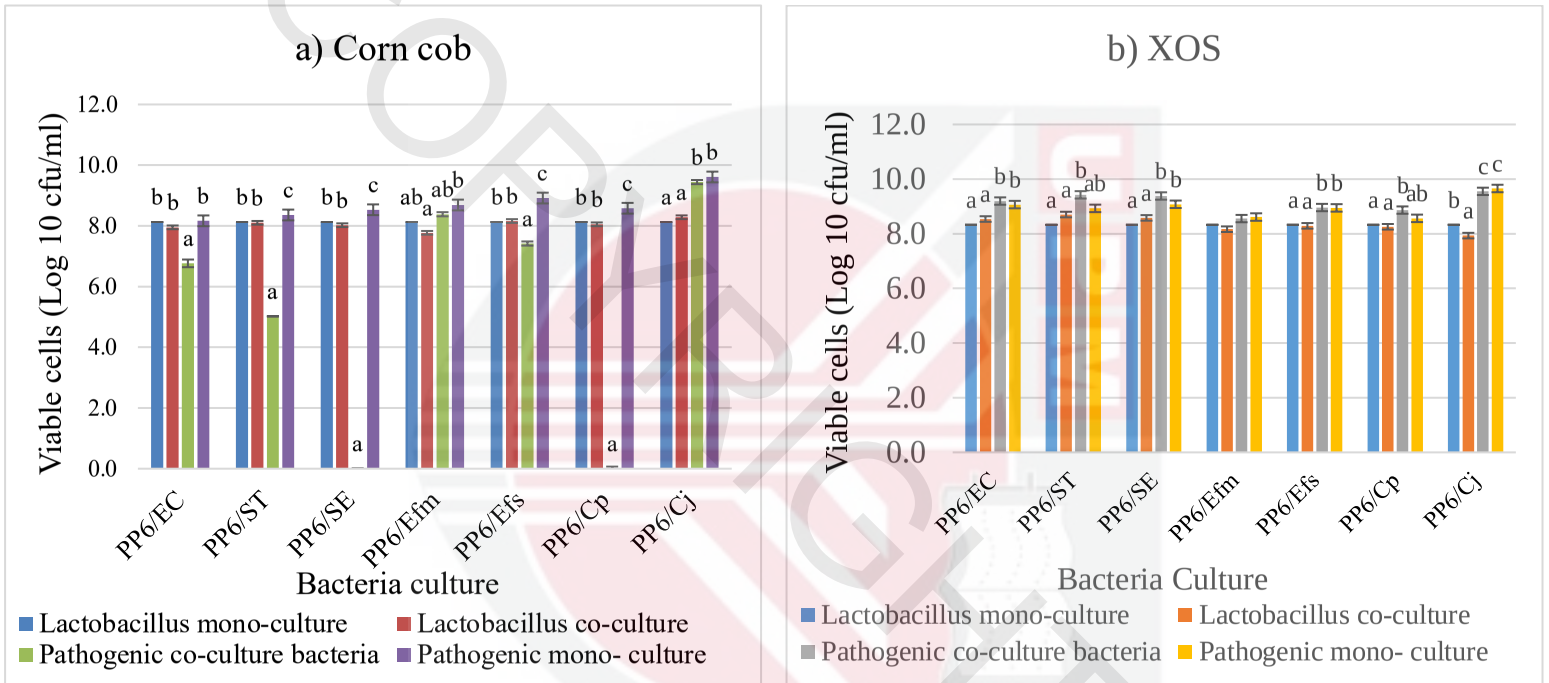


Figure 3.5: The viability (log 10 cfu/ml) of mono- and co-culture of *Pediococcus pentosaceus PP6* and 7 pathogenic strains.
Escherichia coli (EC), Salmonella thyphimurium (ST), Salmonella enterica (SE), Enterococcus faecium (EFm), Enterococcus feacalis (EFs), Clostridium perfringens (CP), Campylobacter jejuni (CJ).
(a) corn cob extract and (b) xylooligosaccharides (XOS).
All bacteria culture were inoculated at standardized 6 log cycles.
[a-c] The viability (log cfu/ml) of specific Lactobacillus / pathogenic strains in mono- or co-culture ($P < 0.05$).

Table 3.7: Summary of antagonistic effect of *Lactobacillus* strains toward pathogenic bacteria in co-culture on different substrates.

Non-beneficial bacteria	Co-culture with beneficial bacteria in corn cob or XOS							
	I24		I25		I26		PP6	
	Corn cob	XOS	Corn cob	XOS	Corn cob	XOS	Corn cob	XOS
E. coli 01:K1	+/-	+	+	+/-	+/-	+	+/-	+
S. Typhimurium ATCC® 81205™	-	+	+	+/-	-	+	+/-	+
S. enterica serovar Enteritidis 692/06	+/-	+	+	+/-	+/-	+	+/-	+
E. feacium (VRE) FM3	+	+	+	+	+	+	+/-	+
E. feacalis ATCC29212	+	+	+	+	+/-	+	+/-	+
C. perfringens (71)	+	+	+	+/-	+/-	+	+/-	+
C. jejuni	+	+	+	+/-	+/-	+	-	+

+: positive growth of non-beneficial bacteria;
-: growth of non-beneficial bacteria inhibited;
+/-: growth of non-beneficial bacteria suppressed (reduced by 0.36-3.85 log cfu/ml)

3.4 Discussion

One of the physiological properties of low molecular weight oligosaccharides is that they are soluble in water. Hence, the aqueous extraction method managed to obtain oligosaccharides from all the plant waste. In addition of sonification, the particle size of the plant waste was further reduced due to the disruptions of the cell wall throughout the process. This enhanced the oligosaccharide from the cell content to breakthrough and dissolved in the solvent caused by the collapse of the bubbles produced by cavitation (Soria and Villamiel, 2010). Thus, the extraction is further improved compared to the conventional solely shaking method. The yield of total carbohydrates from corn cob, pineapple peel and banana peel are significantly higher ($P < 0.05$) than coconut shred, rice bran and oil palm frond. This might due to the composition of lignin is reported 6 times higher in woody plants compared to vegetative plants (Madsen and Gamstedt 2013). The rice bran, oil palm frond and coconut shred were reported to contained lignocelluloses (Baig et al., 2005; Barlianti et al., 2015; Priyanga et al., 2018), which consist of lignin, hemicellulose and cellulose. Lignin is structurally complex that provides the rigidity of the plant cell wall. In the contrary, corn cobs containing more cellulose and hemicellulose compared to lignin in the dry matter (Pointner et al., 2014). Hemicellulose contained some sugar monomers with a

shorter polymer structure. The monosaccharides that form structures of hemicelluloses are composed of hexose sugars namely glucose, mannose and galactose and pentose, comprising of arabinose and xylose, and can also provide varying amounts of uronic acids and deoxy- hexose in some types of vegetables. The chemical structure of xylans present in corn cob is commonly formed by 4- O- methyl- D -glucuronic acid, L- arabinose and D- xylose in the ratio 2 : 7 : 19 (Silva et al., 1998).

In addition, this also explained the oil palm frond extract resulted in significantly lower growth of both beneficial and pathogenic bacteria ($P < 0.05$) compared to corn cob, banana peel and pineapple peel (Figure 3.1). The lignin, which occupies around 30% of the total composition of lignocellulosic residues, forms the barrier for the microorganism to utilize cellulose as carbohydrate for its growth. Corn cob extract showed bifidogenic properties as the relative growth out of 3 *Lactobacillus* strains, including I16, I24 and C1 are significantly enhanced ($P < 0.05$) in the extract compared to other plant crude extracts, especially I16 which has relative growth of 139.45 %.

Autohydrolysis of lignocellulosic biomass in liquid hot water has been widely studied owing to its high efficiency and relatively low cost. Corn cobs contained most hemicellulose that are easily breakdown compared to lignocellulose. The hemicellulose are made up of xylose, mannose, arabinose, glucose, and glucouronic acid that form carbohydrates polymers (Farhat et al., 2017). These saccharides can be extracted using dilute acid pretreatments, alkaline extraction, alkaline peroxide extraction, liquid hot water extraction, steam treatment, microwave treatment, ionic liquid extraction, and other methods (Farhat et al., 2017).

The latest study reported that the total carbohydrates of corn cob contained polymers of glucose (47.1%), xylose (28.0%), arabinose (5.4%), galactose (2.2%), mannose (0.2%), relative to the dry weight (da Silva et al. 2015). This study also discovered that the lignin content (%) is varied from 18.8% - 9.4% compared to previous findings (van Dongen et al., 2011; Wang et al., 2011; Garrote et al., 2004). This might due to the different species of corn will lead to the different content of lignin. The corn cob used in this study were supplied by Nelson's Franchise (M) Sdn. Bhd., which derived from a new generation of hybrid corn called 'Nelson's 28'. The Nelson's 28 corn cob is very small and soft (easily break into half), compared to other sweet corn. Therefore, autoclaving hydrolysis method is sufficient to breakdown the hemicellulose cell wall for oligosaccharide extraction.

A good functional prebiotic required not only selectively enhanced the growth but also the activity of the *Lactobacillus* strains. Thus, the study of the corn cob extract on *Lactobacillus* strains and antagonized properties of the *Lactobacillus* strains toward pathogenic bacteria were conducted. In this study, commercial xylooligosaccharides (XOS) was used as a positive control. This is due to corn cob was extensively studied for the production of XOS (Yang et al., 2005; Kabel

et al., 2002; Carvalho et al., 2013). However, all of the Lactobacillus strains supplemented with commercial XOS neither suppress nor inhibit the growth of pathogenic bacteria ($P < 0.05$). XOS is reported to be more favorable to *bifidobacteria* but not *Lactobacillus* (Finegold et al., 2014). The antagonistic effects of each *Lactobacillus* strains supplemented with corn cob extract were different for each pathogenic strain. Total inhibition of *S. typhimurium* NCTC13348 were recorded in I24, I26 and PP6. *C. jejuni* is totally inhibited by PP6. In addition, PP6 also suppressed all of the remaining 7 pathogenic bacteria stains. This result proved that corn cob extracts enhanced the number of *Lactobacillus* cells and the activity of the bacteria. The corn cob extract contained prebiotic properties by selectively fermented by *Lactobacillus* strains, thus competitive exclusion of pathogenic bacteria. The fermentation of corn cob extract resulted in lactic acid production, lead to a less favorable environment for pathogenic bacteria. Some *Lactobacillus* strains can also produce anti-microorganism substances and thus further inhibit the growth and proliferation of other pathogenic bacteria.

3.5 Conclusions

In conclusion, corn cob extract exhibited prebiotic properties by selectively enhanced the growth of *Lactobacillus* strains and led to suppression or inhibition of pathogenic bacteria. The combination of sonification and mild autohydrolysis resulted in a better yield of total carbohydrate, probably by breaking the hemicellulose cell wall.

CHAPTER 4

DETECTION OF PREBIOTICS IN PLANT EXTRACT VIA NUCLEAR MAGNETIC RESONANCE AND LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY

4.1 Introduction

Mono-, Oligo-, and polysaccharides are carbohydrates according to the number and linkage of saccharide molecule. Monosaccharides are polyhydroxy aldehydes or polyhydroxy ketones. Oligosaccharides are polymers formed by fewer than ten monosaccharide units, whereas polysaccharides are more complex polymers made up of more than ten monosaccharide units with high molecular weight (Bhavbhuti et al., 2012). Monosaccharides such as glucose, fructose, mannose, galactose, and xylose combination can form different oligosaccharides. Oligosaccharides can be found naturally in fruits, herbs, plants, and food in our daily lives, including arabinoxylan-oligosaccharides derived from cereal grains (Vaneeckhaute et al., 2020; Ranjan and Deepak, 2017; Wende et al., 2016). Fructooligosaccharides (FOS) are most prominently found in the edible part of plants and fruits (Benkeblia, 2013), while galactooligosaccharides (GOS) such as raffinose, stachyose and verbascose are found primarily in vegetables (Ahnen et al., 2020).

Over the last two decades, various detection and identification methods of oligosaccharides have been studied for more precise findings, including high-performance liquid chromatography (HPLC) (Churms, 1990). The HPLC analysis involving two steps: 1) separation of oligosaccharides and 2) detection of oligosaccharides. The separation of oligosaccharides is based on different approaches such as size exclusion (degree of polymerization) using permeation chromatography, ligand conversion using Ligand exchange chromatography (LEC), partition (normal phase) using Normal phase chromatography, anion exchange via Anion-exchange chromatography, and borate complex anion exchange by forming a complex with borate to exchange anions. The oligosaccharide mixture will be separated according to the set-up conditions in the HPLC system resulted in different elucidate times through the column toward the detector known as retention time (RT). The modes of detection by HPLC are limited due to the characteristics of oligosaccharide compound which is not volatile as well as lack of chromophores in their molecule structure which optical detector is not acceptable for the analysis (Gimeno et al., 2020). The refractive index detector has low sensitivity, whereas the evaporative light scattering detector required high costing (Xu, 2002). Thus, the selection of a detector is crucial to achieving reliable detection of oligosaccharide compounds.

The Nuclear Magnetic Resonance (NMR) technique has evolved over the past years, and today, it is one of the most utilized methods in research in various fields of study. The NMR spectroscopy technique relies on the nuclear spin theory. The nuclei are usually positively charged, and when they spin on an axis, they create a small magnetic field. However, not all nuclei are suitable for the NMR spectroscopy except for the 1H and 13C. When a molecule is placed on a magnetic field in the NMR, the nuclear spin results in the molecule aligning to the same direction or the magnetic field's opposite direction. The molecule's alignment in two opposite directions results in two different states separated by the energy change ΔE, reflecting the resonance frequency. Besides energy change, the resonance frequency is also dependent on the chemical environment in which the nucleus exists in a molecule. The effect arising from the chemical environment of seats is known as a chemical shift. Since the resonance frequency is usually dependent on the magnetic field strength, the chemical shift is traditionally recalculated in ppm.

The LC-MS is an analytical technique that applies both the HPLC capabilities and mass spectrometry in nuclear analysis. While the HPLC technique is known to distinguish components of a particular mixture, the mass spectrometry technique helps in the structural identification of the individual components based on the mass to charge ratio of specific molecules and their sensitivity. Besides combining the two processes, the LC-MS technique is efficiently designed and has an interface responsible for transferring the distinguished components from the LC area to the area containing the MS ion (Dass, 2007). The interface is vital since the two devices are incompatible. The liquid chromatography's mobile phase is usually pressurized liquid, while the mass spectrometry operates under a vacuum (Pitt, 2009).

This study aimed to identify oligosaccharides present in the corn cob extract based on several approaches, namely Nuclear Magnetic Resonance (NMR), High-performance liquid chromatography (HPLC), and Ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis.

4.2 Materials and Methods

4.2.1 Preparation and Separation of Mono- and Oligosaccharide via High-performance Liquid Chromatography (HPLC)

4.2.1.1 Standard and Samples Preparation for HPLC Injection

The monosaccharide content of corn cob extract was determined by High-Performance Liquid Chromatography (HPLC-Reflective Index Detector) using xylose, fructose, mannose and glucose as standard. The elution of the peak after the monosaccharide will be the oligosaccharide. Each monosaccharides standard, including xylose, fructose, mannose and glucose (ACS reagent,

Sigma-Aldrich, US) were prepared at a concentration of 1 mg/mL in 65% acetonitrile (HPLC grade, Fisher Scientific). The 65% acetonitrile mixture was prepared by mixing 350 mL of distilled water and 65 mL of HPLC grade acetonitrile in the 1 L Scoot bottle. In order to determine the retention time, each monosaccharide was labelled via the Empower® Chromatography Data Software. Thus, the specific retention time of each monosaccharide will be detected after the mixture of all monosaccharides were injected into the HPLC machine. The extracted sample was extracted and freeze-dried as mention in Chapter 3 (Section 3.2.9). All HPLC samples are prepared at a concentration of 1 mg/mL in 65% of acetonitrile (HPLC grade, Merck Millipore, US) and centrifuged at 18 000×g for 15 min, to remove protein. The supernatant was filtered via syringe filter (Nylon, 0.22µm) into the HPLC clear vial (2 mL, Agilent, US) and stored in 4 °C before injection. All samples, including standard were prepared in three replicates.

4.2.1.2 HPLC

The monosaccharides were performed on WATERS e2695 Separation Module equipped with Refractive Index 2414 detection (WATERS Corporation, US). XBridge BEH Amide column (4.6 mm × 250 mm × 3.5µm) (Figure 4.1), which is the type of Cosmosil Sugar D column, was used for the monosaccharides and oligosaccharides separations. The samples were chromatographed using an isocratic mobile phase (65% acetonitrile/35% water (v/v) with 0.2% Triethylamine (TEA) at a flow rate of 1.3 mL/min. The column temperature was maintained at 40 °C. The injection volume was 20 µL and 30 minutes per run. Each solvent, including HPLC grade acetonitrile and distilled water, degassed via the ultrasonic method for 1 hour before use as the mobile phase of the HPLC system.

Before injecting the samples, the XBridge BEH Amide column was conditioned for at least 30 minutes to flush out the storage solvent and equilibrate the mobile phase. This step is critical to ensure a stable baseline before running the samples. Instrument commands and data acquisition were performed via Empower2 software.

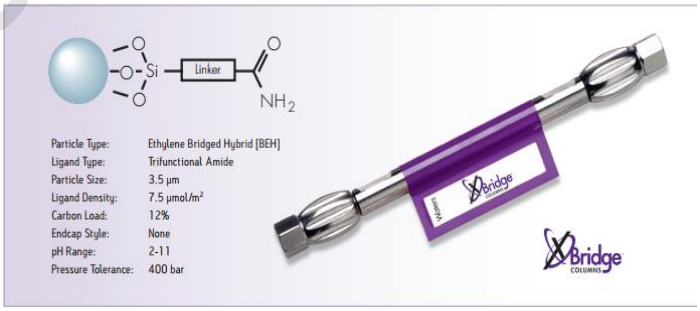


Figure 4.1: XBridge BEH Amide column (4.6 mm × 250 mm × 3.5 µm) consisted of trifunctional bonded amide phase that retains carbohydrates and sugars, which are known as extremely polar compounds.

4.2.2 NMR

4.2.2.1 Sample Preparation, Chemical and Reagent for NMR Analysis

10 mg of corn cob extract were dissolved in 375 µL of CD_3OD (Merck, Germany) solvent and KH_2PO_4 (Merck, Germany) buffer in 99.9% deuterium oxide (D_2O) (Merck, Germany) at pH 6.0 with 0.1% of 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt (TTP) added. The sample mixture was vortexed for 1 minute and then undergo ultra-sonification for 15 min at room temperature. The clear supernatant was obtained by centrifugation at 13000 rpm for 10 min and transferred into NMR tube for analysis. All the measurements setting of NMR were pre-set. The 1H NMR and 2D (J-resolved) NMR determinations were performed using 500 MHz Varian INOVA NMR spectrometer (Varian Incorporation, US), functioning at a frequency of 499.887 MHz at room temperature (25 °C). The CD_3OD was used as an internal lock. Chemical shifts were expressed in ppm relative to TTP used as an internal chemical shift reference at 0 ppm. All NMR experiments comprising ^{1}H, ^{13}C, HSQC, HMBC, and COSY were recorded in D2O at 37 ºC. ^{1}H NMR measurements using a pre-set setting for all of the samples. The required time for each ^{1}H NMR spectrum was 3.53 min which involved 64 scans with a width of 20 ppm. For the structure confirmation, 2D heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC) spectra were obtained using 64 scans, which was achieved in 6 h, 9 min and 9 s. In addition, ^{1}H-^{1}H COSY analysis was also performed. MestReNOVA (Version 6.0, EU) and Chenomx software (version 5.1, Canada) were used for the spectra processing.

4.2.3 Ultra-high Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC-MS/MS)

4.2.3.1 UHPLC-MS/MS Analysis

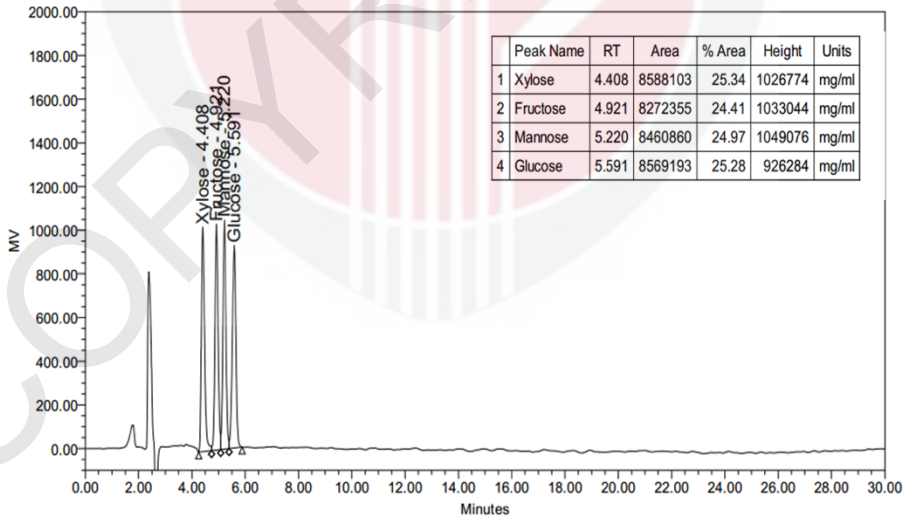
Oligosaccharides from all extracts (enriched, enzyme digest or non-processed) were analyzed based on Mediani et al. (2015) with slight modifications. The samples and standards were prepared by dissolving 2 mg of the extract in 1 ml of 80% methanol following centrifugation and filtration through a 0.22 m nylon membrane into a 2-ml screw-capped sample vial. The sample was injected into the system using Prevail Carbohydrate ES column (150 mm × 2 mm, 5 µm; Alltech Associates Inc., Deerfield, IL, US) coupled to the mass spectrometer. Column: TSK-GEL Amide-80 Series (Merck, US), size: 2.0 × 150 mm packed with 3-micron spherical silica particles that are covalently bonded with carbamoyl groups. The LC-MS system used in this work consisted of three Shimadzu HPLC pumps (LC-20AD) coupled to a Waters LCT Premier Time-of-Flight Mass Spectrometer (TOF-MS). Gradient elution was performed based on solvent A (0.15% aqueous formic acid) and B (acetonitrile) over 30 min. The total solvent flow was maintained at 250 mL/min, and gradient elution was performed using the following solvent compositions: initial, 95% B, held for 1 min; linear gradient

to 70% B at 8 min and then to 50% B at 18 min; hold at 50% B until 25 min, sudden increase to initial condition at 25.01 min and final hold at this composition until 30 min. Positive and negative mode electrospray ionization was performed along with multiplexed collision-induced dissociation (CID) by switching among three different AP1 voltages (15, 40, and 65 V). Processing of LC-MS data was accomplished using MassLynx v. 4.1 software.

Crude extracts from corn cob analyses were performed using UHPLC system of Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, US) attached to TSK-GEL Amide-80 Series column (2.0 mm × 150 mm, 3 µm) (Merck, US) for possible oligosaccharide detection. Gradient elution was performed based on solvent A (0.15% aqueous formic acid) and B (acetonitrile) over 45 min with the total solvent flow was maintained at 250 mL/min and run for 45 minutes in negative polarity mode. Besides, 70,000 full MS resolution, scan ranges from 133.4-2000 m/z, flow rate of 3 µL/min, inner diameter 2.303 mm, volume 250 µL. Instrument commands and data acquisition were performed via HP ChemStation software version 10.02 (Hewlett-Packard, US) and Thermo Xcalibur Roadmap version 2.2 (Thermo Scientific, US).

4.3 Results

4.3.1 HPLC



	Peak Name	RT	Area	% Area	Height	Units
1	Xylose	4.408	8588103	25.34	1026774	mg/ml
2	Fructose	4.921	8272355	24.41	1033044	mg/ml
3	Mannose	5.220	8460860	24.97	1049076	mg/ml
4	Glucose	5.591	8569193	25.28	926284	mg/ml

Figure 4.2: Retention time of monosaccharide standard.

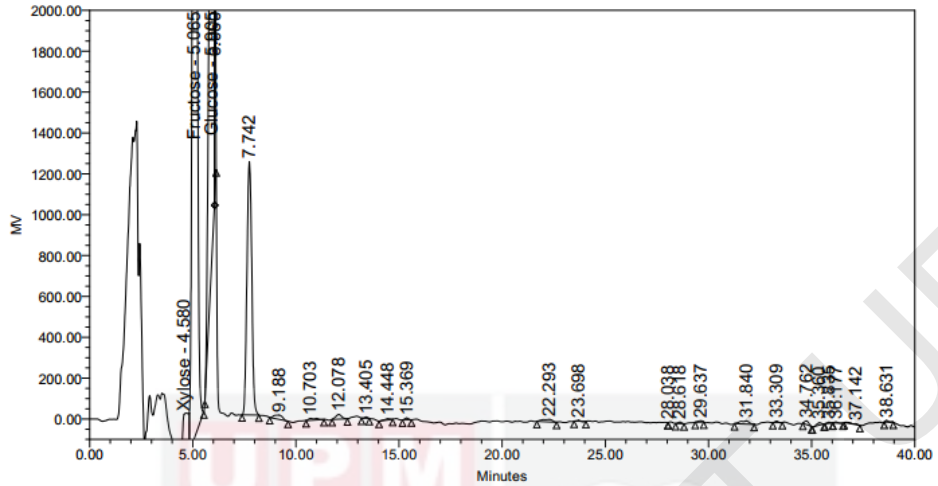


Figure 4.3: The potential saccharide content in 1 mg/mL corn cob extract.

The monosaccharide standard (Figure 4.2) consisted of 1 mg/mL xylose, fructose, mannose and glucose. The retention time (RT) was input into the analysis software as a standard RT of monosaccharides. Peaks appeared after the monosaccharide standard's RT considered as oligosaccharides peaks.

Figure 4.2 showed that glucose had the highest retention time, followed by mannose, fructose, and xylose, respectively. This indicates that glucose is the most non-polar monosaccharide as compared to the rest. Xylose is the most polar monosaccharide.

Based on Figure 4.3, corn cob extracts contain xylose, fructose and glucose. A high area peak emitted at retention time 7.742 proved that the extract contained oligosaccharides. Several small peaks also were detected in the extract. The structure of the sugars available in the HPLC was further investigated via NMR.

4.3.2 NMR

The NMR emitted signals that belongs to a mixture of monosaccharides, disaccharides and oligosaccharides. There are 3 ^{1}H signals in the proton anomeric region at δ 5.40, δ 5.18, and δ 4.58. The chemical shift of H-1 track at δ 4.60 residue A with the beta structure of the sugar compound. There is a total of 6 ^{13}C signals in the anomeric region at δ 106.77, δ 104.61, δ 101.07, δ 99.16, δ 95.25, δ 95.20. The NMR showed that the chemical shift residue A (H-1 track, δ5.20) was in agreement with an internal (1 → 4)-α-D-Glcp-(1 → 4)- unit; that of residue B (H-1 track, δ 5.40) with a) with a terminal α-D-Glcp-(1 → 4)- unit; that of residue C′ (H-1 track, δ 5.40). The structures of the alpha of sugars were illustrated in Figure 4.4, which consists of 3 sugars. The uneven ^{1}H and ^{13}C

chemical shift revealed the presence of oligosaccharides and their derivatives based on the uneven

Table 4.1: ^{1}H and ^{13}C NMR chemical shifts (ppm) of corn cob extract.

		Chemical shift δ (ppm)			
Residues		1	2	3	4
A(α-re)	H	5.20	3.47	4.16	3.20
	C	95.30	72.61/ 74.65	79.73	77.43
A(β-re)	H	4.60	3.19	4.58	3.50
	C	99.22	77.43	99.17	74.35
B	H	5.40	3.51	4.58	3.30
	C	95.25	74.35	99.17	72.81
C	H	5.40	3.69	4.00	3.80

A-C represent the number of residues.
α-re and β-re are the alpha and beta structure of the sugar.

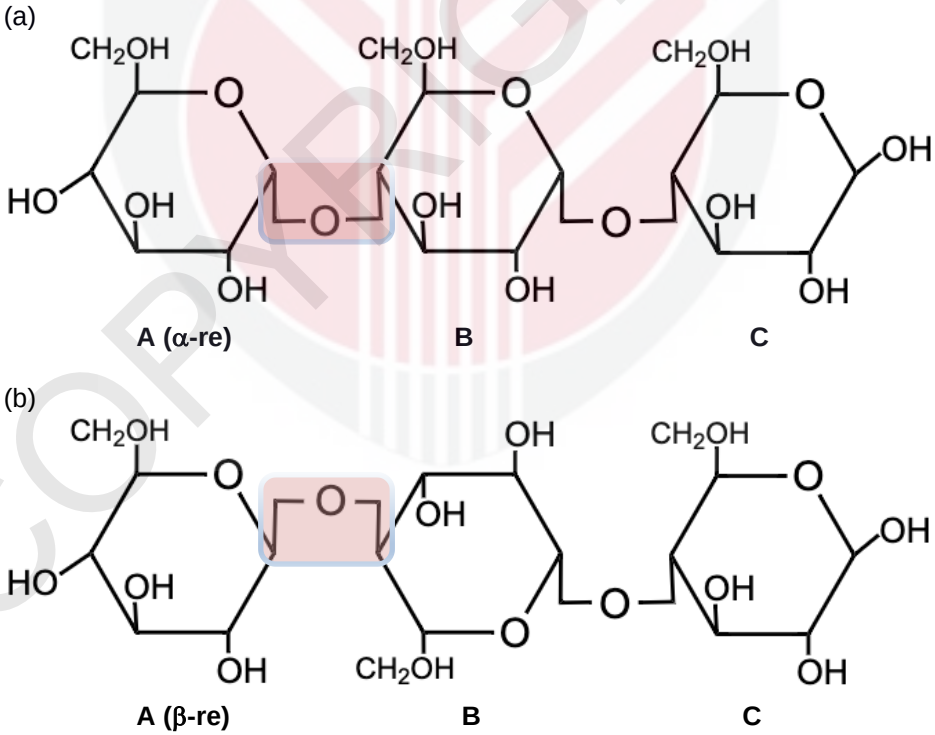


Figure 4.4: The alpha (a) and beta (b) structure of the sugar molecules present in corn cob extract based on the chemical shift resulted in NMR.

4.3.3 UHPLC-MS/MS analysis

The molecular weight of one glucose compound is 180.16 g/mol, whereas xylose is 150.13 g/mol. Sucrose molecular weight is 342.3 g/mol. The ionized value is where molecular weight minus 18, thus the ionized ion of sugar will show as m/z 161 ±1. Saccharides and maltotriose compounds were detected at RT 5.96 and 12.87 minutes, respectively (Figure 4.5 and Figure 4.6). The ion at m/z 503.1 was subsequently isolated and fragmented by CID at low excitation energy (Figure 4.6), which is consistent with the NMR structure. Proton H-1 caused a signal at 3.1 ppm with a ddd ~ dt multiplicity with one unresolved coupling. The two triplet values of 6.3 Hz are consistent with average coupling constants to the neighbouring CH2 group (free rotation).

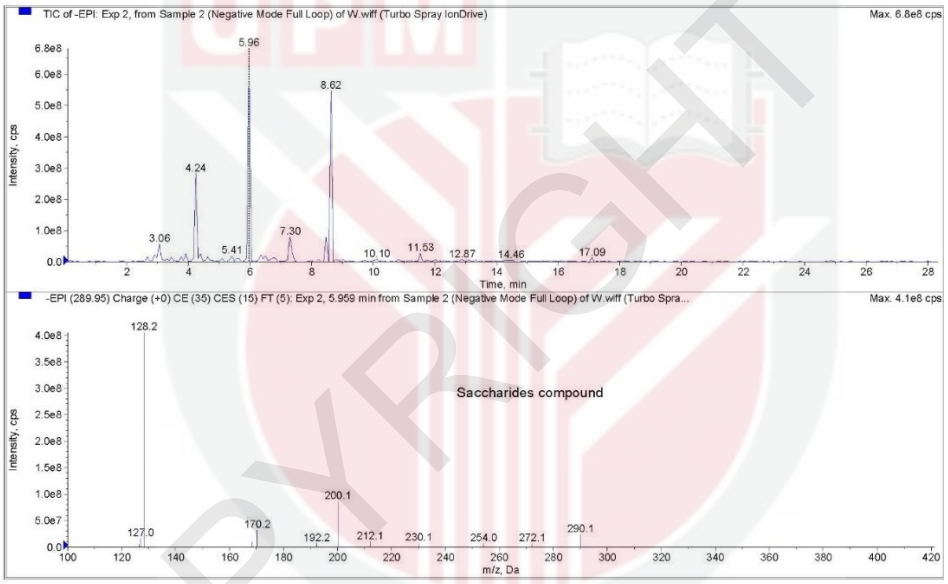


Figure 4.5: Saccharides compound were found in corn cob extracts at RT 5.96 minutes.

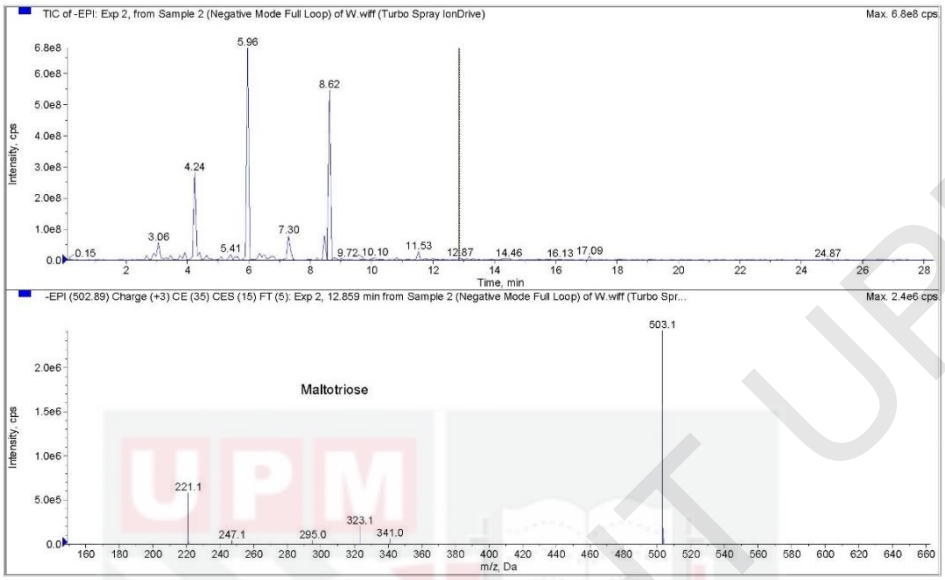


Figure 4.6: Maltotriose compounds were detected in corn cob extract at RT 12.87 minutes.

4.4 Discussion

The first step towards detecting oligosaccharides in the corn cob extract is determining the corn cob's monosaccharide content. In extracting the monosaccharides content, the HPLC technique was used. The HPLC technique used four monosaccharides (xylose, fructose, mannose, and glucose) as standard. The four monosaccharide standards were prepared at a concentration of 1 mg/ml in 65% acetonitrile. A mixture of the standard monosaccharides was then put into the HPLC machine to determine the retention time (RT). The retention time (RT) of each of the standard monosaccharides was recorded in the chromatography data software.

To separate oligosaccharides from the monosaccharides, a Cosmosil Sugar D column was used. From the process, each of the four monosaccharides had four different retention times. Glucose had the highest retention time of 5.59, followed by mannose at 5.22, fructose at 4.82, and xylose at 4.40. Once the retention times were obtained from the chromatography, the RTs acted as the standard retention times for the monosaccharides. The figures were input into the software used for analysis, and the resultant peaks were considered the oligosaccharide peaks. Based on the experiment's nuclei, glucose is the only present oligosaccharide in the corn cob extract.

The NMR technique has evolved over the past years, and today, it is one of the most utilized methods in research in various fields of study. The NMR spectroscopy technique relies on the nuclear spin theory. The nuclei are usually

positively charged, and when they spin on an axis, they create a small magnetic field. However, not all nuclei are suitable for the NMR spectroscopy except for the ^{1}H and ^{13}C. When a molecule is placed on a magnetic field in the NMR, the nuclear spin results in the molecule aligning to the same direction or the magnetic field's opposite direction. The molecule's alignment in two opposite directions results in two different states separated by the energy change ΔE, reflecting the resonance frequency. Besides energy change, the resonance frequency is also dependent on the chemical environment in which the nucleus exists in a molecule. The effect arising from the chemical environment of seats is known as a chemical shift. Since the resonance frequency is usually dependent on the magnetic field's strength, the chemical shift is traditionally recalculated in ppm.

Interpretation of the NMR spectra of carbohydrates requires considering the numerous structural permutations that might give rise to resonances in their NMR spectra. On the other hand, 3 hexoses (glucose) can theoretically yield 38,016 different trisaccharides.

The difference between alpha and beta glucose in 3-dimensions is according to the position of -OH and $-CH_2OH$ group. A trans arrangement (alpha) is where -OH is attached to C-1 and the $-CH_2OH$ at C-5 on the opposite sides of the ring's plane, whereas a cis arrangement (beta) is where they are on the same side of the ring's plane.

In the experiment conducted, 10 mg of the cob extract was dissolved in 375 microliters of CD_3OD solvent and KH_2PO_4, a buffer in 99.9% D_2O. The experiment was conducted at a pH of 6.0 with a 0.1% concentration of TTP, a propionic sodium salt. One of the main problems arising from the application of NMR in biology is low sensitivity. The sensitivity of the NMR signal largely depends on the quantity of material within the spectrometer's sensitive area. For the achievement of a higher resolution, the magnetic field should be homogenous. Also, to achieve homogeneity, the 10 mg cob extract was dissolved inadequate solvent and a buffer.

Usually, a structural analysis of an oligosaccharide begins with sugar analysis. Once the monosaccharides have been identified, the next step is to determine the number of sugars present in the recurring chain and how they are connected. Depending on the oligosaccharides ^{1}H NMR, the number of monosaccharides can be identified in the repeating units by counting the number of resonance in the numeric region. The estimated resonance for ^{1}H ranges between 4.4-5.5 ppm while for ^{13}C ranges between 95-110 ppm.

Additionally, due to the likelihood of the majority of polysaccharides to suffer an overlap at the peak, especially in the ring region of (δH 3.1-4.4), a 2D NMR is preferable. This technique enables the linkage of the protons to the carbon with the help of HSQC NMR. Based on the three residues from the experiment, the

results show oligosaccharides in corn cob extract. Three ^{1}H signals exist in the proton anomeric region. The ppm ranges from 5.40, 5.18, and 4.58. Additionally, a total number of 6 ^{13}C signals exist in the anomeric areas at 106.77, 104.61, 101.07, 99.16, 95.25, 95.20 ppm. However, based on the uneven distribution of the ^{1}H and ^{13}C signals, it can be concluded that the extract contains a mixture of monosaccharides, polysaccharides, and oligosaccharides.

Another disadvantage of the NMR technique is its low sensitivity. The technique cannot detect minor compounds, and so the samples should be more concentrated to achieve better results. Additionally, when interpreting the carbohydrates' NMR spectra, it is important to take note of other structural permutations likely to result in resonance in the spectra (Bubb, 2003).

Other than the concentration, according to Bubb (2003), interpretation of the NMR spectra of carbohydrates requires considering the numerous structural permutations that might give rise to resonances in their NMR spectra. To break this contingency of interpretation with a close comparison of 27 peptides given out by three amino acids is done. It is evident that from the analysis, only three units of hexoses yielded 38,016 different trisaccharides (Laine,1997). Also, Monosaccharides are always assigned to L or D according to the series of the configuration of the highest numbered chiral center.

One of the most likely oligosaccharides identified from the corn cob extract based on the mass is isomaltooligosaccharide (IMO) which is comprised of short-chain carbohydrates. One property of this oligosaccharide is that it is resistant to digestion. IMO occurs naturally in some foods but can also be manufactured for commercial purposes.

4.5 Conclusion

In conclusion, corn cob extract consists of monosaccharides, disaccharides and oligosaccharides. Glucose is the most non-polar monosaccharide with the highest retention time, followed by mannose, fructose, and xylose, which has the most non-polar monosaccharide compared to the rest. The UHPLC-MS/MS showed that the monosaccharide compound is at retention time before 5 min. The NMR data has been supported by the UHPLC-MS/MS results, where the probable oligosaccharide found in the corn cob extract is isomaltooligosaccharide (IMO).

CHAPTER 5

EFFECTS OF SUPPLEMENTED DIETARY PROBIOTIC, CRUDE EXTRACT AND THEIR COMBINATIONS AT DIFFERENT DOSAGES ON GROWTH PERFORMANCE AND HEALTH OF BROILERS

5.1 Introduction

It has been shown that the incorporation of antibiotics in the poultry feed not only promotes growth but also prevents diseases for poultry production (Barton, 2000; Snel et al., 2002). However, increasing resistance of pathogens to antibiotics and the accumulation of antibiotic residues in poultry products had raised the awareness of consumers toward antibiotic-free approaches in farming. To avoid the occurrence of cross-resistance bacteria from poultry products, the EU hassled total removal of antibiotics used as antimicrobial growth promoters (AGP) from poultry farming (Casewell et al., 2003; Castanon, 2007; Huyghebaert et al., 2011). Consequently, alternative substances that can optimize broiler performance and minimize profit loss while ensuring the elimination of foodborne pathogens of broiler meat are very important for the highly intensive broiler production sector.

A study revealed that the inclusion of AGP enhanced the growth performance and health of broilers by altering the microbial population in the gut (Danzeisen et al., 2011). Therefore, diversifying the population of beneficial bacteria in the broilers' gut might affect their growth performance and health.

A living microorganism that exerts positive effects to the animal host by maintaining normobiosis in the gut is defined as "probiotic" (FAO/WHO, 2002). The predominate of probiotics in number over potentially harmful bacteria results in an improved gut microbiota balance, host immune response and intestinal function, and overall host health; thus, the host will be unlikely prone to diseases. Effective probiotics can stabilize the gastrointestinal barrier function, produce bacteriocins, induce absorption of nutritions, enhance immunomodulatory effects, inhibit procarinogenic enzymes and competitive exclusion of pathogens colonization that infect mucosa (Salminen et al., 1996; Mazmanian et al., 2008; Hooper et al., 2002; Timmerman et al., 2005; Salzman et al., 2003; Gill, 2003). Nevertheless, there are several limitations to be an effective probiotic: (i) shelf life, (ii) survival rate in the gut, (iii) adhesion on the gut wall, (iv) colonize and modulate the expression of the gene to create a favorable condition in host gut (Gaggia et al., 2010). The administration of new probiotics has to adapt to the new gastrointestinal environment and compete with the pathogenic bacteria and the host existed microflora.

On the contrary, prebiotic is a non-digestible food ingredient that selectively stimulating the growth and/or activity of one or a limited number of bacteria, thus altering the composition and metabolism of the gut microbiota lead to beneficially affect the host's health (Gibson, 1999; Gibson, 2004; Marshall, 2008; Huyghebaert et al., 2011; Das et al., 2012). In addition, prebiotic is also known as oligosaccharide that stimulates beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* by providing energy sources. In this notion, prebiotic has more advantages compared to probiotic as it stimulates commensal bacteria that have adapted to the environment of the gastrointestinal tract that may aid the competitive exclusion of pathogens.
Since both probiotic and prebiotic are proven to confer benefits to the host, the combination of both might create a synergic effect. This formation is called synbiotic. The improvement of growth performance is by enhancing intestinal morphology and nutrient intake of broilers (Awad et al., 2008; Abdel-Raheem et al., 2012; Mookiah et al., 2014; Hassanpour et al., 2013).

In terms of performance, synbiotics were effective in improving broiler growth, which corresponded to the effect of inclusion of either probiotics or prebiotics in the diet of chickens (Abdel-Raheem et al., 2012; Mookiah et al., 2014). The improvement of intestinal morphology and nutrient absorption due to feeding synbiotics seems to contribute to the enhanced performance of broiler chicken (Awad et al., 2008; Hassanpour et al., 2013).

Thus, the study aims to study the effects of corn cob extracts at different dosage with the combination of a commercialized probiotic (Primalac®) on the (i) growth performance, (ii) haematological and serum lipid profiles, (iii) immunological response, (iv) jejuna and ilea villus height and crypt depth and (v) relative organ weight of broilers at 21 days (starter period) and 35 days (finisher period).

5.2 Materials and Methods

5.2.1 Large Scale Production of Corn Cob Extract Powder

The production of corn cob extract solution for the broiler is the same as the method mentioned in Chapter 4. After the corn cob extract solution was obtained, the solvent was frost in the provided steel trays at -20 °C overnight and inserted into the shelves of HK-4 freeze dryer (Frozen in Time Ltd, UK). The freeze dryer is fully automated. Dried corn cob extract was obtained after 3 days of operation.

5.2.2 Ethical Aspects of Regulating Broilers Experiment

In order to ensure the animal trial was carried out in ethical condition, all of the procedures regarding the broilers experiment, including broilers management, supplement to be fed, experimental design, procedures and analyses have been approved by Institutional Animal Care and Use Committee (IACUC) Universiti

Putra Malaysia before the broilers farming was carried out. (UPM/IACUC/AUP-R054/2016)

5.2.3 Broilers and Rearing Management

A total of 240 one-day-old male Cobb 500 broilers were obtained from a local commercial hatchery. The initial bodyweight of the broilers was determined with an average of 35.19 ± 0.12g. They were randomly distributed into 24 stainless steel three-tiered battery cages with raised wire floor space of 103.24 m^2 and 0.46 m height in an open house facility at Animal Research Centre (ARC), Institute of Tropical Agriculture, Universiti Putra Malaysia (UPM). All the cages were disinfected via fumigation 1 week before the feeding trial. Each cage was equipped with a feeding trough hung inside and drinkers inside the pen and also an excreta collection tray beneath the cage floor. All the biosafety measures and strict hygiene were executed throughout the feeding trial. The feeding trough, drinker, and excreta try were cleaned and refilled with fresh feed, water, and new lining paper in a daily basis. During the 14- days of broilers brooding period, a 100 W bulb was provided for each replicates cage. A layer of lining paper was placed in each cage to prevent injuries of broilers' feet stuck in between the holes of the wire cage floor. In addition, each cage was provided with a plastic feeder placed on the floor, which was replaced by a feed trough hung inside when the chickens were 14 days old.

Figure 5.1: The broilers were housed in three tiers battery cages with feeders and drinkers in each cage in an open house facility.

5.2.4 Experimental Design

Four replicates groups, each consisting of 1-day-old broiler chicks were randomly assigned to the six dietary treatment groups, including: (i) basal diet (BD) as control group; (ii) BD + 0.5% corn cob extract (CC05); (iii) BD + 1.0% corn cob extract; (iv) BD + 0.1% probiotic (Primalac®) (PRO) ; (v) BD + 0.1% probiotic + 0.5% corn cob extract (SYN05); (vi) BD + 0.1% probiotic + 1.0 % corn cob extract (SYN10). Mixtures of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium termophilum*, *Enterococcus faecium* and *Aspergillus oryzae* were included in the commercial probiotic (Primalac®) (Starlabs, US) at a concentration of 10^9 cfu/g each. In order to obtain an equal total of 100% ingredients, the corn was expensed for supplemented corn cob extract and probiotic supplemented diet, respectively. All of the corn-soybean meal basal in dietary treatments at starter phase (1-21 d) (Table 5.1) and finisher phase (22-35 d) (Table 5.2) were prepared in mash form (antibiotic-free) and formulated to meet or exceed the nutrient requirements of broiler chickens recommended by the National Research Council (NRC, 1994). The supplemented corn cob extract and commercial probiotic were freshly mixed daily with the corn-soybean meal basal. *Ad libitum* feeding of feeds and water were practiced throughout the trial. Feed proximate analyses (Table 5.1 and Table 5.2) of the formulated starter feed, finisher feed, corn cob extract and probiotic on crude protein, crude fat, crude fiber, calcium, phosphorus and sodium were conducted based on Malaysian Agriculture Research and Development Institute (MARDI) methodology.

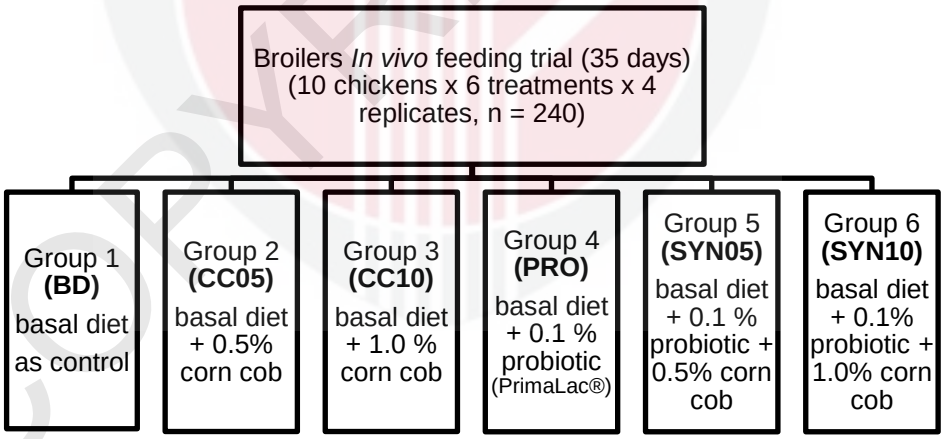


Figure 5.2: The overall experimental design of the broilers feeding trial with a total of four replicates of six treatment groups (10 broilers in each).

Table 5.1: Feed compositions of dietary treatments for starter phase (1 to 21 days).

Ingredients (%)	Basal Diet (BD)	BD + 0.5% Corn cob extracts (CC05)	BD + 1.0% Corn cob extracts (CC10)	BD + Probiotics (PRO)	BD + 0.5% Corn cob extracts + Probiotics (SYN05)	BD + 1.0% Corn cob extracts + Probiotics (SYN10)
Corn	53.18	52.68	52.18	52.18	51.68	51.18
Soybean meal, 48% CP	33.49	33.49	33.49	33.49	33.49	33.49
Fish Meal	4.00	4.00	4.00	4.00	4.00	4.00
Palm oil	6.07	6.07	6.07	6.07	6.07	6.07
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00
Limestone	1.08	1.08	1.08	1.08	1.08	1.08
L-lysine HCL (ADM L-lysine HCL 98.5%)	0.06	0.06	0.06	0.06	0.06	0.06
DL-Methoinine	0.17	0.17	0.17	0.17	0.17	0.17
Vitamin premix[1]	0.26	0.26	0.26	0.26	0.26	0.26
Mineral premix[2]	0.25	0.25	0.25	0.25	0.25	0.25
Common salt	0.35	0.35	0.35	0.35	0.35	0.35
70% Chlorine Chloride	0.10	0.10	0.10	0.10	0.10	0.10
Corn Cob Extract	0.00	0.50	1.00	0.00	0.50	1.00
Probiotic	0.00	0.00	0.00	1.00	1.00	1.00
Total	100.00	100.00	100.0	100.00	100.00	100.00
Calculated Analysis						
Metabolisable Energy (MJ/kg)	13.39	13.39	13.39	13.39	13.39	13.39
Crude protein	23.00	23.00	23.00	23.00	23.00	23.00
Crude fat	3.69	3.69	3.69	3.69	3.69	3.69
Crude Fiber	2.02	2.02	2.02	2.02	2.02	2.02
Methionine + Cystein	0.50	0.50	0.50	0.50	0.50	0.50
Lysine	1.10	1.10	1.10	1.10	1.10	1.10
Calcium	1.08	1.08	1.08	1.08	1.08	1.08
Phosphorus	0.50	0.50	0.50	0.50	0.50	0.50

Table 5.1: Continued

Ingredients (%)	Basal Diet (BD)	BD + 0.5% Corn cob extracts (CC05)	BD + 1.0% Corn cob extracts (CC10)	BD + Probiotics (PRO)	BD + 0.5% Corn cob extracts + Probiotics (SYN05)	BD + 1.0% Corn cob extracts + Probiotics (SYN10)
Chemical Analysis						
Crude Protein	24.48	24.48	24.57	24.49	24.50	24.58
Crude fat	5.79	5.79	5.79	5.80	5.80	5.80
Crude fiber	2.96	2.96	2.97	3.18	3.18	3.18
Calcium	0.47	0.47	0.47	0.63	0.63	0.63
Phosphorus	0.32	0.32	0.32	0.32	0.32	0.33
Sodium	0.06	0.06	0.06	0.06	0.06	0.06

[1] Supplied per kilogram of diet: vitamin A, 50.00 MIU; vitamin B1, 10.00g; vitamin B2, 30.00g; Vitamin B6, 20.00g; Vitamin B12, 0.10g; Vitamin D3, 10.00 MIU; Vitamin E, 75.00g; Vitamin K3, 20.00g; Calcium D-Panthothenate, 60.00g; Nicotinic Acid, 200.00g; Folic Acid, 5.00g; Biotin, 235.00g; Antioxidant, Anti cracking and Carrier.
[2]Supplied per kilogram of diet: Se, 0.20g; Fe, 80.00g; Mn, 100.00g; Zn, 80.00g; Cu, 15.00g; KCl, 4.00g; MgO, 0.60g; $NaCO_3$, 1.50g, I, 1.00g; Co,0.25g.

Table 5.2: Feed compositions of dietary treatments for finisher phase (22 to 35 days).

Ingredients (%)	Basal Diet (BD)	BD + 0.5% Corn cob extracts (CC05)	BD + 1.0% Corn cob extracts (CC10)	BD + Probiotics (PRO)	BD + 0.5% Corn cob extracts + Probiotics (SYN05)	BD + 1.0% Corn cob extracts + Probiotics (SYN10)
Corn	57.34	56.84	56.34	56.34	55.84	55.34
Soybean meal, 48% CP	30.04	30.04	30.04	30.04	30.04	30.04
Fish Meal	3.00	3.00	3.00	3.00	3.00	3.00
Palm oil	6.34	6.34	6.34	6.34	6.34	6.34
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00
Limestone	1.16	1.16	1.16	1.16	1.16	1.16
L-lysine HCL (ADM L-lysine HCL 98.5%)	0.06	0.06	0.06	0.06	0.06	0.06
DL-Methoinine	0.17	0.17	0.17	0.17	0.17	0.17
Vitamin premix 528	0.25	0.25	0.25	0.25	0.25	0.25

Table 5.2: Continued

Ingredients (%)	Basal Diet (BD)	BD + 0.5% Corn cob extracts (CC05)	BD + 1.0% Corn cob extracts (CC10)	BD + Probiotics (PRO)	BD + 0.5% Corn cob extracts + Probiotics (SYN05)	BD + 1.0% Corn cob extracts + Probiotics (SYN10)
Mineral premix	0.25	0.25	0.25	0.25	0.25	0.25
Common salt	0.29	0.29	0.29	0.29	0.29	0.29
Chlorine Cl-70%	0.10	0.10	0.10	0.10	0.10	0.10
Corn Cob Extract	0.00	0.50	1.00	0.00	0.50	1.00
PrimaLac	0.00	0.00	0.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated Analysis						
Metabolisable Energy (MJ/KG)	13.39	13.39	13.39	13.39	13.39	13.39
Crude protein	20.00	20.00	20.00	20.00	20.00	20.00
Crude fat	3.70	3.70	3.70	3.70	3.70	3.70
Crude Fiber	2.02	2.02	2.02	2.02	2.02	2.02
Methionine + Cystein	0.38	0.38	0.38	0.38	0.38	0.38
Lysine	1.00	1.00	1.00	1.00	1.00	1.00
Calcium	0.93	0.93	0.93	0.93	0.93	0.93
Phosphorus	0.45	0.45	0.45	0.45	0.45	0.45
Chemical Analysis						
Crude Protein	20.22	20.22	20.31	20.24	20.24	20.33
Crude fat	5.56	5.56	5.56	5.56	5.56	5.56
Crude fiber	3.71	3.71	3.72	3.93	3.93	3.93
Calcium	0.59	0.59	0.59	0.75	0.75	0.75
Phosphorus	0.48	0.48	0.48	0.48	0.48	0.48
Sodium	0.13	0.13	0.13	0.13	0.13	0.13

[1] Supplied per kilogram of diet: vitamin A, 50.00 MIU; vitamin B1, 10.00g; vitamin B2, 30.00g; Vitamin B6, 20.00g; Vitamin B12, 0.10g; Vitamin D3, 10.00 MIU; Vitamin E, 75.00g; Vitamin K3, 20.00g; Calcium D-Panthothenate, 60.00g; Nicotinic Acid, 200.00g; Folic Acid, 5.00g; Biotin, 235.00g; Antioxidant, Anti cracking and Carrier.

[2] Supplied per kilogram of diet: Se, 0.20g; Fe, 80.00g; Mn, 100.00g; Zn, 80.00g; Cu, 15.00g; KCl, 4.00g; MgO, 0.60g; $NaCO_3$, 1.50g, I, 1.00g; Co,0.25g

5.2.5 Parameters Measured

Every day, feed intake (FI) and mortality rate were checked and recorded, whereas the individual body weight (BW) of broilers was recorded weekly starting from day 1 until 35. The average body weight gain (BWG), the feed conversion ratio (FCR), and the mortality rate of broilers were calculated (Naidoo et al., 2008; Wang and Xu, 2008) as shown following:

$$\text{Average body weight gain (BWG)} = \frac{\text{Final BW} - \text{Initial BW}}{\text{Number of birds}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{FI}}{\text{BWG}}$$

$$\text{Mortality rate} = \frac{\text{Number of dead bird(s) in treatment group}}{\text{Number of initial birds in each treatment group}} \times 100$$

5.2.6 Sampling Procedures

Three broilers per replicate of each dietary treatment were randomly selected, weighted, and euthanized by severing the jugular veins for sampling purposes on day 21 and 35. The blood samples were immediately collected during euthanizing into 10 mL anti-coagulated clot activator tubes (BD, US) for biochemical and serum immunoglobulins analyses, and coagulated K_2EDTA tubes (BD, US) for haematological analyses, respectively. The broilers were then dissected to obtain organs including the thymus, proventriculus, gizzard, pancreas, liver, spleen, bursa of Fabricius and heart and weighed individually. The content in ilea and caeca (gastrointestinal tract) were obtained by cutting it and scraping gently using a sterile microscopic slide. The contents were collected for real-time (PCR) (qPCR) and high-throughput next-generation sequencing (HT-NGS) of 16S rRNA gene amplicons (Chapter 6). The ilea and caeca tissue were further collected for the measurement of the villus height and crypt depth. During the sample collection, all samples were kept on the ice and stored at -80 °C freezer until analysis was completed.

5.2.7 Haematological and Serum Collection and Biochemical Analysis

Serum samples were obtained by centrifugation of the blood samples from anti-coagulated activator tubes (BD, US) using JA-20.1 rotor of Beckman Avanti J-25I Centrifuge (Beckman, US) at 3,000 × g for 10 minutes. Afterward, the serum was carefully pipetted and transferred into two sterile 2 mL microcentrifuge tubes for biochemistry and immunoglobulin analyses, respectively. The serum samples were mixed with COBAS reagents (Roche Diagnostics, US) and analyzed by Hitachi 902 Automatic Biochemistry Analyser (Hitachi, Japan) to obtain the reading of glucose, total protein, albumin, triglycerides, cholesterol level, high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

On the other hand, coagulated blood samples from K_2EDTA tubes (BD, US) were analyzed for the red blood cell (RBC), hemoglobin, packed cell volume (PCV) concentration, mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) using Cell-Dyn 3700 heamatology analyzer (Abbott Laboratories, US). The heterophils, lymphocytes, monocytes, eosinophils and basophils of white blood cell (WBC) were counted manually by smearing the blood stained with Wright's stain (Sigma Chemical, US). The heterophil: lymphocyte ratio (H : L) were also calculated.

5.2.8 Serum Immunoglobulins

Serum samples of two broilers from four replicates cages were selected for IgG, IgA, and IgM analyses of every treatment on day 21 and 35. All serum samples were diluted according to the double–antibody sandwich enzyme-linked immunosorbent assay (ELISA) manufacturer's kit sample preparation IgG, IgA, and IgM (Bethyl Laboratories Inc., US). Standard curve point concentration of the serum was also prepared based on the manufacturer's instruction accordingly. All blank, standard and serum samples were collected and pipetted 100 µL each to the well in duplicate accordingly to reduce technical errors. The plate was covered and incubated at 25 °C for 1 hour and rinsed four times using 1X Wash Buffer. The plates were bloated on paper towels to ensure no excessive buffer on the plate. Detection of antibody were performed by pipetting 100 µL of Detection Antibody reagent and incubated for 1 hour at 25 °C. The reagents were discarded and four times washing step was repeated after incubation. Next, 100 µL of streptavidin-conjugated horseradish peroxidase (SA-HRP) were added into each well as catalyst and incubated for 30 minutes and then carefully discarded. The reagents were discarded and four times washing step was repeated after incubation. Then, 100 µL of chromogenic substrate TMB (3, 3', 5, 5'-tetramethylbenzidine) was added to each well and incubated in a dark room for enzymatic colour reaction to develop blue colour at 25 °C. Finally, the reactions were stopped by adding 100 µL dilute sulfuric acid to each well that turn into yellow colour. The absorbance was measured by using BioTek EL800 Absorbance Reader (BioTek, US) at 450 nm. The 4 parameters logistic (4PL) regression standard curve was plotted from the average absorbance measured versus known concentration of chicken IgG, IgA, and IgM, to determine the concentration of unknown samples. The data was then multiplied by the dilution factor used earlier to determine the amount of antibody present in the undiluted sample.

5.2.9 Villus Height and Crypt Depth of Broilers Small Intestine (Jejuna and Ilea)

The 1 cm middle section of ilea and caeca were cut and collected for measurement of the villi height and crypt depth of three broilers from four replicate cages of each diet treatment for day 21 and 35. The jejuna and ilea

segments were fixed with 10% buffered formalin overnight. The tissue was then dried in Leica ASP 3000 fully-enclosed tissue processor for 16 hours and embedded in paraffin wax by using Leica EG1160 paraffin embedding station (Leica, Japan). All histological studies were performed on 4 µm sections (two cross-sections for each sample) using Leica RM2155 microtome (Leica, Japan), stained by hematoxylin and eosin, and examined by Nikon Eclipse 80i microscope (Nikon Corporation, Japan) with built-in camera. The villus height and crypt depth image and data were acquainted by using NIS-Elements D3.2 (Nikon Corporation, Japan). The villus height was measured from the top end to the bottom of the villus-crypt junction, whereas the crypt depth from the basement to the villus-crypt junction, as shown in Figure 5.3. An average of three villus height and crypt depth were measured for each sample. The villus to crypt ratio were calculated as follows:

$$\text{Villus to crypt ratio} = \frac{\text{Villus height}}{\text{Crypth depth}}$$

5.2.10 Relative Weights of Organs

Eight chicken organs, including thymus, proventriculus, gizzard, pancreas, liver, spleen, bursa of Fabricius, and heart were collected from three broilers from four replicate cages of each diet treatment for day 21 and 35 during the dissection. Excessive fats were removed and cleaned thoroughly before weighing. The relative weight of organ (RWO) were calculated as shown below:

$$\text{Relative weight of organ (\%)} = \frac{\text{Weight of organ}}{\text{Body weight}} \times 100$$

5.2.11 Statistical Analysis

All of the data were analyzed based on one-way analysis of variance (ANOVA) and paired *t-test* using Statistical Package for Social Science (SPSS) Statistic version 22 (IBM, US). Significant means ± standard error $P < 0.05$ were compared within samples by Duncan's multiple range test (Duncan, 1955).

5.3 Results

5.3.1 Temperature and Humidity

The ambience temperature and relative humidity of the chicken farm in the morning were ranged between 25.4 °C- 33.3 °C and 50%-99%; whereas in the evening were ranged between 24.6 °C - 32.6 °C and 51% - 94%, respectively.

5.3.2 Broilers Growth Performance and Mortality Rate

During the starter period (1-21 days), the body weight gain (BWG) of broilers supplemented with CC10 diet has a significant ($P < 0.05$) higher body weight compared to CC05 and PRO diet. During the finisher period (22-35 days), the BWG of broilers with all supplemented diet groups, except SYN10 were significantly ($P < 0.05$) enhanced compared to the basal diet. Overall, the broilers with supplemented diet throughout the feeding trial, except SYN10 showed significant ($P < 0.05$) higher BWG compared to BD.

The feed intake (FI) of broilers supplemented SYN10 were significantly ($P < 0.05$) lesser than the CC10 diet during the starter period and significantly ($P < 0.05$) lesser than basal diet and CCO5 during the finisher period. Broilers supplemented with PRO diet have significant ($P < 0.05$) lower FI compared to CC10 diet during the starter period, but no significant difference during the finisher period. The overall FI of broilers supplemented with SYN10 was significantly lesser than BD, CC05 and CC10 diet.

The feed conversion ratio (FCR) of broilers supplemented with SYN10 diet was significantly ($P < 0.05$) lower than BD, CCO5 and SYN05 diet during the starter period. During the finisher period, broilers with all of the supplement diet had significant ($P < 0.05$) lower FCR compare to the control (BD) group. Thus, the overall FCR of all supplemented treatment groups is significantly ($P < 0.05$) lower than BD.

Throughout the study, some dead birds were detected due to nonspecific causes where the mortality rate of the broilers is between 2.5 % (BD, CC10 and PRO diet group) to 5.0 % (SYN05 and SYN10 diet group). All of the growth performance and mortality rate of broilers results were shown in Table 5.3.

Table 5.3: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on growth performance and mortality rate of broilers.

Category[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
Average BWG (g/bird/d)						
1 to 21 days	784.24 ± 5.29^{ab}	767.62 ± 3.09^{a}	825.71 ± 22.19^{b}	769.11 ± 14.09^{a}	782.85 ± 20.56^{ab}	789.90 ± 24.36^{ab}
22 to 35 days	887.25 ± 49.21^{a}	1079.20 ± 12.07^{c}	1071.23 ± 42.52^{c}	1086.78 ± 33.13^{c}	1012.11 ±13.32^{bc}	957.68 ± 20.23^{ab}
1 to 35 days	1671.49 ± 45.61^{a}	1846.82 ± 10.48^{bc}	1896.93 ± 47.72^{c}	1855.89 ± 43.04^{bc}	1794.96 ± 30.17^{bc}	1747.58 ± 26.85^{ab}

Table 5.3: Continued

Category[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
FI (g/bird/day)						
1 to 21 days	1144.00 ±10.16^{ab}	1107.50 ± 13.87^{ab}	1161.00 ± 14.91^{b}	1085.75 ± 22.35^{a}	1144.75 ± 27.16^{ab}	1089.75 ± 27.76^{a}
22 to 35 days	1918.22 ± 37.50^{b}	1960.34 ± 83.29^{b}	1892.01 ± 48.86^{ab}	1850.90 ± 78.44^{ab}	1809.70 ± 28.86^{ab}	1705.06 ± 58.98^{a}
1 to 35 days	3062.00 ± 42.58^{b}	3068.00 ± 84.47^{b}	3053.25 ± 41.87^{b}	2936.50 ± 91.42^{ab}	2954.50 ± 53.06^{ab}	2794.75 ± 67.89^{a}
FCR (feed/gain)						
1 to 21 days	1.46 ± 0.01^{b}	1.44 ± 0.02^{b}	1.41 ± 0.02^{ab}	1.41 ± 0.01^{ab}	1.46 ± 0.02^{b}	1.38 ± 0.02^{a}
22 to 35 days	2.18 ± 0.11^{b}	1.83 ± 0.09^{a}	1.77 ± 0.03^{a}	1.71 ± 0.04^{a}	1.79 ± 0.02^{a}	1.78 ± 0.03^{a}
1 to 35 days	1.84 ± 0.05^{b}	1.67 ± 0.05^{a}	1.61 ± 0.02^{a}	1.58 ± 0.02^{a}	1.65 ± 0.02^{a}	1.60 ± 0.02^{a}
Mortality rate (%)						
1 to 35 days	2.50	0	2.50	2.50	5.00	5.00

Each data represents mean ± standard error of 8 broilers per treatment.
[a-c] Means within a row with no common superscript differ significantly ($P < 0.05$).
[1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.; BWG= body weight gain; FI= feed intake; FCR= feed conversion ratio.

5.3.3 Haematological and Serum Biochemistry Analysis

There are no significant differences in total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride among all diet groups at 21 days. However, at 35 days, the concentration of LDL in the SYN05 group was significantly ($P < 0.05$) lower than the control (BD) group. In addition, the concentration of triglycerides in the SYN10 group was significantly ($P < 0.05$) lower than the control and CC10 group.

In general, there are no significant differences among the broilers with supplemented diet treatment group on the serum heamatology compared to the basal diet fed broilers at 21 and 35 days (Table 5.5). The red blood cell (RBC) count of broilers in the SYN05 diet group is significantly ($P < 0.05$) higher than the CC05 and CC10 diet groups on day 21. The hemoglobin count as well as pack cell volume (PCV) count of broilers in the PRO diet group is significantly ($P < 0.05$) higher than the CC10 diet group. Besides that, broilers in the SYN10 diet group also showed significant ($P < 0.05$) higher eosinophils concentration compared to the CC10 diet group. Besides, the thrombocytes count of broilers

in the SYN05 and SYN10 also were significantly ($P < 0.05$) higher than the CC05 and CC10 diet groups.

On day 35, although there are no significant differences of RBC and hemoglobin count among the diet group, but the mean corpuscular volume (MCV) is significantly ($P < 0.05$) higher in the SYN05 diet group compared to the CC10 diet group. On the other hand, the mean corpuscular hemoglobin concentration (MCHC) was significantly ($P < 0.05$) higher in the PRO diet group than the SYN05 diet group.

The heterophils : lymphocytes ratio (H : L) of the CC10 and SYN10 diet groups is significantly ($P < 0.05$) higher than the CC10, PRO, and SYN05 diet groups, but not significantly different compared to control (BD) at 21 days. At 35 days, the H : L of control (BD) diet is significantly ($P < 0.05$) higher than the CC05 diet group.

Table 5.4: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on serum lipid concentration (mmol/L) of broilers at 21 and 35 days.

Category[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
21 days of age						
Total cholesterol (mmol/L)	3.58 ± 0.23	3.89 ± 0.24	3.81 ± 0.22	3.39 ± 0.10	3.61 ± 0.10	3.36 ± 0.10
HDL (mmol/L)	2.89 ± 0.13	3.29 ± 0.20	3.16 ± 0.20	2.79 ± 0.07	2.96 ± 0.06	2.77 ± 0.10
LDL (mmol/L)	0.40 ± 0.02	0.37 ± 0.04	0.42 ± 0.05	0.40 ± 0.02	0.43 ± 0.04	0.43 ± 0.05
Triglycerides (mmol/L)	0.38 ± 0.02	0.28 ± 0.01	0.39 ± 0.05	0.29 ± 0.02	0.30 ± 0.02	0.33 ± 0.04
35 days of age						
Total cholesterol (mmol/L)	3.44 ± 0.14	3.28 ± 0.14	3.50 ± 0.09	3.28 ± 0.18	3.06 ± 0.14	3.49 ± 0.15
HDL (mmol/L)	2.59 ± 0.08	2.57 ± 0.06	2.79 ± 0.12	2.66 ± 0.15	2.62 ± 0.14	2.90 ± 0.14
LDL (mmol/L)	0.81 ± 0.09^{b}	0.68 ± 0.09^{ab}	0.70 ± 0.07^{ab}	0.54 ± 0.06^{ab}	**0.48 ± 0.03^{a}**	0.60 ± 0.07^{ab}
Triglycerides (mmol/L)	0.55 ± 0.07^{b}	0.45 ± 0.07^{ab}	0.54 ± 0.06^{b}	0.51 ± 0.08^{ab}	0.39 ± 0.05^{ab}	**0.27 ± 0.02^{a}**

Each data represents mean ± standard error of 8 broilers per treatment.

$^{a-b}$ Means within a row with no common superscript differ significantly ($P < 0.05$).

[1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob. HDL=high density lipoprotein; LDL= low density lipoprotein.

Table 5.5: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on blood haematology of broilers at 21 and 35 days.

Category[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
21 days of age						
RBC ($x10^{12}$/L)	2.21 ± 0.05^{ab}	2.14 ± 0.05^{a}	2.15 ± 0.03^{a}	2.29 ± 0.13^{ab}	2.38 ± 0.05^{b}	2.28 ± 0.08^{ab}
Hb (g/L)	117.38 ± 2.28^{ab}	114.63 ± 2.31^{ab}	112.75 ± 1.24^{a}	121.50 ± 2.98^{b}	121.13 ± 2.17^{ab}	117.25 ± 4.15^{ab}
PCV (L/L)	0.29 ± 0.01^{abc}	0.28 ± 0.01^{ab}	0.28 ± 0.00^{a}	0.30 ± 0.01^{c}	0.31 ± 0.01^{c}	0.30 ± 0.01^{bc}
MCV (fL)	130.88 ± 4.51	131.00 ± 0.93	127.88 ± 1.62	135.38 ± 7.82	128.63 ± 1.64	131.13 ± 2.33
MCHC (g/L)	409.75 ± 15.50	409.13 ± 2.70	410.63 ± 5.67	400.13 ± 5.44	397.38 ± 4.06	393.50 ± 5.20
WBC ($x10^{9}$/L)	18.69 ± 1.70^{ab}	19.44 ± 1.19^{ab}	16.58 ± 0.71^{a}	25.05 ± 5.27^{b}	22.28 ± 1.87^{ab}	22.54 ± 1.79^{ab}
Heterophils ($x10^{9}$/L)	11.37 ± 1.01	11.47 ± 0.83	10.18 ± 0.82	15.24 ± 3.44	12.95 ± 1.16	14.35 ± 1.54
Lymphocytes ($x10^{9}$/L)	3.42 ± 0.32	2.90 ± 0.33	3.29 ± 0.30	5.29 ± 1.22	4.10 ± 0.40	3.47 ± 0.39
H : L	3.39 ± 0.24^{ab}	4.12 ± 0.46^{b}	3.31 ± 0.41^{a}	3.01 ± 0.23^{a}	3.18 ± 0.15^{a}	4.17 ± 0.22^{b}
Monocytes ($x10^{9}$/L)	1.91 ± 0.33	2.56 ± 0.42	1.38 ± 0.11	1.99 ± 0.40	2.28 ± 0.39	2.04 ± 0.32
Eosinophils($x10^{9}$/L)	0.88 ± 0.22^{ab}	0.73 ± 0.15^{ab}	0.37 ± 0.04^{a}	1.17 ± 0.29^{ab}	0.72 ± 0.18^{ab}	1.41 ± 0.28^{b}
Basophils ($x10^{9}$/L)	1.12 ± 0.32	1.79 ± 0.41	1.37 ± 0.21	1.37 ± 0.29	2.22 ± 0.27	1.27 ± 0.38
Thrombocytes ($x10^{9}$/L)	6.33 ± 1.18^{ab}	3.10 ± 0.59^{a}	3.05 ± 0.70^{a}	5.91 ± 1.21^{ab}	8.69 ± 1.3^{b}	10.85 ± 2.03^{b}
35 days of age						
RBC ($x10^{12}$/L)	2.47 ± 0.04	2.32 ± 0.06	2.38 ± 0.04	2.36 ± 0.11	2.41 ± 0.08	2.44 ± 0.04
Hb (g/L)	124.38 ± 2.36	117.00 ± 2.95	117.13 ± 2.36	119.00 ± 5.22	120.88 ± 2.61	122.50 ± 1.71
PCV (L/L)	0.30 ± 0.00	0.28 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.00
MCV (fL)	121.88 ± 1.20^{ab}	122.50 ± 1.45^{ab}	119.50 ± 1.21^{a}	121.75 ± 1.44^{ab}	125.00 ± 1.15^{b}	122.25 ± 2.03^{ab}

Table 5.5: Continued

Category[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
MCHC (g/L)	412.75 ± 5.23^{ab}	412.88 ± 4.21^{ab}	411.25 ± 2.95^{ab}	416.50 ± 4.30^{b}	401.63 ± 4.56^{a}	410.50 ± 5.66^{ab}
WBC (x10^{9}/L)	43.96 ± 5.26	46.30 ± 3.47	41.51 ± 2.53	39.26 ± 2.31	41.36 ± 3.62	40.30 ± 3.73
Heterophils (x10^{9}/L)	29.16 ± 3.93	25.80 ± 2.30	25.31 ± 1.88	24.69 ± 1.70	23.49 ± 2.02	26.87 ± 2.61
Lymphocytes (x10^{9}/L)	6.19 ± 0.52	8.41 ± 0.85	7.62 ± 0.88	6.96 ± 0.50	7.58 ± 1.19	6.69 ± 0.77
H : L	4.64 ± 0.43^{b}	3.18 ± 0.26^{a}	3.63 ± 0.46^{ab}	3.65 ± 0.30^{ab}	3.56 ± 0.58^{ab}	4.19 ± 0.34^{ab}
Monocytes (x10^{9}/L)	4.16 ± 1.00^{ab}	6.43 ± 1.23^{b}	3.76 ± 0.68^{ab}	3.80 ± 0.42^{ab}	4.99 ± 1.04^{ab}	2.65 ± 0.29^{a}
Eosinophils (x10^{9}/L)	1.91 ± 0.35	2.07 ± 0.53	1.88 ± 0.51	1.41 ± 0.24	1.95 ± 0.31	1.94 ± 0.40
Basophils (x10^{9}/L)	2.54 ± 0.81	3.59 ± 0.68	2.95 ± 0.69	2.42 ± 0.57	3.35 ± 0.88	2.16 ± 0.38
Thrombocytes (x10^{9}/L)	4.32 ± 2.50	2.55 ± 0.56	2.81 ± 0.39	3.25 ± 0.58	3.92 ± 1.18	3.56 ± 0.83

Each data represents mean ± standard error of 8 broilers per treatment.

$^{a-b}$ Means within a row with no common superscript differ significantly ($P < 0.05$).

[1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob; RBC= red blood cell; Hb= hemoglobin; PCV= pack cell volume; MCV= mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; WBC = white blood cell; H : L = heterophils : lymphocytes.

5.3.4 Serum Immunoglobulins

During the starter period, broilers supplemented with SYN10 diet showed significant ($P < 0.05$) higher IgA and IgM concentration compared to the basal diet. IgG concentration is numerically highest in broilers supplemented with PRO diet. During the finisher period, broilers supplemented with the CC10 diet had a lower IgA concentration ($P < 0.05$). In contrast, broilers supplemented with PRO and SYN05 diet had a lower IgG concentration ($P < 0.05$) compared to BD. There are no significant differences of IgM concentration observed among the broilers of all diet treatments.

Table 5.6: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on serum immunoglobulins of broilers at 21 and 35 days.

Category[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
21 days of age						
IgA (g/L)	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}	0.04 ± 0.00^{a}	0.06 ± 0.01^{a}	0.08 ± 0.01^{b}
IgG (g/L)	5.73 ± 0.72	5.59 ± 0.85	3.81 ± 0.50	5.88 ± 0.90	4.07 ± 0.42	5.53 ± 0.44
IgM (g/L)	0.18 ± 0.01^{a}	0.19 ± 0.01^{ab}	0.20 ± 0.01^{ab}	0.17 ± 0.01^{ab}	0.18 ± 0.02^{ab}	0.23 ± 0.01^{b}
35 days of age						
IgA (g/L)	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}	0.05 ± 0.01^{a}	0.11 ± 0.02^{b}	0.07 ± 0.00^{ab}	0.09 ± 0.01^{b}
IgG (g/L)	14.67 ± 0.52^{c}	13.88 ± 0.88^{bc}	11.92 ± 0.68^{abc}	11.11 ± 0.89^{ab}	10.07 ± 0.81^{a}	11.57 ± 0.83^{abc}
IgM (g/L)	0.70 ± 0.09	0.74 ± 0.24	0.40 ± 0.04	0.42 ± 0.06	0.55 ± 0.10	0.38 ± 0.03

Each data represents mean ± standard error of 8 broilers per treatment.

$^{a-c}$ Means within a row with no common superscript differ significantly ($P < 0.05$).

[1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

5.3.5 Villus Height and Crypt Depth of Broilers Small Intestine (Jejuna and Ilea)

On day-21, the villus height of jejuna in SYN05 is significantly higher than BD ($P < 0.05$), but no significant difference in crypt depth. At 35 days, the villus height of jejuna in CC05, CC10, and SYN05 showed numerically higher villus height than the BD, which are 29913.26 µm, 35014.56 µm, and 37796.08 µm, respectively. The ilea villus height of SYN05 was 466.23 µm higher and 2284.47 µm thicker crypt depth ($P < 0.05$) than BD at 21 days. However, the villus height is numerically shortest in SYN05 at 35 days with no significant difference in crypt depth in ilea than BD on day-35.

In general, there are no significant differences of villus to crypt ratio in broilers' jejuna among treatment diet at 21 and 35 days. However, in ilea, basal diet showed a significantly higher ($P < 0.05$) villus to crypt ratio than SYN05 at 21 days. There are no significant differences in the villus to crypt ratio in ilea among the dietary treatment groups.

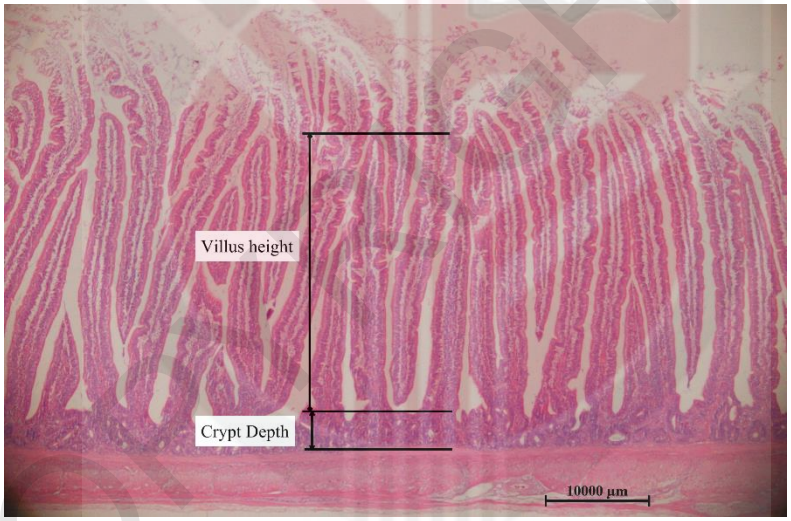
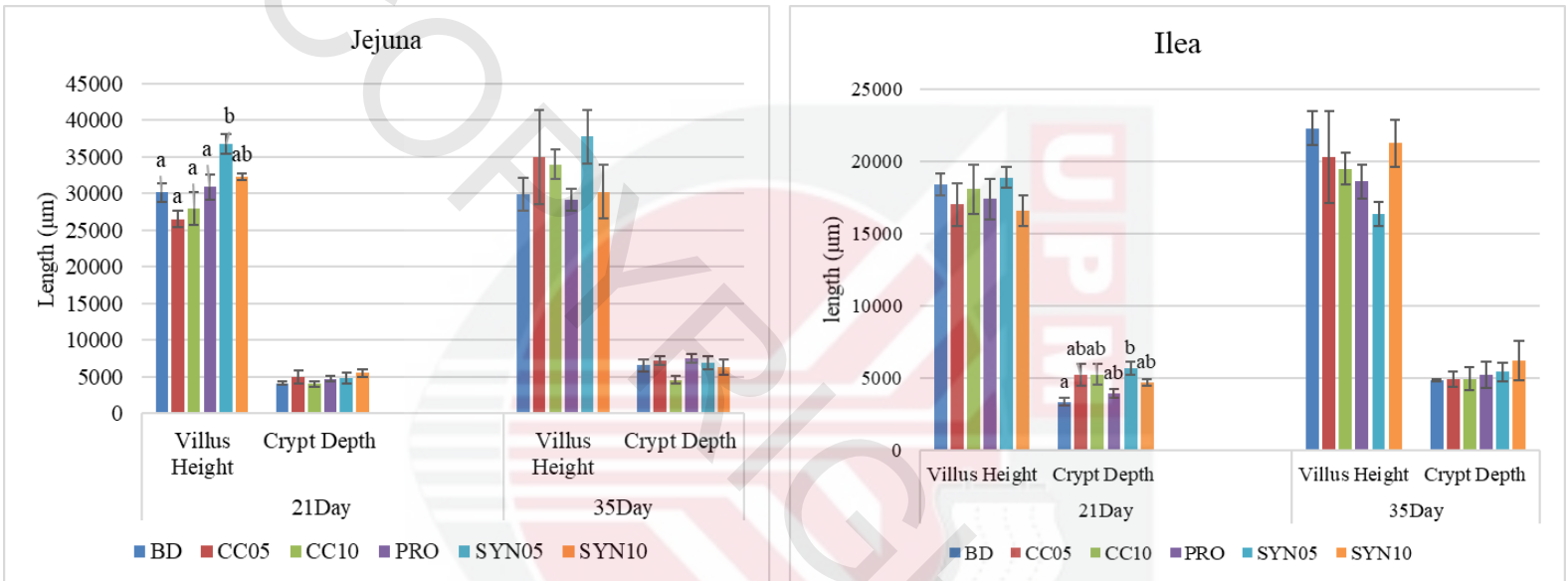


Figure 5.3: Villus height and crypt depth of broilers jejunum fed with 0.5% corn cob extract at 35 days.



(a) (b)

Figure 5.4: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on villi height and crypt depth of broilers jejuna and ilea at 21 and 35 days.

Each bar is mean ± standard error of villus height and crypt depth of 4 replicate cages with 2 broilers each from (a) jejuna and (b) ilea at 21 and 35 days.

[a-b]Bars that have different superscripts differ significantly ($P < 0.05$).

BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

Table 5.7: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on villus to crypt ratio of broilers jejuna and ilea at 21 and 35 days.

Category[1]	Age	BD (control)	CC05	CC10	PRO	SYN05	SYN10
Jejuna	21d	7.37± 0.57	5.89± 1.08	7.16± 1.00	6.61± 0.45	8.17± 1.07	5.95± 0.51
	35d	4.90± 1.02	4.78± 0.73	7.92± 1.39	3.95± 0.31	5.88± 1.20	5.46± 1.41
Ilea	21d	5.56± 0.32[b]	3.49± 0.66[ab]	3.63± 0.49[ab]	4.62± 0.66[ab]	3.40± 0.23[a]	3.55± 0.27[ab]
	35d	4.62± 0.19	4.10± 0.28	4.21± 0.58	3.94± 0.79	3.10± 0.30	3.91± 0.74

Each data represents mean ± standard error of 8 broilers per treatment.
[a-b] Means within a row with no common superscript differ significantly ($P < 0.05$).
[1] BD = basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

5.3.6 Relative Weight of Organs (RWO)

Overall, there are no significant differences relative weight of organs (RWO) recorded in all dietary treatment groups compared to basal diet at 21 and 35 days.

.

Table 5.8: Effects of dietary treatments of corn cob extract at different dosages, commercial probiotic and their combinations on relative organs weight (%) of broilers at 21 and 35 days.

Treatment[1]	BD (control)	CC05	CC10	PRO	SYN05	SYN10
21 days of age						
Thymus (%)	0.14 ± 0.00	0.11 ± 0.01	0.15 ± 0.04	0.12 ± 0.01	0.11 ± 0.02	0.14 ± 0.01
Proventriculus (%)	0.62 ± 0.01	0.65 ± 0.03	0.71 ± 0.12	0.60 ± 0.01	0.59 ± 0.04	0.58 ± 0.05
Gizzard (%)	1.70 ± 0.19	1.82 ± 0.05	1.66 ± 0.11	1.83 ± 0.04	1.83 ± 0.04	1.80 ± 0.04
Pancreas (%)	0.31 ± 0.02	0.32 ± 0.00	0.29 ±0.01	0.31 ± 0.01	0.33 ± 0.06	0.31 ± 0.13
Liver (%)	2.62 ± 0.03	2.66 ± 0.03	2.59 ± 0.06	2.45 ± 0.10	2.52 ± 0.06	2.57 ± 0.13
Spleen (%)	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.08 ± 0.01
Bursa of Fabricius (%)	0.17 ± 0.02	0.19 ± 0.01	0.20 ± 0.02	0.21 ± 0.02	0.19 ± 0.02	0.18 ± 0.02
Heart (%)	0.57 ± 0.01	0.55 ±0.01	0.53 ± 0.12	0.54 ±0.06	0.50 ±0.08	0.53 ±0.10
35 days of age						
Thymus (%)	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.02	0.12 ± 0.02	0.17 ± 0.01
Proventriculus (%)	0.38 ± 0.03	0.35 ± 0.02	0.33 ± 0.01	0.39 ± 0.03	0.33 ±0.01	0.36 ± 0.02
Gizzard (%)	1.27 ± 0.05	1.17 ± 0.04	1.23 ± 0.08	1.27 ± 0.05	1.24 ± 0.02	1.25 ± 0.04
Pancreas (%)	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.18 ± 0.02
Liver (%)	2.10 ± 0.17	2.12 ± 0.08	1.94 ± 0.04	2.11 ± 0.06	1.93 ± 0.08	1.99 ± 0.06
Spleen (%)	0.14 ± 0.03	0.18 ± 0.01	0.15 ± 0.03	0.22 ± 0.03	0.16 ± 0.00	0.17 ± 0.03
Bursa of Fabricius (%)	0.10 ± 0.02	0.10 ± 0.02	0.13 ± 0.02	0.12 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
Heart (%)	0.40 ± 0.01	0.40 ± 0.02	0.37 ± 0.01	0.43 ± 0.02	0.36 ± 0.02	0.42 ± 0.03

Data represent the mean values of 8 broilers per treatment.

[1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (Primalac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

5.4 Discussion

Studies have revealed that the addition of probiotics to the supplemented feed has led to improved performances and better gut microflora in broilers, layers and domestic pigs (Mountzouris et al., 2010). It also has been shown to improve the absorption of minerals and vitamins and prevents diarrhea, thus maximizing digestion of resorption of nutrients. Some synbiotics are much more efficient when used in combinations (Alloui et al., 2013). The combination of probiotics and prebiotic called synbiotic has also demonstrated an increase in the antimicrobial, anticarcinogenic and antiallergic characteristics. Research has confirmed that supplementation of probiotics and synbiotics has had a considerable positive impact on the productive performance of broilers which helps in improving their intestinal and immunological conditions.

Feed composition:
Feed compositions of the dietary treatments were assessed for both the starter phase and the finisher phase. The composition of the basal diet and its addition to the different concentrations of corn cob extracts, only probiotics, a mixture of corn cob extracts and probiotics were chemically analysed. Metabolizable energy levels remained approximately the same in both phases. The crude protein content was observed to be higher in the feed of the starter phase than the feed of the finisher phase. However, the crude fiber levels were estimated to be higher in the feed used in the finisher phase than the starter phase. There was no notable change in the levels of crude fat in both feeds. Calcium, potassium was seen to be slightly elevated in the feed of the starter phase and finisher phase, respectively. Sodium levels were also higher in the feed of the finisher phase. These slight variations were due to the compositional changes in the natural product, i.e., corn extracts used in this study.

Growth performance and mortality rates:
The growth performance and the effects on overall body weight gain of all the broilers were calculated and noted. The results were in line with some studies demonstrating that supplementation of probiotics alone resulted in improvement of BWG during the starter phase (Bai et al., 2013; Alkhalf et al., 2010; Ghasemi et al., 2014). Mixed results were also observed showing no positive effects of a modified diet than the control diet (Taheri et al., 2014; Afsharmanesh et al., 2011; Al-Zenki et al., 2009). Studies have also reported that diet supplemented with 0.1% probiotic resulted in a significant increase in the BWG and decrease FCR of broilers starting from the 2nd week (starter period) of feeding, but no significant effect was observed on the overall BWG and FCR of broilers supplemented with probiotic (Alkhalf, et al., 2010; Bai et al., 2013). Besides, the broilers supplemented with prebiotic had enhanced overall BWG (Afsharmanesh et al., 2011; Salianeh et al., 2011), yet no significant increase of BW was reported by Midilli et al., (2008). The current studies also demonstrated a similar effect on BWG analysis where diet supplemented with neither probiotic nor prebiotic significantly affects the body weight and body weight gain except for an improved feed conversion ratio. Alloui et al. (2013) suggested that synbiotic feed may represent a two-fold increase in the synergistic effects, the data obtained in the

current study did not show a significant impact on the overall body weight gain (BWG) of broilers. This result was almost similar to what Taheri et al. (2014) confirmed about no considerable increase in the BWG analysis of broilers and layers. However, the total FCR is significantly lesser in broilers supplemented with prebiotic, probiotic as well as synbiotic. The BWG and FCR of broilers supplemented with 0.3% of prebiotic resulted in significantly enhanced of the BWG and less FCR in broilers compared with the control diet, whereas no effect was observed with supplemented probiotic diet (Salianeh et al., 2011).

Haematology:

Haematological studies evaluated various haematological parameters such as the levels of cholesterol, RBC and WBC count, Hb concentration and PCV percentage and Erythrocyte Sedimentation Rate (ESR) levels. No notable significant difference was calculated in total cholesterol, low-density lipoprotein (LDL), and triglyceride among all diet groups at 21 days. On day 35, the concentration of LDL in the SYN05 group was significantly ($P < 0.05$) lower than the control (BD) group, while the concentrations of triglycerides in the SYN10 group was estimated to be significantly ($P < 0.05$) lower than the control and CC10 group. These results were in line with the findings of Al-Kassie et al. (2008) who found that the modified probiotic diet lowered the triglyceride content in the broilers. The estimated cholesterol levels were in accordance with the findings of Panda et al. (2006), who concluded that there was a significant reduction in serum cholesterol and LDL concentration of broilers due to the dietary supplementation of different concentrations of probiotics as compared to the control group diet. Paryad and Mahmoudi (2008) also found a significant reduction in serum cholesterol levels. Cholesterol analysis studies performed by Ashayerizadeh et al. (2009) revealed that the probiotic, prebiotic and synbiotic addition to the diet of the broilers displayed no significant effect on the serum LDL levels. Another interesting study by Safalaoh (2006) did not significantly differ in the serum cholesterol levels when the probiotics were added to the drinking water of broiler chicks. The serum haematology analysis revealed no significant difference among the broilers with supplemented diet treatment group when compared to the basal diet-fed broilers at 21 and 35 days (Table 5.5). The red blood cell (RBC) count of broilers subjected to the SYN05 diet group was estimated to be significantly ($P < 0.05$) higher than the CC05 and CC10 diet groups. The hemoglobin concentration and the pack cell volume (PCV) broilers subjected to the PRO diet group were estimated to be significantly ($P < 0.05$) higher than the CC10 diet group. In addition to this, broilers subjected to the SYN10 diet group also showed significant ($P < 0.05$) higher eosinophils concentration compared to the CC10 diet group. The volume of thrombocytes of broilers subjected to SYN05 and SYN10 also were observed to be significantly ($P < 0.05$) higher than the CC05 and CC10 diet groups. Studies to check the blood glucose levels and the levels of triglycerides were found to be similar in all the groups provided with varying concentrations of supplements. It was also reported that some of the microorganisms present in the probiotics could utilize the cholesterol present in the gastrointestinal tract and carry out metabolism, thus reducing the absorption of cholesterol from the GI tract (Gurram et al., 2018). In contrast, the mean corpuscular hemoglobin concentration (MCHC) was significantly ($P < 0.05$) higher in the PRO diet group than the SYN05 diet group. The H : L ratio is an established indicator of stress in poultry as responses to any

stressors cause (Gross and Siegel, 1983; Zulkifli et al., 1999). According to Gross and Siegel (1983), the H : L ratio might be stimulated through the adrenal gland that secretes hormones during stress. The H : L ratio of broilers subjected to all supplemented groups are not significantly different compared to control (BD) at 21 days and 35 days, except the CC05 diet group that was significantly ($P < 0.05$) lower at 35 days. Hence, this proved that corn cob extracts and probiotic inclusion did not cause any physiological manifestations of stress in broilers. This finding was in line with the study conducted by Karoglu and Drudag, (2005), that a supplementary probiotic diet might inhibit stress associated with nutrition by assisted in nutrient absorption and strengthening microbial balance in the broiler digestive tract.

Serum Immunology:

The immunoglobulin concentrations were calculated to assess the contribution of probiotic supplement feed. Broilers supplemented with SYN10 diet resulted in a significant ($P < 0.05$) higher IgA and IgM concentration compared to basal diet during the starter phase. IgG concentrations were notably highest in broilers supplemented with PRO diet. Broilers augmented with the CC10 diet represented lower concentrations of IgA ($P < 0.05$) in the finisher phase. In contrast, broilers supplemented with PRO, and SYN05 diet demonstrated lower IgG concentration ($P < 0.05$) levels as compared to BD. There are no significant differences in the IgM concentration levels broilers subjected to all the different diets. Studies by Mountzouris et al. (2010), showed that the immunoglobulin concentration increases due to the availability of nutrients and minerals available in the probiotic diet.

Small Intestine Morphology:

Dietary supplementation of broilers subjected to SYN05 diet showed a significant increase in the villi height of jejuna than broilers subjected to BD ($P < 0.05$) on day 21. However, there was no significant difference in crypt depth. At 35 days, the villus height of jejuna in broilers subjected to CC05, CC10, and SYN05 showed notably higher villus height compared to the BD, of 29913.26 µm, 35014.56 µm, and 37796.08 µm, respectively. The ilea villus height of SYN05 was estimated to be 466.23 µm higher and 2284.47 µm thicker for crypt depth ($P < 0.05$) than BD at 21 days. Awad et al. (2009) reported that synbiotic supplementation had reduced the crypt depth of the ileum compared to the control. However, the villus height is notably shortest in SYN05 at 35 days with no significant difference of crypt depth in the ileum than the crypt depth of broilers subjected to BD on day 35. In general, no significant difference was recorded with respect to villus to crypt ratio in broilers jejuna among treatment diet at 21 and 35 days. Analysis of the ileum morphology revealed that the broilers subjected to basal diet showed a significantly higher ($P < 0.05$) villus to crypt ratio than SYN05 at 21 days. The synbiotic diet improved the villus height in ilea and also the thickening of the depth of the crypt, which led to a lower villus to crypt ratio. The higher villus may have an impact on better absorption, whereas the thinner crypt is one of the indicators of the health and functional status of the intestine in the broilers. This result is aligned with Rehman et al. (2007) findings, where the inulin affects the morphology of the intestine by enhancing villi height and also deeper crypt compared to the control group. Therefore, the

supplemented diet did improve the absorption by increase the height of villus but not intestinal epithelial cell renewal processes by having thinner crypt depth. There was no considerable difference in the villus to crypt ratio in the ileum in all the dietary treatment groups.

Relative organ weight (ROW):
There was no significant difference in the percentage of the relative weight of the organs in the supplemented dietary treatment group compared to basal diet on day 21 and day 35. Studies by Awad et al. (2009), estimated the weights of broilers individually after they arrived from the hatchery to the experimental farm (initial weight) and on day 35. Feed consumption led to no difference in the initial and final weights of the broilers. Besides, there was no notable positive effect of synbiotic diet on the growth performance of the broilers which in line with the study by Mookiah et al. (2014).

5.5 Conclusion

In brief, the key findings of this study are:

(1) all supplemented diets significantly ($P < 0.05$) improved the body weight gained with a significantly ($P < 0.05$) lower FCR ratio compared to BD on the overall 35 day trials.
(2) the supplementation of 1.0% of corn cob extract and 1.0% of probiotics (SYN10) resulted in the reduction of triglyceride level and enhanced the antibody production ($P < 0.05$) compared to BD.
(3) the supplementation of 0.5% of corn cob extract and 1.0% of probiotics (SYN05) significantly lowered the LDL level ($P < 0.05$) compared to BD.
(4) there is no physiological stress in broilers with supplementation of both corn cob extracts and probiotic or singly during the starter period, yet significant ($P < 0.05$) lower H : L was observed in CC05 diet at 35 days, whereas other supplemented diets were numerically lower compared to BD.
(5) there is no significant improvement on the villus height to crypt depth ratio and RWO compared to BD by supplementing corn cob extract and probiotic or singly with different dosages throughout the feeding period.

Overall analysis indicated that the diet supplemented with corn cob extract, PrimaLac® or a combination of both on boilers growth and health performance showed promising results in varying degrees compared to BD. Thus, the effect of corn cob extract supplemented diet on broilers gut microbiota will be further studied in Chapter 6.

CHAPTER 6

EVALUATION OF CEACAL AND ILEAL MICROBIAL COMMUNITY IN RESPONSE TO DIETARY PROBIOTIC, CRUDE EXTRACT AND THEIR COMBINATIONS AT DIFFERENT DOSAGES BASED ON HIGH-THROUGHPUT SEQUENCING OF 16S rRNA GENE AMPLICONS

6.1 Introduction

The gut plays an essential role in animals, where digestion and uptake of nutrients take part. It is easily affected by environmental pathogens (Yegani and Korver, 2008). Therefore, the optimal performance of the broilers is closely related to the gut conditions. If the gut is well-functioning and in a healthy state, the microbiota aid the assimilation of nutrients from foods that produce vitamins and amino acids, consequently boosting the health and performance of broilers (Apajalahti, 2005).

Besides the digestibility of the gut lumen, the intestinal mucosa of the broiler also acts as an effective barrier that separates the luminal content and the internal tissues. This concept showed the importance of intestinal mucosa on the gut health and performance of broilers (Rinttiläand Apajalahti, 2013). Schenk and Mueller (2008) reported the dynamic balance between the mucus layers, epithelial cells, microbiota and immune cells in the intestine are related to the intestinal mucosal barrier activity. Several studies have proven that the diet of broilers affected the microbiota balance that leads to the growth performance and health condition of broilers (Yan et al., 2017; Arrieta et al., 2014; Stanley et al., 2013). Thus, the health of the broiler can be determined by investigating the diversity of the gut microbiota in the broiler.

The findings via cultivation of gut microbiota are very limited due to their requirement of an anaerobic condition similar to the gut environment. Therefore, a culture-independent approach via metagenomics is more reliable in studying the diverse gut microbiota nutritional and ecological roles. Microbial metagenomics is a powerful tool that can rapidly reveal a vast microbial diversity profile by directly collecting the genetic materials from the gut. In conventional studies for more than 20 targets, the Sanger sequencing method, which is more costly and less time effective with low coverage, has provided low-throughput data that limited the metagenomics studies of gut microbiota. On the contrary, real-time quantitative PCR (qPCR) provides high accuracy and throughput on quantifying specific bacteria in gut microbiota. However, this method is specific that the least abundance to study such complex microbes communities in the gut. Thus, high-throughput next-generation sequencing (HT-NGS) was implied to provide the accurate census of a bacteria community by defining the type and microbial presence in the gut with their relative abundance (Liu et al., 2012).

HT-NGS is a convenient, rapid, and accurate tool to investigate the biological and ecological role of microbiota. Lozupone et al., 2012 found out in broilers, the number of microbes in the gut declined during antibiotic treatment or nutrient imbalance. Therefore, both richness and evenness of the microbial diversity are important to indicate the health of broilers. There are currently several HT-NGS platforms, in this study, the Illumina MiSeq platform was used for 16S metagenomic sequencing.

Based on Chapter 5, the supplemented corn cob extract, PrimaLac® probiotics and combination of both at different dosages on broiler growth performance were evaluated, where the supplemented diet of CC05, CC10, PRO and SYN05 resulted in significantly higher body weight gain during the finisher and overall day. Therefore, this chapter investigates the effect of supplemented corn cob extract, PrimaLac® probiotics, and a combination of both at different dosages on broilers gut microbiota. The ilea and caeca population of 5 targeted bacteria, including *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Clostridium perfringens* and *Salmonella* spp. were determined via real-time quantitative PCR (qPCR) microbiota on day 21 and 35 of broilers, where the whole microbiota population was determined via high-throughput sequencing of 16S rRNA gene amplicon. The study of both targeted bacteria and whole microbiota at different parts of the intestine is important to demonstrate the relationship between diet and microbial toward the growth performance of broilers.

6.2 Materials and Methods

6.2.1 Caeca and Ilea Collection

The collection of caeca and ilea on day 21 and 35 described in Section 5.2.6, where eight broilers per treatment; two broilers per replicate were collected to elucidate the ilea and caeca microbiota in broilers fed with six diets: (i) basal diet (BD) as control group; (ii) BD + 0.5% corn cob extract (CC05); (iii) BD + 1.0% corn cob extract (CC10); (iv) BD+ 0.1% probiotic (Primalac®) (PRO) ; (v) BD + 0.1% probiotic + 0.5% corn cob extract (SYN05); (vi) BD + 0.1% probiotic + 1.0 % corn cob extract (SYN10).

6.2.2 Genomic DNA Extraction

The bacterial genomic DNA was extracted from each ileum and caecum sample according to the manual of QIAampFast DNA Stool Mini Kit (Qiagen, Hilden, Germany) for pathogen detection with some modification to optimize the yield. The collected caeca and ilea content were weighed between 180-220 mg and treated with lysis buffer and incubated for 30 minutes at 37 °C to break down the Gram-positive bacteria cell wall that aided the genomic extraction. Each lysis

buffer were prepared fresh for every batch of extraction by mixing the following ingredients: 25 mg/mL lysozyme (Vivantis, Malaysia); 20 mM Tris-Cl (pH 8.0); 2 mM EDTA (pH 8.0); 1% Triton X-100. After incubation, 1 mL Inhibit buffer was added into each sample and vortexed continuously for 1 minute. The mixtures were again heated at 95 °C for 5 minutes followed by 10 seconds vortexed. The intestinal debris was removed by centrifuging at 16,100 × *g* for 1 minute. Next, 4 µL of 5 µg/mL RNase A (Epicentre, US) were added into the supernatant and incubated for 30 minutes at 37 °C to get rid of RNA. Subsequently, new 1.5 mL centrifuge tubes were prepared and 15 µL of proteinase K followed by pipetting 200 µL lysate and 200 µL of Buffer AL. The mixture were vortexed for 15 seconds and again incubated at 70 °C for 10 minutes. The precipitation of genomic DNA was aided by adding 200 µL of 100% ethanol (Fisher Scientific, US) into the lysate and vortex until the mixture was homogenized. QIAamp spin columns were prepared and labelled and 600 µL of lysate were added into the spin columns accordingly. The columns were centrifuged at 16,100 × *g* for 1 minute, followed by washing by adding Buffer AW1 and AW2. Solutions that resulted from the both buffer washing were discarded after centrifugation 16,100 × *g* for 1 and 3 minutes, respectively. Lastly, the genomic DNA were eluted by adding 100 µL of Buffer ATE directly onto the QIAamp membrane, incubated for 5 minutes and centrifuged at 16,100 × *g* for 1 minute. All the genomic DNA were stored in -20 °C freezer for subsequent study.

6.2.3 Genomic DNA Quantification

The extracted genomic DNA were quantified via Quantus Fluorometer (Promega, US) according to the Quantiflour ONE dsDNA system (Promega, US) manual. Each standard, blank, and samples were prepared by adding 1 µL of 400 mg/µL QuantiFlour ONE Lamda DNA, 1 µL of 1 × TE Buffer and 1 µL of unknown extracted genomic DNA sample into 199 µL of QuantiFlour ONE dsDNA Dye in separated 0.5 mL (Axygen, US) PCR tubes, respectively. Next, each mixture was vortexed for 1 minute, followed by 5 minutes incubation at room temperature (25 °C - 30 °C) in the dark room. Calibration of the Quantus Fluorometer was performed by using blank and standard. Lastly, the concentration of extracted genomic DNA samples were quantified and recorded accordingly.

6.2.4 Real-time Quantitative PCR (qPCR)

Total of five targeted gut microbes population which are *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Clostridium perfringens* and *Salmonella* spp. in ilea and caeca contents on day 21 and 35 were quantified in real-time qPCR. The *Escherichia coli*, *Clostridium perfringens* and *Salmonella* spp. were quantified as are common pathogens indicators in broilers, whereas PrimaLac® probiotic dietary treatment (PRO, SYN05, and SYN10) contained *Lactobacillus* spp., *Bifidobacterium* spp. that might affect the gut population.

6.2.4.1 Genomic DNA Extraction and PCR Amplification of Targeted Pure Culture

Genomic DNA of all five targeted bacteria were extracted according to DNeasy Blood and Tissue Kit (Qiagen, Germany) manufacturer instructions. *Lactobacillus* spp., *Bifidobacterium* spp. and *Clostridium perfringens* were cultivated in anaerobic conditions, whereas *Salmonella* spp. and *Escherichia coli* were in aerobic condition at 37 °C, overnight as mentioned in Chapter 3 (Section 3.2.10.1). The bacteria cells were collected by centrifugation at 5,000 × *g* for 10 min. ATL buffer (180 µL) were added into the pallet and resuspended. The total removal and lysis of protein were performed by adding 200 µL of Proteinase K and further incubated at 56 °C for 1 hour. A total of 200 µL of Buffer AL was added into the sample tube, followed by 200 µL of absolute ethanol for DNA precipitation. The sample was washed in DNeasy Mini Spin columns with 500 µL buffer AW1 and Buffer AW2 and centrifuged at 16,100 × *g* for 1 minute. Lastly, 100 µL of Buffer AE was added for the elution of genomic DNA. The genomic DNA were quantified as mentioned in Section 6.2.3.

The extracted genomic DNA of each bacteria was used as the template for PCR amplification. A total of 50 µL reaction mixture were prepared by mixing the components (iNtRON, Korea) listed in Table 6.1 using SureCycler 8800 (Agilent, US). The lists of reverse and forward primers for each bacteria were shown in Table 6.2. The initial denaturation was set at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at variable temperature for 1 minute, extension at 65 °C for 8 minutes and a final extension at 65 °C for 8 minutes, lastly the PCR product held at 4 °C.

Table 6.1: Reaction set-up for PCR.

Component	Volume per reaction (µL)	Final Concentration
10X iTaq Buffer	5.0	1X
50mM $MgCl_2$	1.5	1.5mM
10mM dNTPs	1.0	200µM
iTaq DNA Polymerase	0.25	1.25U
Reverse Primer	2	2µM
Forward Primer	2	2µM
Deionized sterile water	36.25	
DNA template	2	

6.2.4.2 Absolute Quantification of Ilea and Caeca Sample via Standard Curve Method

The absolute quantification via the standard curve method compares the unknown concentration of targeted bacteria in ilea and caeca genomic DNA samples to the respective standard curve and extrapolates a value. The standard

curve of *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Clostridium perfringens* and *Salmonella* spp. were first constructed from the amplicons of the bacteria as mentioned in Section 6.2.2. Each sample was quantified using Quantus Fluorometer (Section 6.2.3) and serially diluted accordingly, between 10^2 – 10^7 16S rDNA copy number (Appendix I) based on the formula (Staroscik, 2004):

$$\text{Number of copies} = \frac{\text{Amount of DNA } \left(\frac{\mu g}{mL}\right) \times 6.022 \times 10^{23}}{\text{Length (bp)} \times 10^9 \times 650}$$

The standard curves were plotted of quantification cycle (Cq) versus log 16S rDNA copy number (Appendix III). The known copy number of diluted samples were run along with the unknown samples for every run to ensure the amplification efficiency (E %) were between 95-99%, calculated from the formula:

$$\text{E \%} = [10^{(\frac{1}{\text{slope}})} - 1] \times 100$$

A total of eight genomic DNA samples isolated from the ilea and caeca on day 21 and 35 for each treatment (four cages with two broilers) were quantified for the real-time qPCR study using CFX96 Real-Time PCR Detection System (Bio-Rad, US). The 20 µL qPCR reaction mixture of SensiFAST SYBR No-ROX Kit (Bioline, UK) consisted of 10 L SensiFAST SYBR No-ROX mix, normalized genomic DNA template at 15 ng/µL, 1 L of forward and reverse primer each (10 pmol/µL) (Table 6.2) and sterile deionized distilled water (Milli-Q® Integral system, US). Both non-template control (NTC) and standard curve samples were included in every run to ensure no cross-contamination with high E%. Each sample was run in duplicate to minimize technical error. The initial denaturation was set at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 5 seconds, annealing at various temperatures (Table 6.2) and extension at 72 °C for 20 seconds. The specificity of amplicons was verified based on the melting curve analysis (Appendix II) by increasing 0.5 °C from 65 °C to 95 °C and hold for 5 seconds. The results were obtained by using Bio-Rad CFX Manager Version 3.1 (Bio-Rad, US).

Table 6.2: Lists of reverse and forward primers for each bacteria.

Targeted Bacteria	Forward (F) and Reverse (R) Primers	Amplicon size (bp)	Annealing temperature (°C)	Annealing time (seconds)	Reference
Lactobacillus spp.	(F) 5' AGC ACT AGG GAA TCT TCC A 3' (R) 5' CAC CGC TAC ACA TGG AG 3'	341	58	10	Walter et al. (2001)
Bifidobacterium spp.	(F) 5' GGG TGG TAA TGC CGG ATG 3' (R) 5' CCA CCG TTA CAC CGG GAA 3'	510	59	30	Kok et al. (1996)
Escherichia coli	(F) 5' GTT AAT ACC TTT GCT CAT TGA 3' (R) 5' ACC AGG GTA TCT AAT CCT GT 3'	340	60	30	Kim et al. (2011)
Clostridium perfringens	(F) 5' AAA GAT GGC ATC ATC ATT CAA C 3' (R) 5' TAC CGT CAT TAT CTT CCC CAA A 3'	279	53	30	Wang et al. (1994)
Salmonella spp.	(F) 5'TCG TCA TTC CAT TAC CTA CC 3' (R) 5'AAA CGT TGA AAA ACT GAG GA 3'	119	49.3	30	Rezaei et al. (2015)

6.2.5 PCR Amplification of V3-V4 Region of 16S rRNA Gene via High-throughput Next-generation Sequencing (HT-NGS)

Previously, the extracted genomic DNA (Section 6.2.2) of caeca and ilea at 21 and 35-day broilers, which four replicate cages per treatment (two broilers per cage) were randomly selected. The 16S rRNA gene sequencing libraries were prepared via the amplification of V3-V4 hypervariable region. The forward and reverse primer (Integrated DNA Technologies (IDT), Singapore) referred to Klindworth et al. (2013) study with some modification, where the underlined N were the four degenerated bases to maximize the diversity for unique clusters identification.

i. Forward primer: 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN CCT ACG GGN GGC WGC AG-3'
ii. Reverse primer: 5'-GTC TCG TGG GCT ATA AGA GAC AGG ACT ACH VGG GTA TCT AAT CC-3'

The diversity of unique cluster identification can be enhanced by inserting four degenerate bases (N). A total of 50 µL PCR were prepared using NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolab (NEB), US) as shown in Table 6.3. The master mix consisted of 2 mM $MgCl_2$, 200 µM of deoxynucleotide triphosphates (dNTPs), and DNA polymerase. The PCR cycles were set using SureCycler 8800 Thermal Cycler (Agilent, US), including initial denaturation at 98 °C for 30 seconds, 25 cycles of 98 °C for 10 seconds, 60 °C for 10 seconds, 65 °C for 30 seconds, and finally extension step at 65 °C for 5 minutes. The amplicons with a product size of 469 bp were confirmed by electrophoresis on 1.5% agarose gel. Lastly, the concentration of amplicons was determined using Quantus Fluorometer (Section 6.2.3).

Table 6.3: PCR reaction mixtures.

Component	Volume per reaction (µL)	Final Concentration
NEBNext Q5 Hot Start HiFi PCR Master Mix	25	1X
Reverse Primer	5	10µM
Forward Primer	5	10µM
DNA template	15	10ng

Before second PCR amplification were performed, purification steps were carried out via E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, US), following the manufacturer's instructions. The PCR amplicon samples were transferred into a new 1.5 mL microcentrifuge tube together with five volumes of CP buffer. The mixture was vortexed for 30 seconds and centrifuged briefly to collect the drops inside the lid. The mixture was pipetted into a HiBind® DNA Mini Column provided and inserted into 2 mL collection tube. After centrifuged at 16,100 × g for 60 seconds at 37 °C, the filtrate was discarded. Next, 700 L DNA Wash Buffer (diluted with 100% ethanol) were added into the collection tube and centrifuged

again. The washing steps were repeated. An additional centrifugation step was performed for 2 minutes in order to get rid of trace ethanol that will affect the downstream application. The HiBind® DNA Mini column was transferred into a sterilized 1.5 mL microcentrifuge tube. The purified amplicon was collected by adding 50 µL of Elution Buffer directly to the center of the column matrix and sited for 2 minutes, lastly centrifuged at 16,100 × g for 60 seconds. The purified amplicons were stored in -20 °C before sent in for next-generation sequencing.

Subsequently, the high-quality library was produced in Monash University Malaysia Genomic Facility. Nextera XT (Illumina, US) sequencing adapters and 8-index unique barcodes were added to the PCR amplicons. The amplification cycles were conditioned at 98 °C (30 s) for initial denaturation, followed by eight cycles of extension step at 98 °C (10 s), and 65 °C (75 s) and 65 °C (2 min) for the final extension. The amplicons were again purified via 0.8 volumes of Agencourt AMPure XP bead x0.8 (Beckman Coulter, US) and lastly washed with 80% ethanol. Electrophoresis of the amplicon product were performed and purified based on E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, US) manufacturer instruction. The amplicons were then quantified using Qubit Fluorometer and normalized to 2 nM. Next, the amplicons were pooled and sequenced and conditioned at paired 300 bp reads using Miseq Reagent Kit v3 (600 cycle) (Illumina, US).

6.2.6 Trimming and Merging of Illumina Pair-end Reads for Bioinformatics Analysis

The Illumina Miseq sequencing generated FASTQ formatted files were then analyzed using the mother software package (v.1.38.1) (Schloss et al., 2009). The trimming and merging were done according to the Miseq SOP as provided in the updated website (https://www.mothur.org/wiki/MiSeq_SOP) (Kozich et al., 2013).

6.2.7 Bioinformatics Analysis

The trimmed microbiome data were then analyzed using Microbiome Analyst, which is a comprehensive online software. The analysis provided including visual, meta and also statistical analysis. The microbiome data were uploaded together with Operational Taxonomic Unit (OTU) and taxonomy table using Greengenes as references. Next, the data were scanned for integrity check in order to exclude identical values across the samples or unique data that only resulted in one sample, which is a possible error caused by techniques, equipment or conditions. The subsequent low count (at least 10% of its values should contain a minimum of one count) and low variance (interquartile range of 5%) data filtration were aimed to omit low quality or ambiguous data for better downstream statistical analysis. Lastly, normalization of data was performed using the cumulative sum scaling (CSS) with no data rarefying and transformation. The further analysis of the processed microbiome data are

divided into two major analysis including alpha and beta-analysis. The alpha analysis grouped the gut microbial accordingly into defining taxonomy groups (phylum, class and family). Subsequently, the beta analysis which is the similarities of gut microbial communities were plotted via principal coordinate analysis (PCoA) based on the Bray-Curtis similarity index. The phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) 1.1.4 were performed to analyze the functional potential of caeca microbiota (Langille et al., 2013) using the OTU table generate which were uploaded and processed using the online Galaxy terminal (http://huttenhower.sph.harvard.edu/galaxy/). The functional prediction OTU of the gene were compared between treatments based on KEGG orthologs functions and further analyzed via STAMP software package (Parks et al., 2014).

6.2.8 Statistical Analysis

The real-time PCR results were plotted using each broiler as an individual experimental unit by all parameters to obtain the means ± standard error. The Duncan's multiple range test (Duncan, 1955) was used to compare the means within samples which significant $P < 0.05$. The alpha diversity (richness and diverseness) of the gut microbial samples were compared via One-way analysis of variance (ANOVA) using SPSS software version 22 (IBM, US). The vegan package R Studio version 2.4-1 were commanded to plot the rarefaction curve (Oksanen et al., 2016). The significant difference among the treatment group of principle coordinate analysis (PCoA) was measured using PERMANOVA via R studio (Paulson, 2014). The R studio package of Storey's False Discovery Rate (FDR) multiple test correction generates p-values for pairwise and multiple comparisons of predicted functional metagenomes.

6.2.9 Nucleotide Sequence Accession Numbers

The BioSample Accession Numbers (PRJNA509303) are V3-V4 region of 16s rRNA gene sequences from the study that deposited in the National Center for Biotechnology Information (NCBI) sequence (https://submit.ncbi.nlm.nih.gov/sra).

6.3 Results

6.3.1 Real-time Quantification PCR (qPCR) Standard Curve of Targeted Gut Microbial Populations

Real-time detection of PCR products is enabled by the inclusion of a fluorescent reporter molecule in each reaction well that yields increased fluorescence with an increasing amount of product DNA. The fluorescence chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-

specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence signal as amplification occurs. The measured fluorescence is proportional to the total amount of amplicon; the change in fluorescence over time is used to calculate the amplicon produced in each cycle.

The number of *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Clostridium perfringens*, *Salmonella* spp. was investigated by using real-time qPCR. The specific primers of each targeted bacteria (Table 6.2) with the inclusion of fluorescence molecules enable to report of the signals during the amplification of DNA. The measured fluorescence is directly proportional to the total copy number (Cq) of amplicons. The standard curve of each targeted bacteria was generated with known starting Cq with ten-fold dilution to obtain the slope equations accordingly (Appendix I and Appendix III). The known number of copies for *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Clostridium perfringens*, *Salmonella* spp. were 8.37+E9, 6.01+E9, 5.29+E9, 1.11+E10 and 2.30+E10, respectively. Thus, the starting Cq of each targeted bacterium in the collected sample can be determined via calculation according to the respective slope equation (Table 6.4).

Table 6.4: Standard curve of the targeted gut bacteria for real-time PCR assay.

Target Bacteria	Slope Equation	PCR efficiency (E %)	Correlation coefficient (R^2)
Lactobacillus spp.	y= -3.242 x + 37.873	104.5	0.999
Bifidobacterium spp.	y= -3.283 x + 35.395	101.7	0.990
Escherichia coli	y= -3.508 x + 40.658	92.8	0.998
Clostridium perfringens	y= -3.538 x + 42.839	91.7	0.997
Salmonella spp.	y= -3.585 x + 52.221	90.1	0.997

The standard graph of each bacteria can refer to Appendix III.

On day 21, there were no significant differences between dietary treatments for *Bifidobacterium* spp. population (Figure 6.1) in caeca. The 1.0% corn cob (CC10) supplemented diet has a significantly ($P < 0.05$) higher *Lactobacillus* spp. and lower *E. coli* population than the basal diet (BD). The SYN10 diet has a significantly ($P < 0.05$) lower *C. perfringens* population compared to BD and other supplanted diets. The population of *Salmonella* spp. is significantly ($P < 0.05$) higher with PRO diet compared to BD.

On day 35, the caeca *Bifidobacterium* spp. population (Figure 6.2) with CC10 supplemented diet are significantly ($P < 0.05$) higher compared to the basal group. The *C. perfringens* population was significantly ($P < 0.05$) lower in the

PRO supplement diet compared to the basal group. There are no significant differences between dietary treatments for the *Lactobacillus* spp., *E. coli* and *Salmonella* spp. population on day 35 in caeca. . In overall, the caeca population fed with CC10 diet significantly ($P < 0.05$) enhanced the *Lactobacillus* during the starter period and *Bifidobacteria* during the finisher period, as well as suppressed the growth of *E. coli*.

On day 21, the ilea microbial population (Figure 6.3) resulted that both PRO and SYN10 supplemented diet had significantly higher ($P < 0.05$) *Lactobacillus* spp. population compared to the basal diet. All supplemented groups had no significant difference of *Bifidobacterium* spp. population compared to basal diet, except SYN10 supplemented diet which is significantly lower ($P < 0.05$). The population of *E. coli* was ($P < 0.05$) lowest in SYN10, follow by PRO, and CC10 and CC05. The population of *C. perfringens* showed significantly ($P < 0.05$) lower in the CC05 supplemented diet compared with the basal group. PRO supplemented diet also resulted in the reduction of *Salmonella* spp. population (6.4 $\log_{10}$ DNA copy number).

On day 35, the ilea microbial population (Figure 6.4) resulted in both population of *Lactobacillus* spp. and *Bifidobacterium* spp. with all supplemented diet were significantly ($P < 0.05$) enhanced, whereas *E. coli* population was significantly ($P < 0.05$) lower compared to the basal diet. Conversely, the population of *C. perfringens* and *Salmonella* spp. did not have any significant differences among the diets. In overall, on day 21, the ilea population fed with PRO diet significantly ($P < 0.05$) enhanced the population of *Lactobacillus* and reduced the population of *E. coli* and Salmonella. On day 35, all supplemented diets significantly ($P < 0.05$) enhanced the population of *Lactobacillus* and *Bifidobacterium* and suppressed the population of *E. coli*.

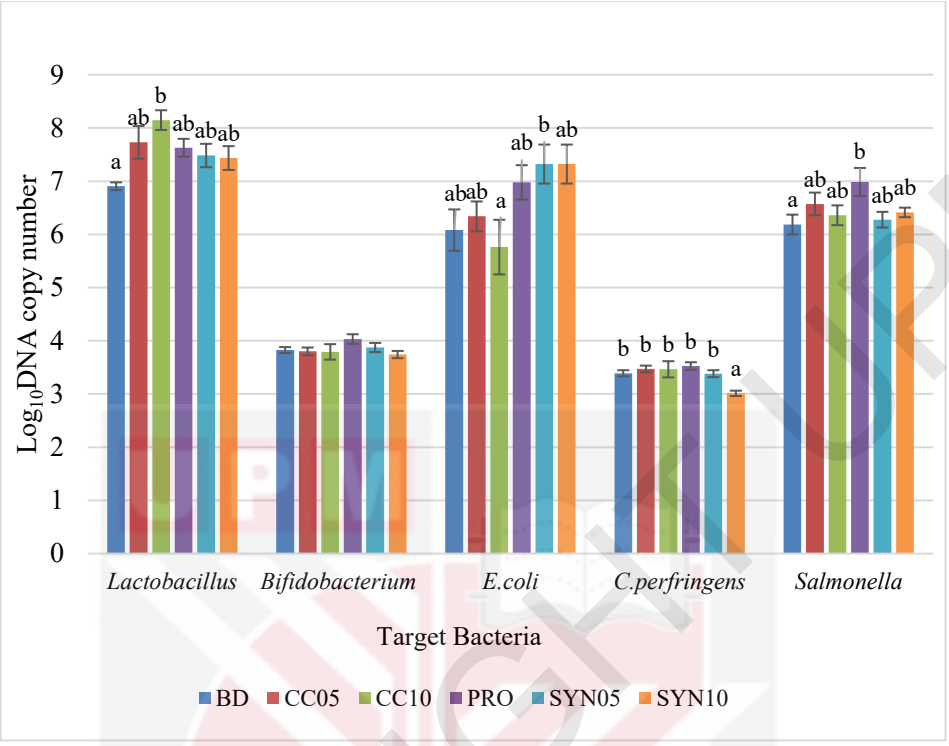


Figure 6.1: Effects of dietary treatments of corn cob extract, PrimaLac® probiotics and combination of both at different dosage on gut microbial populations of *Lactobacillus* spp., *Bifidobacterium* spp., *E. coli*, *C. perfringens* and *Salmonella* spp. in caeca on day 21.

Each bar is the mean of 4 replicate cages with 2 broilers each.

Error bar represents standard error.

[a-b]Bars that have different superscripts differ significantly ($P < 0.05$). [1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

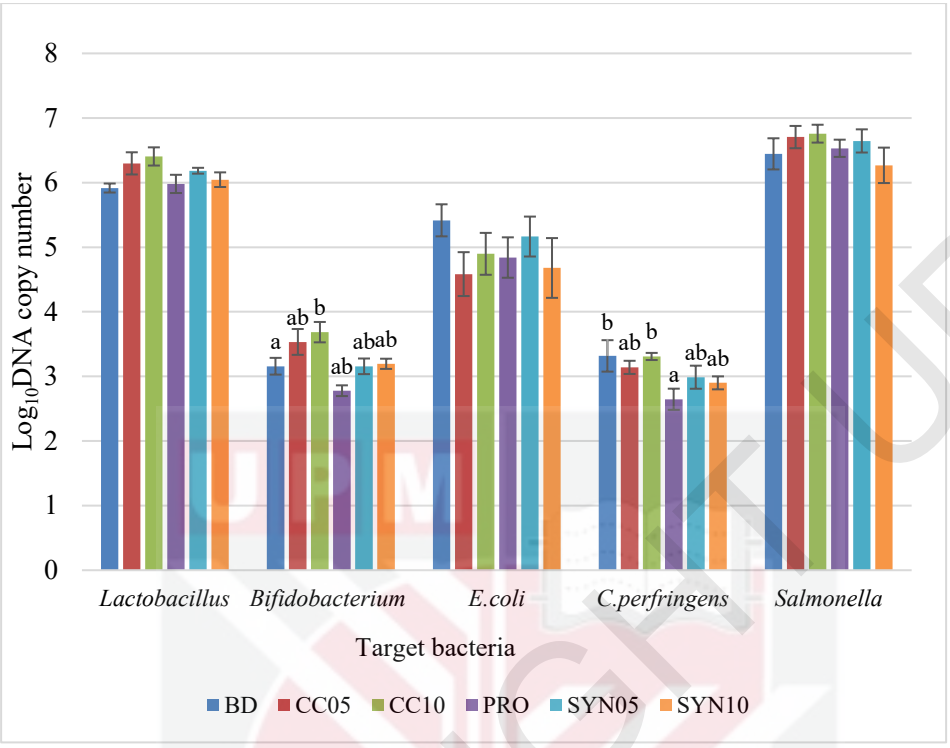


Figure 6.2: Effects of dietary treatments of corn cob extract, PrimaLac® probiotics and combination of both at different dosage on gut microbial populations of *Lactobacillus* spp., *Bifidobacterium* spp., *E. coli, C. perfringens* and *Salmonella* spp. in caeca on day 35.

Each bar is the mean of 4 replicate cages with 2 broilers each.

Error bar represents standard error.

[a-b]Bars that have different superscripts differ significantly ($P < 0.05$). [1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

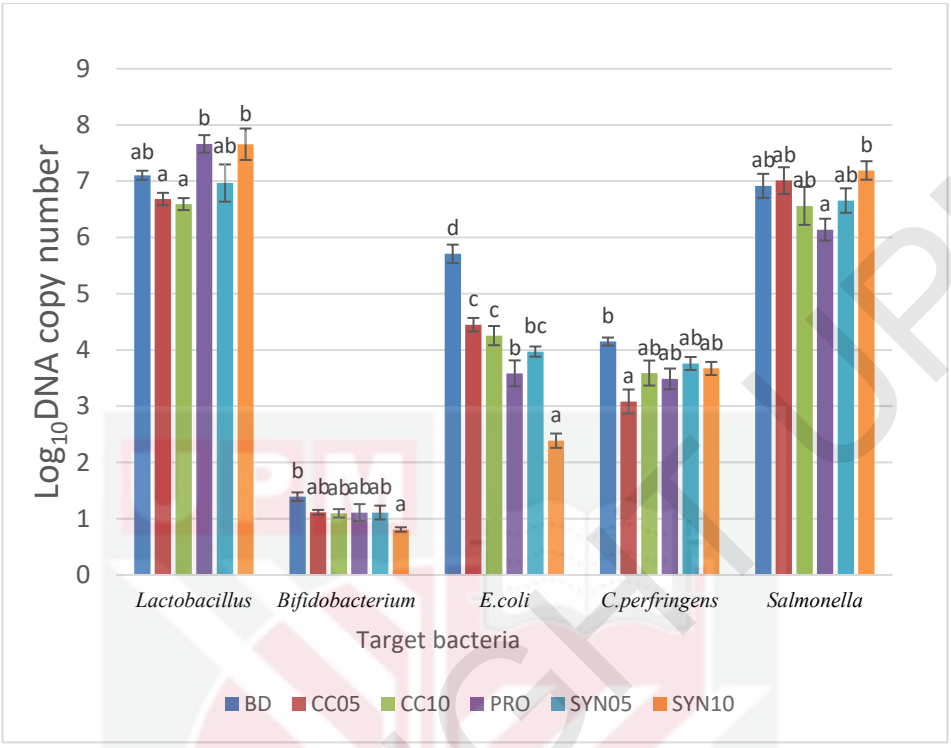


Figure 6.3: Effects of dietary treatments of corn cob extract, PrimaLac® probiotics and combination of both at different dosage on gut microbial populations of *Lactobacillus* spp., *Bifidobacterium* spp., *E. coli, C. perfringens* and *Salmonella* spp. in ilea at 21 days.

Each bar is the mean of 4 replicate cages with 2 broilers each.

Error bar represents standard error.

[a-b]Bars that have different superscripts differ significantly ($P < 0.05$). [1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

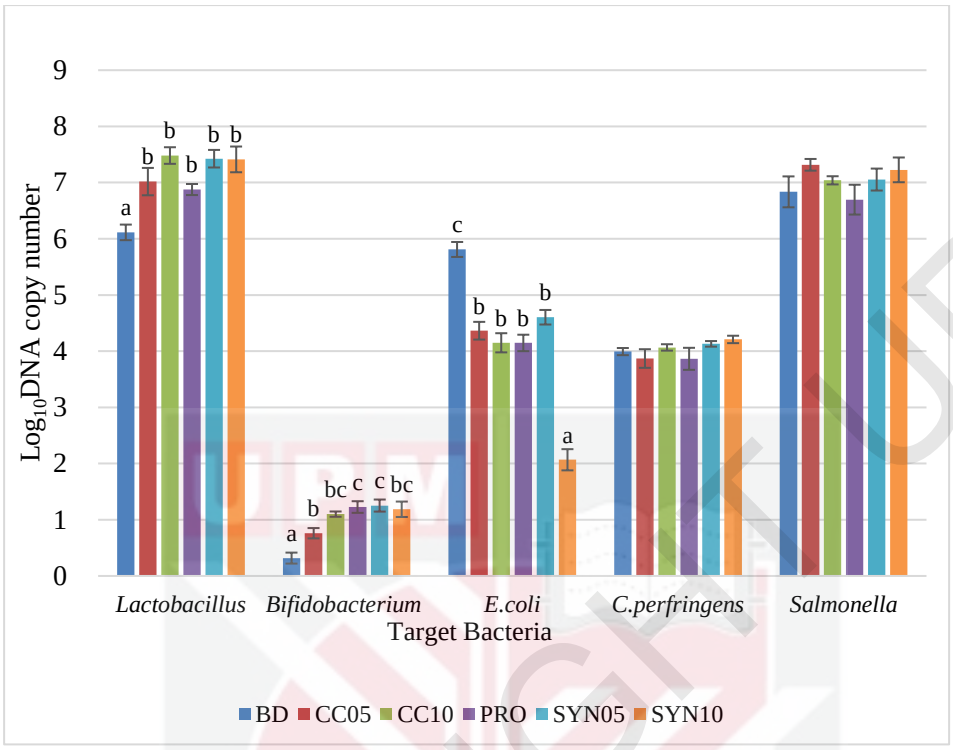


Figure 6.4: Effects of dietary treatments of corn cob extract, PrimaLac® probiotics and combination of both at different dosage on gut microbial populations of *Lactobacillus* spp., *Bifidobacterium* spp., *E. coli*, *C. perfringens* and *Salmonella* spp. in ilea on day 35.

Each bar is the mean of 4 replicate cages with 2 broilers each.

Error bar represents standard error.

[a-b]Bars that have different superscripts differ significantly ($P < 0.05$). [1]BD= basal diet; CC05 = basal diet + 0.5% corn cob; CC10 = basal diet + 1.0 % corn cob; PRO = basal diet + 0.1 % probiotic (PrimaLac®); SYN05 = basal diet + 0.1 % probiotic + 0.5% corn cob; SYN10 = basal diet + 0.1% probiotic + 1.0% corn cob.

6.3.2 Alpha Diversity of the Gut Microbiota Based on Illumina Sequencing of 16S rRNA Gene

The final filtered and processed sequences were comprised of 12,481,451 sequences with a mean length of 459 bp. Among them, 3,640,069 were unique sequences followed by clustered based on 97% homology cut-off, a total of 1586 OTUs were acquired. Furthermore, normalization of the resulted dataset to 6055 sequences, with an average of 99% sequence coverage (Table 6.5, Table 6.6, and Figure 6.5). Therefore, most of the bacteria populations in this study were covered in depth by the NGS in the samples. The Shannon and Simpson method simplified the complex microbial population into two components, which are the species richness and their relative abundance in broiler gut (Kim et al., 2017; Stanley et al., 2016). Based on the comparison of the values between Table 6.5

and Table 6.6, ilea has a much lower microbial species richness and diversity compared to caeca. Apparently, the richness and diversity of ilea microbiota were not affected by the supplemented diets. The caeca microbiota were affected on 35 days, with 0.5% corn cob extract and 1% probiotic (SYN05) diet resulted in significantly ($P < 0.05$) higher bacteria richness (ACE, Chao1, and Sobs) than other treatments in caeca on 35 days (Table 6.6). In addition, the bacterial diversity reflected in Fisher also shown a significant ($P < 0.05$) increase with SYN05 diet compared to the basal group.

Table 6.5: The broilers' ilea microbial species richness and diversity of measured number of observed OTUs (Sobs), ACE, Chao1, Fisher, Shannon, and Simpson from sequences that were normalized to 6055.

Group	ACE	Chao1	Sobs	Fisher	Shannon	Simpson
21I_BD	21.34 ± 8.53	21.26 ± 8.46	21.25 ± 8.45	2.61 ± 1.19	0.51 ± 0.21	0.21 ± 0.09
21I_CC05	40.61 ± 15.68	40.50 ± 15.71	40.50 ± 15.71	5.58 ± 2.46	0.95 ± 0.21	0.40 ± 0.09
21I_CC10	57.50 ± 23.82	57.50 ± 23.82	57.50 ± 23.82	8.38 ± 3.74	0.80 ± 0.29	0.27 ± 0.09
21I_PRO	14.50 ± 2.80	14.50 ± 2.80	14.50 ± 2.80	1.60 ± 0.36	0.52 ± 0.18	0.26 ± 0.09
21I_SYN05	37.68 ± 12.06	37.52 ± 12.02	37.50 ± 12.01	5.05 ± 1.83	0.95 ± 0.18	0.42 ± 0.07
21I_SYN10	32.54 ± 20.82	32.38 ± 20.79	32.38 ± 20.79	4.81 ± 3.60	0.61 ± 0.32	0.22 ± 0.09
35I_BD	36.41 ± 12.76	36.25 ± 12.76	36.25 ± 12.76	5.17 ± 2.06	1.11 ± 0.18	0.51 ± 0.06
35I_CC05	15.63 ± 2.24	15.63 ± 2.24	15.63 ± 2.24	1.66 ± 0.22	0.62 ± 0.13	0.31 ± 0.08
35I_CC10	14.68 ± 1.66	14.63 ± 1.65	14.63 ± 1.65	1.65 ± 0.21	0.58 ± 0.09	0.28 + 0.05
35I_PRO	18.14 ± 3.05	18.14 ± 3.05	18.14 ± 3.05	2.14 ± 0.44	0.68 ± 0.14	0.32 + 0.08
35I_SYN05	39.74 ± 16.42	39.63 ± 16.39	39.63 ± 16.39	5.11 ± 2.41	0.63 ± 0.15	0.25 + 0.06
35I_SYN10	54.92 ± 20.78	54.75 ± 20.74	54.75 ± 20.74	7.27 ± 3.31	1.12 ± 0.20	0.47 + 0.05

Each value is mean ± standard error of 4 replicate cages with 2 chickens each.
[ab] Means within the same column that have different superscripts differ significantly ($P < 0.05$).
For treatment (BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO), age (21= 21-day-old; 35=35-day-old) and part of intestine (I=ilea). Sobs= number of observed OTUs.

Table 6.6: The broilers' caeca microbial species richness and diversity of measured number of observed OTUs (Sobs), ACE, Chao1, Fisher, Shannon, and Simpson from sequences that were normalized to 6055.

Group	ACE	Chao1	Sobs	Fisher	Shannon	Simpson
21C_BD	205.68 ± 16.56^{a}	205.50 ± 16.54^{a}	205.50 ± 16.54^{a}	31.32 ± 2.51^{a}	3.76 ± 0.10	0.95 ± 0.01
21C_CC05	208.31 ± 16.94^{a}	208.13 ± 16.93^{a}	208.13 ± 16.93^{a}	32.68 ± 2.87^{a}	3.57 ± 0.14	0.93 ± 0.01
21C_CC10	235.97 ± 10.59^{a}	235.88 ± 10.59^{a}	235.88 ± 10.59^{a}	37.43 ± 1.67^{ab}	3.80 ± 0.09	0.95 ± 0.01
21C_PRO	198.89 ± 23.82^{a}	198.75 ± 23.86^{a}	198.75 ± 23.86^{a}	30.58 ± 3.93^{a}	3.52 ± 0.13	0.93 ± 0.01
21C_SYN05	201.91 ± 16.34^{a}	201.88 ± 16.32^{a}	201.88 ± 16.32^{a}	31.08 ± 2.86^{a}	3.70 ± 0.11	0.95 ± 0.01
21C_SYN10	215.39 ± 21.57^{a}	215.25 ± 21.53^{a}	215.25 ± 21.53^{a}	33.52 ± 3.59^{a}	3.77 ± 0.09	0.95 ± 0.01
35C_BD	257.96 ± 15.91^{ab}	257.75 ± 15.91^{ab}	257.75 ± 15.91^{ab}	40.22 ± 2.65^{ab}	3.71 ± 0.05	0.94 ± 0.00
35C_CC05	257.55 ± 14.57^{ab}	257.38 ± 14.55^{ab}	257.38 ± 14.55^{ab}	42.52 ± 2.54^{ab}	3.84 ± 0.08	0.94 ± 0.00
35C_CC10	259.15 ± 13.98^{ab}	258.88 ± 14.05^{ab}	258.88 ± 14.05^{ab}	40.70 ± 1.93^{ab}	3.77 ± 0.08	0.94 ± 0.01
35C_PRO	262.09 ± 15.05^{ab}	262.00 ± 15.05^{ab}	262.00 ± 15.05^{ab}	41.30 ± 2.38^{ab}	3.72 ± 0.03	0.94 ± 0.00
35C_SYN05	317.13 ± 16.18^{b}	317.00 ± 16.18^{b}	317.00 ± 16.18^{b}	48.60 ± 2.37^{b}	3.77 ± 0.07	0.93 ±0.01
35C_SYN10	260.07 ± 9.84^{ab}	259.74 ± 9.80^{ab}	259.71 ± 9.79^{ab}	41.00 ± 1.73^{ab}	3.68 ± 0.08	0.93 ± 0.01

Each value is mean ± standard error of 4 replicate cages with 2 chickens each.
^{ab} Means within the same column that have different superscripts differ significantly ($P < 0.05$).
For treatment (BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO), age (21= 21-day-old; 35=35-day-old) and part of intestine (I=ilea). Sobs= number of observed OTUs.

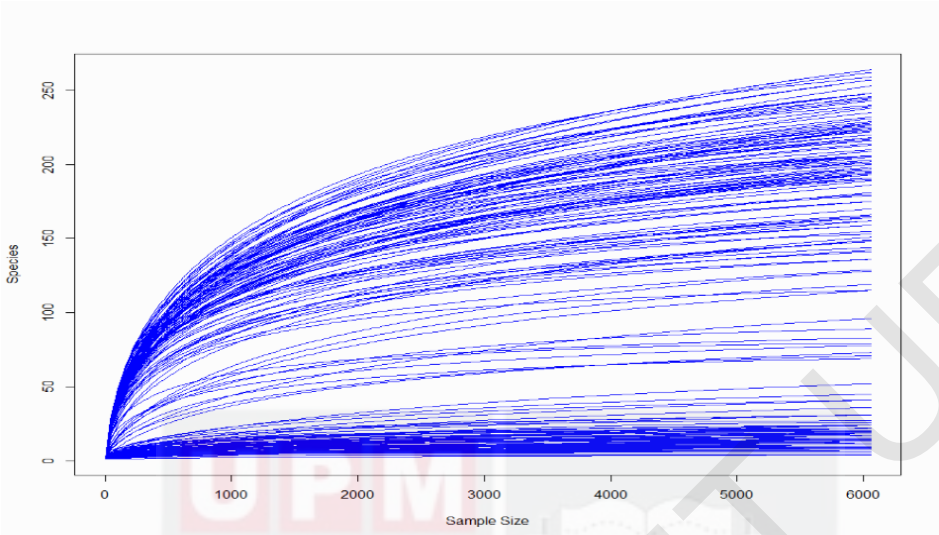


Figure 6.5: Rarefaction curves of species (OTUs) versus sample size (number of sequences) plotted at 99% sequences identity.
A total of 192 samples (6 treatments x 8 replicates x 2 part of intestine x 2 time point) were normalised to 6055 sequences per sample.
The samples were sufficiently sequenced based on the plots that reached a plateau towards the end.

6.3.3 Total Microbiome Diversity in Broilers' Gut

In this study, taxonomic characterization of both caeca (Figure 6.6) and ilea (Figure 6.7) bacteria communities was based on the relative abundance of OTUs. All treatments were compared to the control group (basal diet). Figure 6.6 showed the overall caeca bacteria diversity in broilers, which majority of the caeca bacteria belongs to phylum *Firmicutes* (58.65%), followed by *Bacteroidetes* (35.98%) and unclassified bacteria (4.25%). On the contrary, in ilea, there are significant ($P < 0.05$) higher phylum *Firmicutes* which is 98.94% and only 0.90% of *Bacteroidetes* (Figure 6.7). The phylum *Firmicutes* in caeca were dominated by the genus *Clostridia* (86.10%), followed by *Bacilli* (9.20%). However, in ilea, the phylum *Firmicutes* were dominated by 96.5% of genus *Bacilli* and only 3.5% of *Clostridia*. Based on both relative percentage results in caeca and ilea, ~95% of the microbiome diversity in the broiler gut was represented by phyla *Firmicutes* and *Bacteroidetes.*

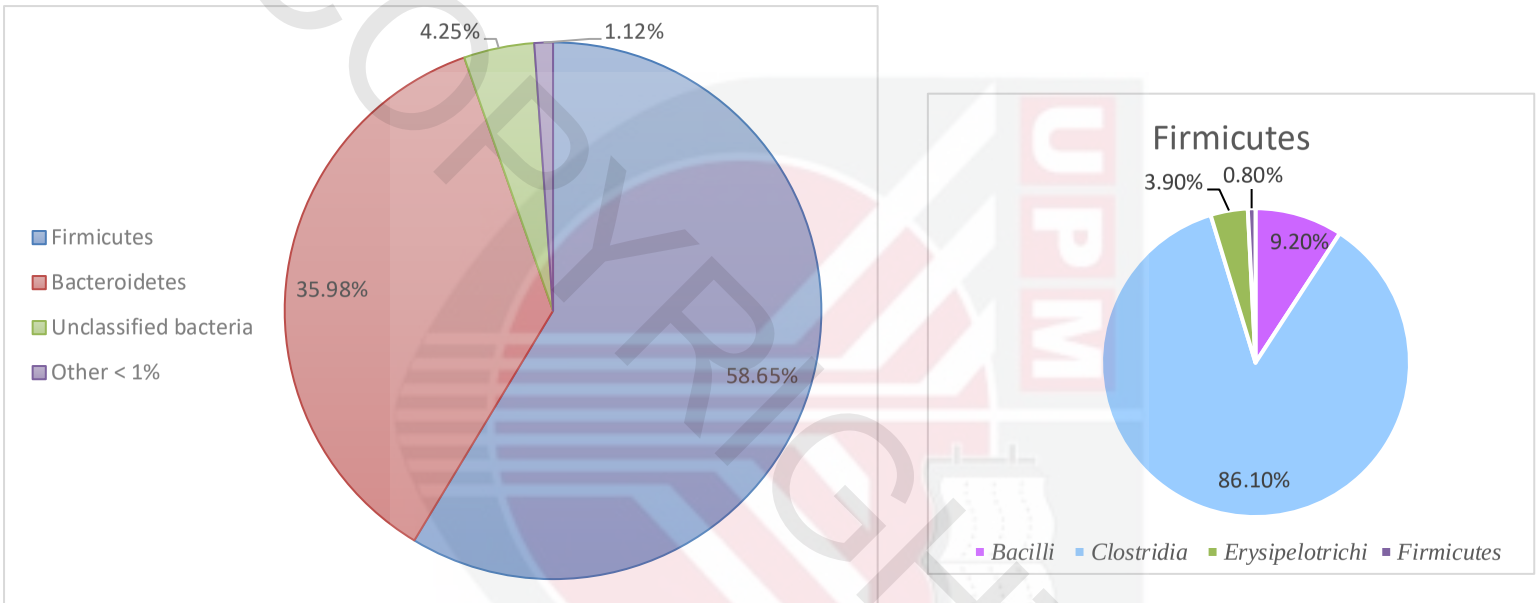


Figure 6.6: The overall total microbiome diversity in caeca based on 6 broilers of basal diet (control) treatment groups at phylum and class level.

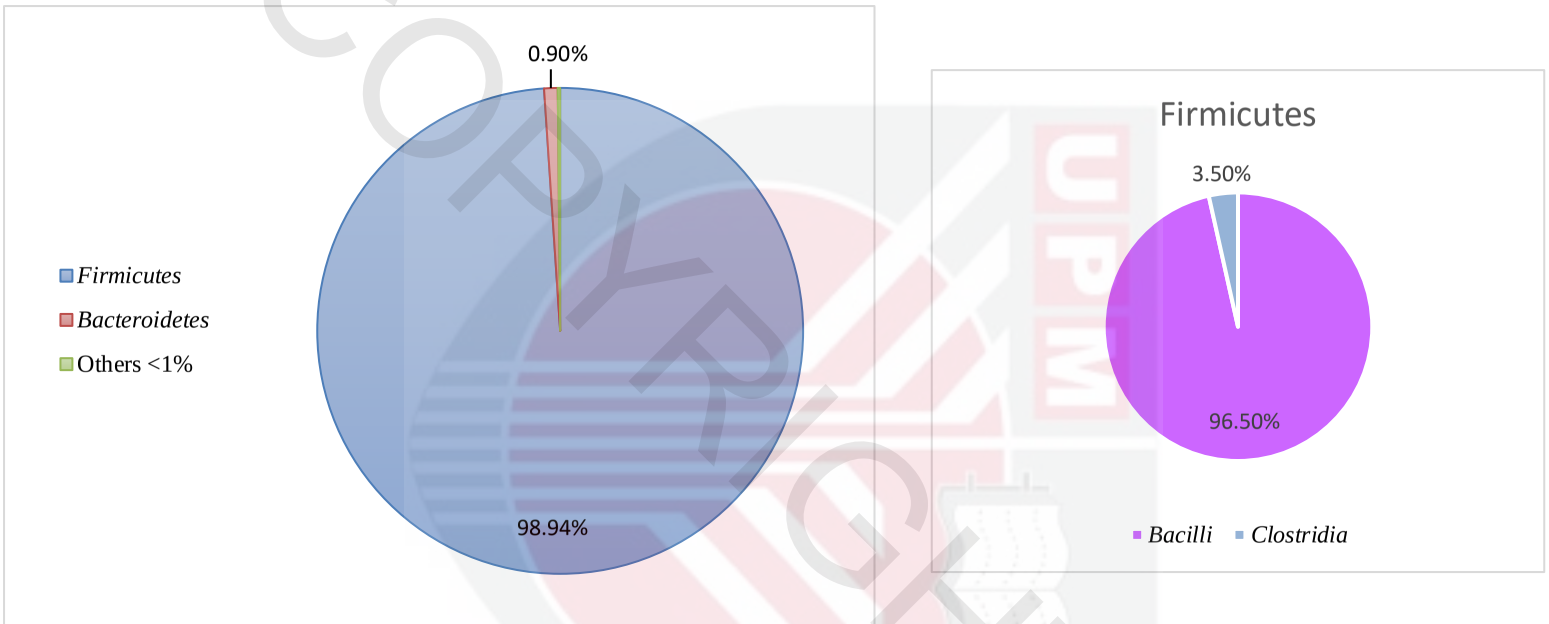


Figure 6.7: The relative percentage microbiome diversity in ilea based on 6 broilers of basal diet (control) treatment groups at phylum and class level.

6.3.3.1 Broilers' Gut Microbiota Relative Abundance in Control Group (Basal Diet) at 21 Day and 35 Day

The shifting pattern of caeca's and ilea's bacteria communities at lower taxonomical levels was performed to determine the distribution of different bacteria in control (basal diet) broilers (Figure 6.8 and Figure 6.9).

At 21 days caeca's bacteria communities were ~80% dominated by phylum *Firmicutes* followed by *Bacteroidetes* (19.04%). The *Firmicutes* are mainly composed of order *Lachnospiraceae* (class *Clostridia*, order *Clostridiales*). However, on day 35, the caeca's bacteria communities were composed of 3 phyla which were *Bacteroids*, *Firmicutes*, and *Proteobacteria*. Both phyla *Bacteroids* and *Firmicutes* shared similar abundance, 48.96% and 40.34%, respectively. *Ruminococcacea* (phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*) has a higher quantity compared to *Rikenellaceae* (phylum *Bacteroidetes*, class *Bacteroidia*, order *Bacteroidales*).

The ilea's bacteria communities at 21 days, ~99% of phylum *Firmicutes* were dominated by genus *Lactobacillus* (class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*). The remaining bacteria were classified in phylum *Bacteroidetes*. On day 35, 6.46% of the genus *Lactobacillus* abundance had dropped compared to day 21.

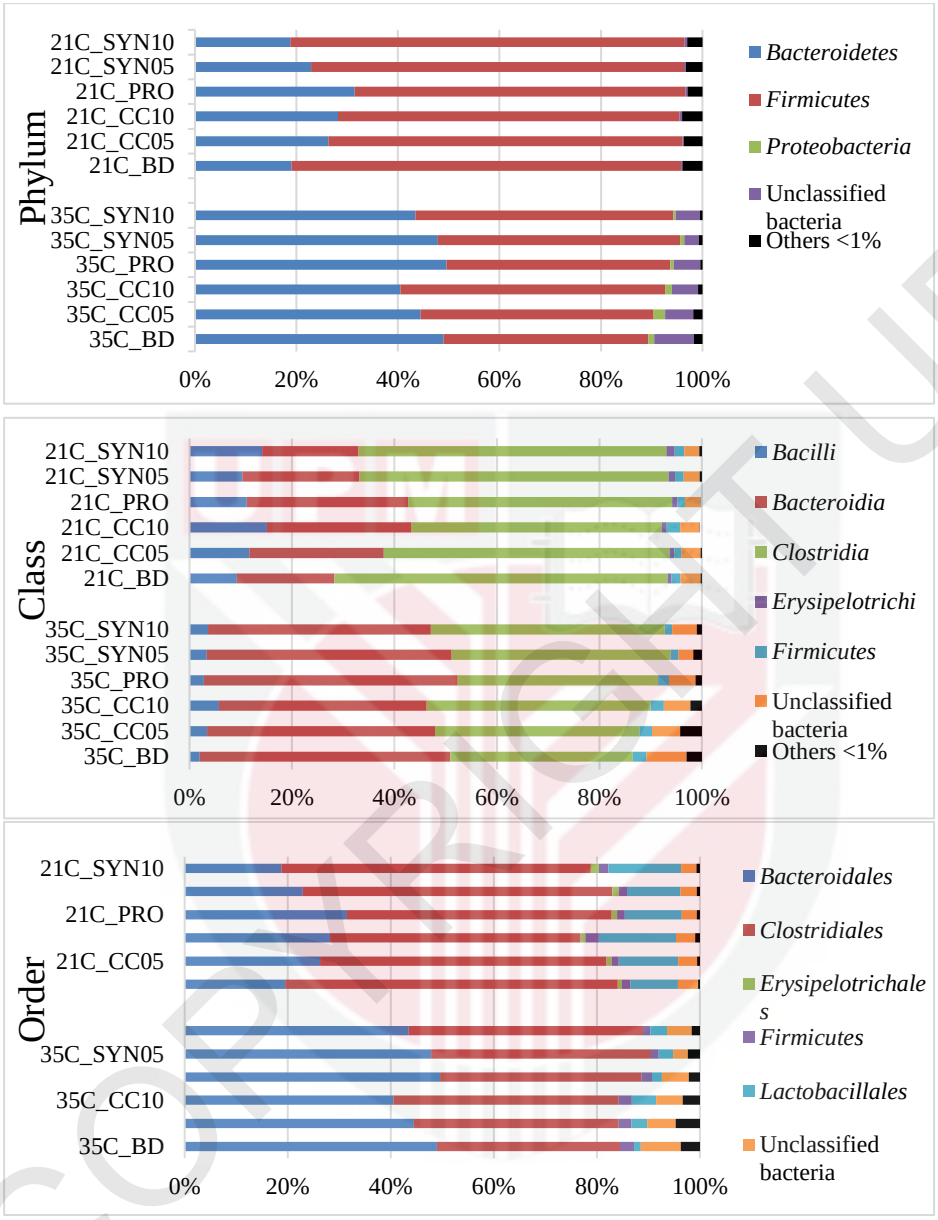


Figure 6.8: The relative abundance of caeca bacteria at phylum, class and order level for each diet treatment group at 21 and 35 days-old broilers. Each data is mean ± standard error of 4 replicate cages with 2 broilers each. The diet treatments including BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO.

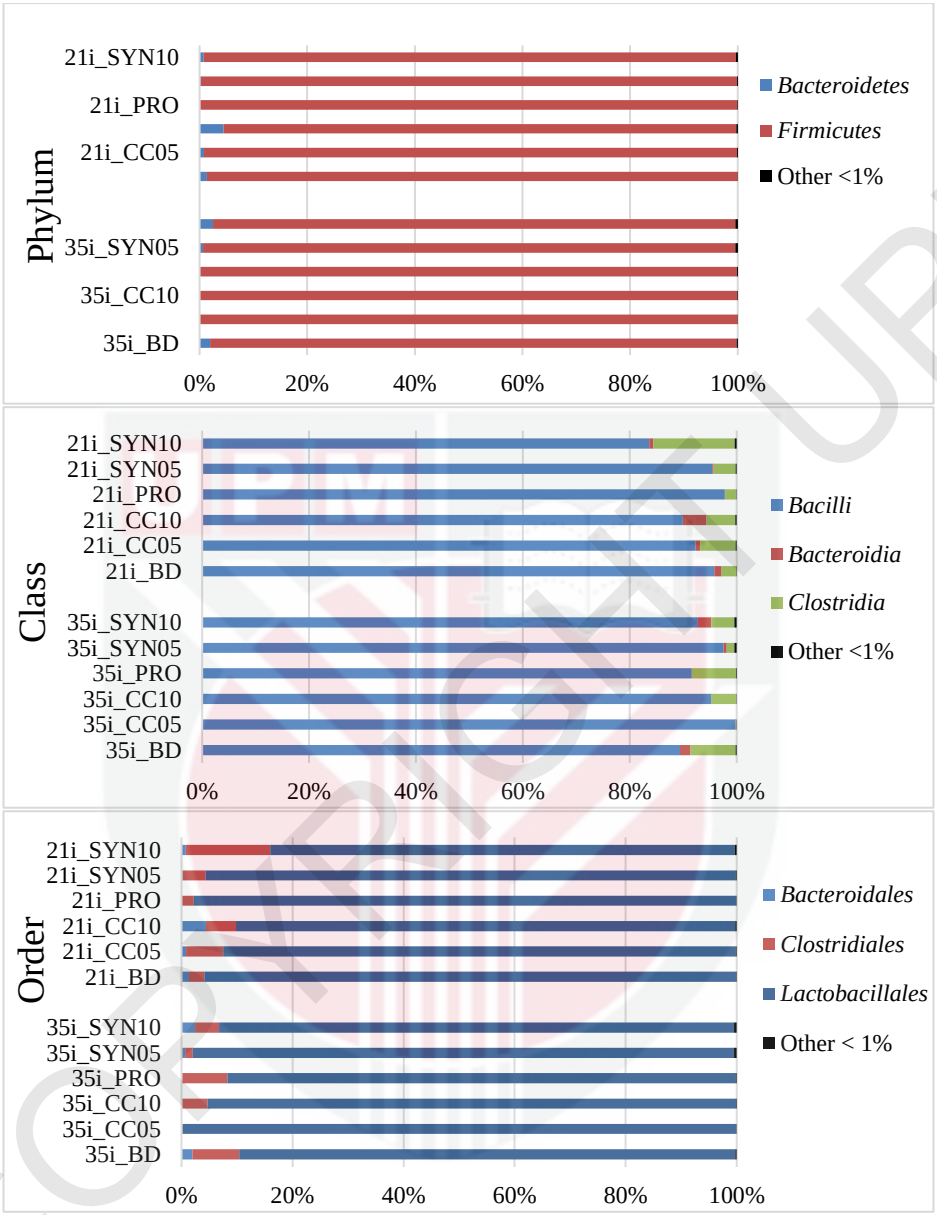


Figure 6.9: The relative abundance of ilea bacteria at phylum, class and order level for each diet treatment group at 21 and 35 days-old broilers. Each data is mean ± standard error of 4 replicate cages with 2 broilers each. The diet treatments including BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO.

6.3.3.2 Comparison of Broilers' Gut Microbiota Relative Abundance among Treatment at 21 Day and 35 Day

The comparison of bacteria communities between treatments from day 21 to day 35 with an OUT definition at a similarity cut-off of 97% were analysed for significant enhancement or reduction in treatment based on the abundance in caeca and ilea (Figure 6.8, Figure 6.9, and Figure 6.10).

Overall, the bacteria communities in all treatment diet resulted significant ($P < 0.05$) enhancement of 0.44% - 2.2.26% in phyla *Proteobacteria* and 18.01% - 29.92% in phyla *Bacteroides* compared from day 21 to day 35. However, phyla *Firmicute* in all diet descent from average 72% to 42%.

Based on Figure 6.10, in caeca (upper diagram), all supplemented diets significantly ($P < 0.05$) enhanced both the *Alistipes* and *Lactobacillus* populations on both 21 and 35 days compared to BD. In addition, significant ($P < 0.05$) suppression of the genus *Clostridium* was also observed in all treatment diets compared to BD on 21 days, but not on day 35, where only CC05 and SYN05 resulted in a lower *Clostridium* population compared to the BD diet. The population of *Bacteroides*, *Butyricicoccus* and *Firmicutes* were not affected by the supplemented diets in broiler's ceca.

In ilea based on Figure 6.10 (lower diagram), the *Alistipes* population were significantly ($P < 0.05$) enhanced with CC05, CC10 and SYN10 diet treatments on 21 days; and SYN10 on 35 days compared to BD. The population of *Lactobacillus* were numerically higher with PRO supplemented diet on 21 days; and significantly ($P < 0.05$) higher with CC05, CC10 and SYN05 on 35 days. The population of *Clostridium* was totally inhibited (0%) by CC10 supplemented diet.

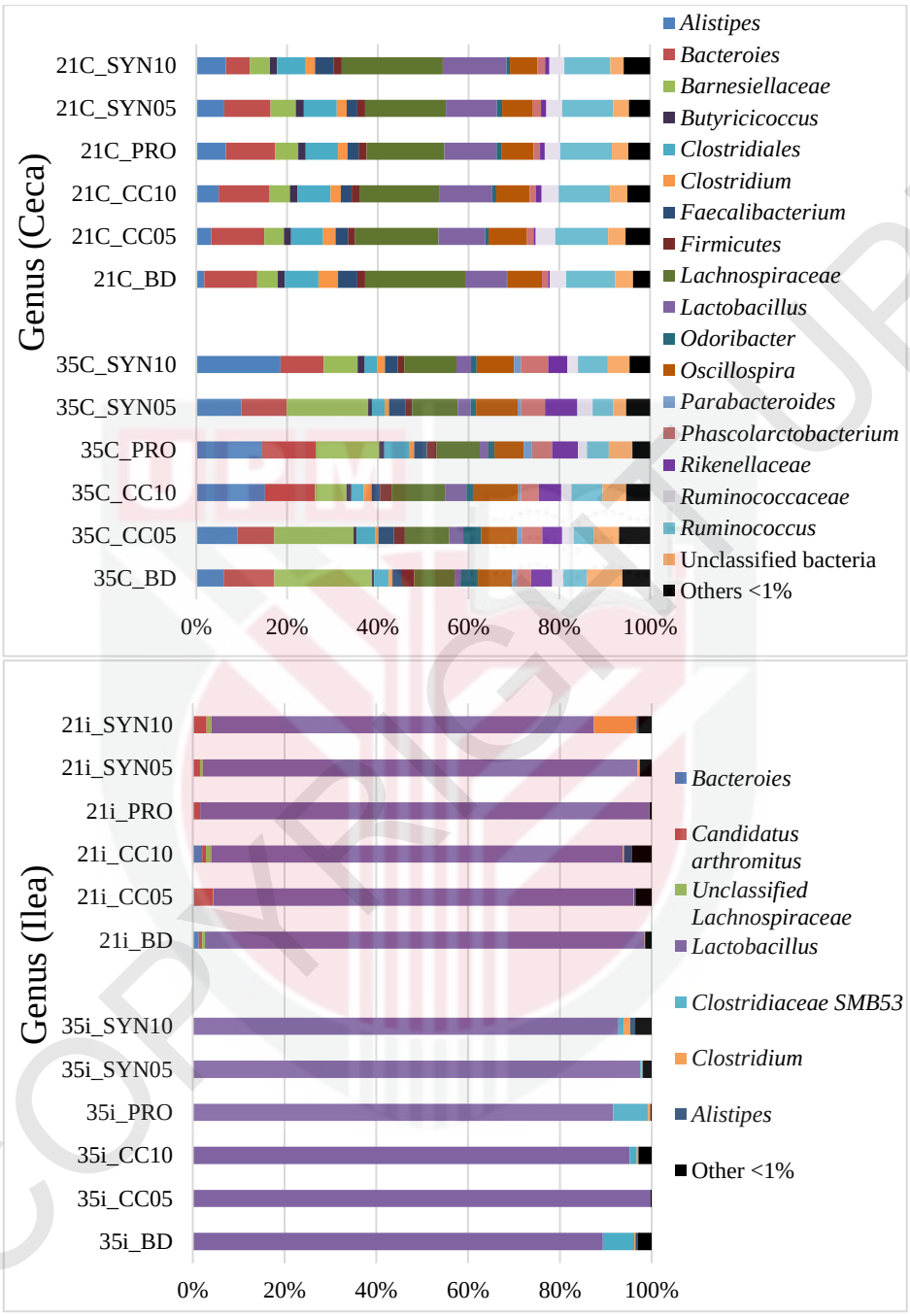


Figure 6.10: The relative abundance of caeca (upper diagram) ilea (lower diagram) bacteria at genus level for each diet treatment group at 21 and 35 days-old broilers.

Each data is mean ± standard error of 4 replicate cages with 2 broilers each. The diet treatments including BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO.

6.3.4 Principle Coordinate Analysis (PCoA) of Gut Microbial Communities Based on Bray-Curtis Similarity Index

The overall similarities of the gut microbiota structure were investigated via the principal coordinate analysis (PCoA) plot of the Bray-Curtis index. The PCoA plot in the data set that best represents the distances between samples via similarities. A closer distance represents higher similarity, and samples that cluster together are composed of similar microbiota. Principal Coordinate Analysis (PCoA) is an approach to sequencing using dimension reduction (Meng et al., 2016). It assumes that where there is data to measure the differences or distances between N number of samples, this method can be used to plot a rectangular coordinate system representing the N samples as N points, and the square of the Euclidean distance between the points is exactly equal to the original differential data. Thus, qualitative data is converted into quantitative data and the most important elements and structures are extracted from the multi-dimensional data. The classification of multiple samples can be achieved through PCoA, which further demonstrates the differences in diversity between samples. As observed in PCO1 (Axis 1) vs. PCO2 (Axis 2) shown in Figure 6.11, samples isolated from caeca and ilea that further from each other in the graph indicate dissimilarity of both intestine parts.

Figure 6.12 showed that broilers fed with CC05 and CC10 diet treatment on day 21 had significant ($P < 0.05$) dissimilarities among the caeca microbiota compared to the BD group. However, on day 35, the dissimilarities among the caeca microbiota were significant ($P < 0.05$) for all diet treatment groups except CC05 compared to the BD group. The PERMANOVA indicated overall significant dissimilarities of samples' caeca microbiota among different diet groups only on day 35. On the other hand,

Figure 6.13 illustrated that the ilea microbial on days 21 and 35 were relatively correlated among the diet treatment groups. Thus, the diet treatments did alter the caeca microbiota but not ilea.

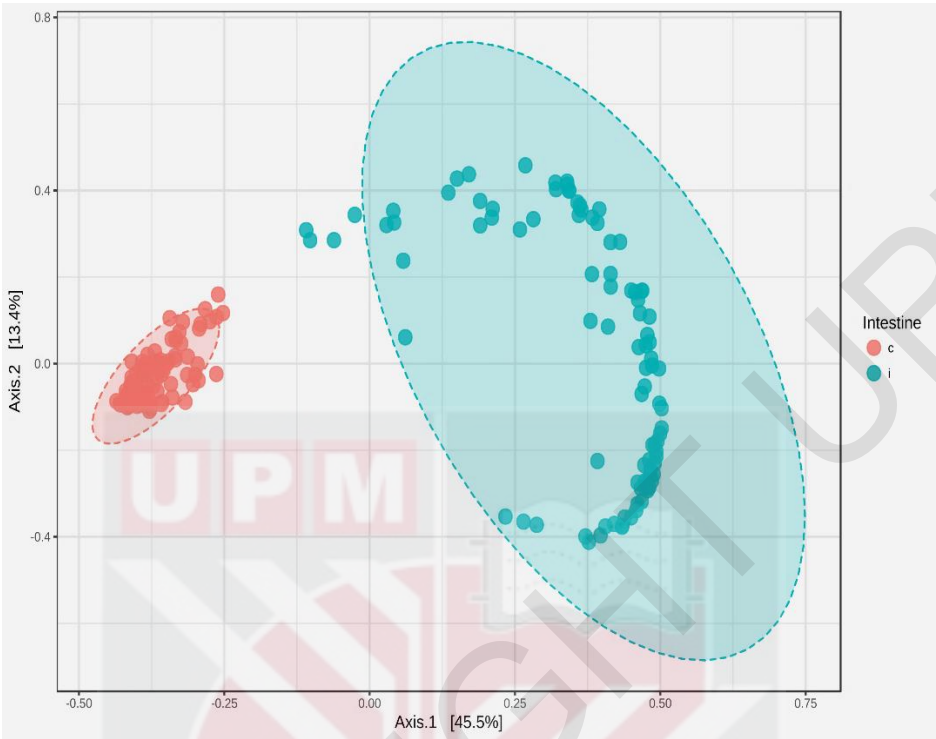


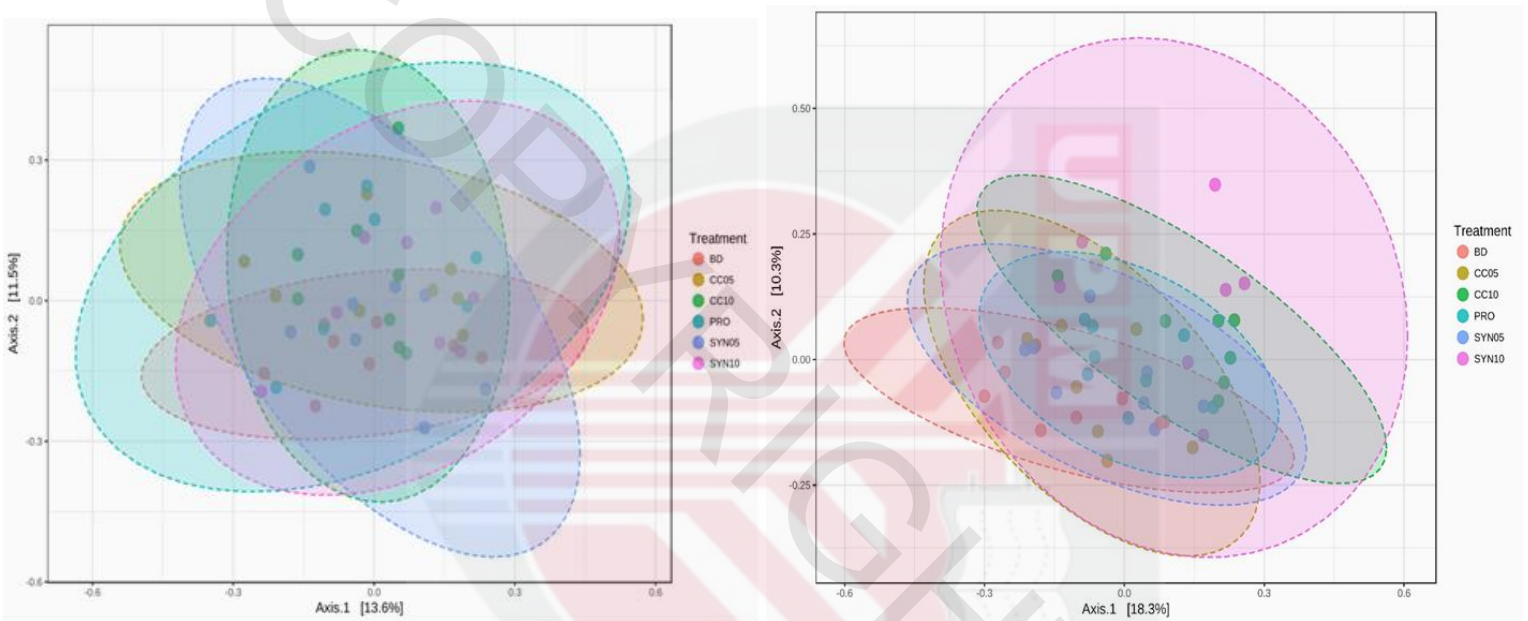
Figure 6.11: Principal coordinate analysis (PCoA) of overall ilea and caeca microbial communities based on Bray-Curtis similarity index at phylum level for all diet treatments.

Red dots are samples extracted from caeca, and blue dots are samples extracted from ilea.

PCO1 (Axis1) vs. PCO2 (Axis2) is the PCoA plot obtained from the first and second main coordinates; the x-axis and y-axis represent the first and second main coordinates, respectively.

The percentage of the main coordinates represents the relative contribution of this coordinate to sample differences, which measure the amount of original information extracted by this main coordinate.

The diagram indicated a clear separation of the gut microbial relationship between parts of the intestine ($P < 0.05$).



21 days

35 days

Figure 6.12: Principal coordinate analysis (PCoA) of caeca communities at 21 and 35 days based on Bray-Curtis similarity index at Phylum level with different diet treatments via Permanova statistical analysis.

PCO1 (Axis1) vs. PCO2 (Axis2) is the PCoA plot obtained from the first and second main coordinates; the x-axis and y-axis represent the first and second main coordinates, respectively.

The different colours represented different diet treatment (BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO).

The diagram showed overlapping of the sample due to similarities of caeca microbial although fed with different diet treatments.

However, the overall dissimilarities of samples' caeca microbiota among different diet treatments are significant ($P < 0.05$) only at 35 days.

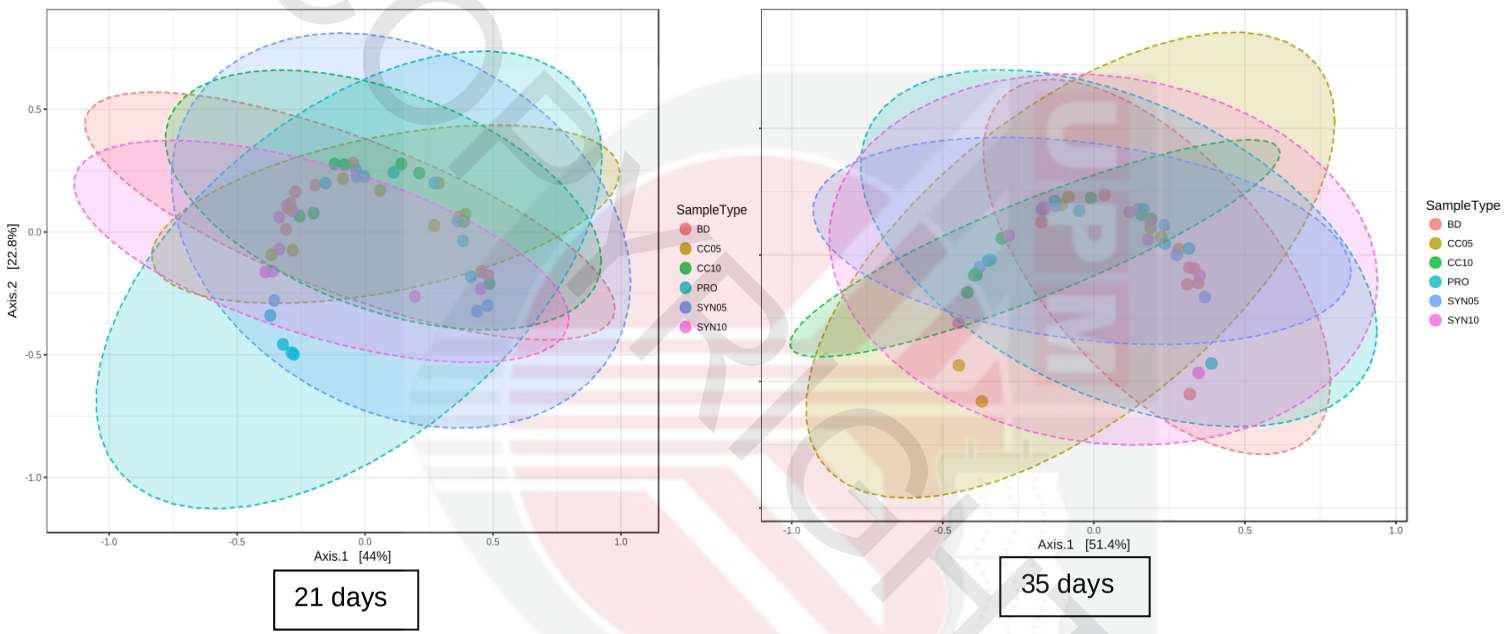


Figure 6.13: Principal coordinate analysis (PCoA) of ilea communities at 21 and 35 days based on Bray-Curtis similarity index at Phylum level with different diet treatments via Permanova statistical analysis.

PCO1 (Axis1) vs. PCO2 (Axis2) is the PCoA plot obtained from the first and second main coordinates; the x-axis and y-axis represent the first and second main coordinates, respectively.

The different colours represented different diet treatment (BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO).

The diagram showed overlapping of the sample due to the similarities of ilea microbial although fed with different diet treatments.

In overall PERMANOVA analysis, the ilea microbial from different diet treatments were not significantly different compared to BD group on day 21 and day 35.

6.3.5 Microbial Predicted Functional Metagenomes

The prediction of microbial functions was analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via PICRUSt 1.1.4 platform. Pairwise comparison of the first level predicted functional metagenomes fed with BD (control) between caeca and ilea (Figure 6.14), among caeca treatments and also among ilea treatment on days 21 and 35 (Figure 6.15 - Figure 6.17), were performed and illustrated separately. The first comparison compares the abundance of microbiome predicted function gene in ilea and caeca fed with BD (control). In contrast, the second is to investigate the effects of diet treatment on the microbiome gene function. The first level predicted functional metagenomes clustered into metabolism, cellular process and signalling, environmental information processing, organismal system, and human diseases. This was followed by an in-depth clustered of the metabolism functions of each treatment in caeca on day 35 (Figure 6.19).

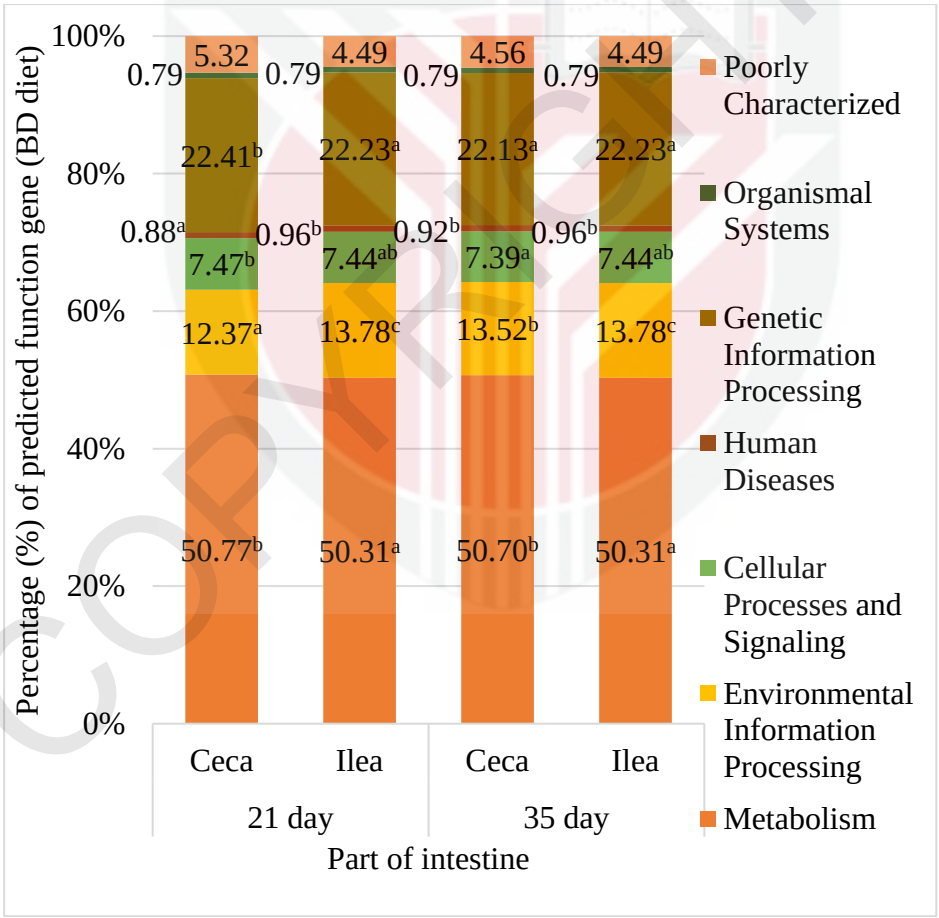


Figure 6.14: Comparison of the control (basal diet) relative abundance of the first level predicted function genes in caeca and ilea microbiome at 21 and 35 days.

The predicted functions were retrieved in the Kyoto Encyclopedia of Genes and Genome (KEGG) database.
The pairwise comparison of predicted functions between caeca and ilea microbiome at both 21 and 35-day (Figure 6.14) illustrated a significant difference in metabolism, environmental information processing, cellular process and signaling, and human diseases and genetic information processing. Around 4.49% - 5.32% of genes were poorly characterized. Ilea microbiome was significantly ($P < 0.05$) enriched with environmental information processing, which is higher (1.41% at 21 days; 0.26% at 35 days) than the caeca microbiome.

This was followed by comparing the predicted functions at the first level of KEGG orthologs among diet treatments in caeca and ilea separately to investigate the effects of diets toward enhancement or depletion of microbiome gene functions. However, there are no significant differences in predicted functional metagenomes in neither caeca nor ilea at 21 days with different diet supplements.

Pie chart (Figure 6.15) showed the metabolism gene of caeca microbiome fed with basal diet at 35-day resulted significant ($P < 0.05$) increased by 0.29%, 0.17% and 0.21% compared to CC10, SYN05 and SYN10, respectively. The genetic information processing function gene is significantly ($P < 0.05$) higher in the caeca microbiome fed with SYN05 (20.62%) and SYN10 (20.60%) diet treatments compared to BD (20.50%) diet. Environmental information processing functional gene supplemented with CC10 diet treatment lead to significant ($P < 0.05$) enhancement compared to BD treatment from 10.39% to 10.55%. The result is supported by the Multiple comparisons of the diet treatment on caeca predicted functional metagenomes using Storey's FDR multiple test correction methods (Figure 6.16).

At 35 days, the ilea microbiome (Figure 6.17 and Figure 6.18) resulted in significant ($P < 0.05$) higher genetic information processing genes in BD treatment compared to CC10 treatment, followed by SYN10 treatment.

The metabolism category in the caeca microbiome at 35 days was further investigated to compare the effects of diet treatments on the second level of predicted functional metagenomes (Figure 6.19). There were no significant differences in carbohydrate metabolism, lipid metabolism, biosynthesis of the secondary metabolites, biosynthesis and metabolism of terpenoids and polyketides, and xenobiotics biodegradation and metabolism, glycan biosynthesis and metabolism and others. Others consisted of poorly characterized genes. SYN05 diet treatment significantly ($P < 0.05$) enhanced the metabolism of cofactors of vitamins (10.02%) and nucleotide metabolism compared to BD. On the other hand, BD treatment managed to elevate ($P < 0.05$) the metabolism of the amino acids at 23.37% compared to PRO, SYN05, SYN10 treatment, but has the least ($P < 0.05$) production of nucleotide production (8.74%) compared to all diet treatment. Besides, BD treatment also

resulted in lower ($P < 0.05$) energy metabolism compared to CC10, which is 15.75%.

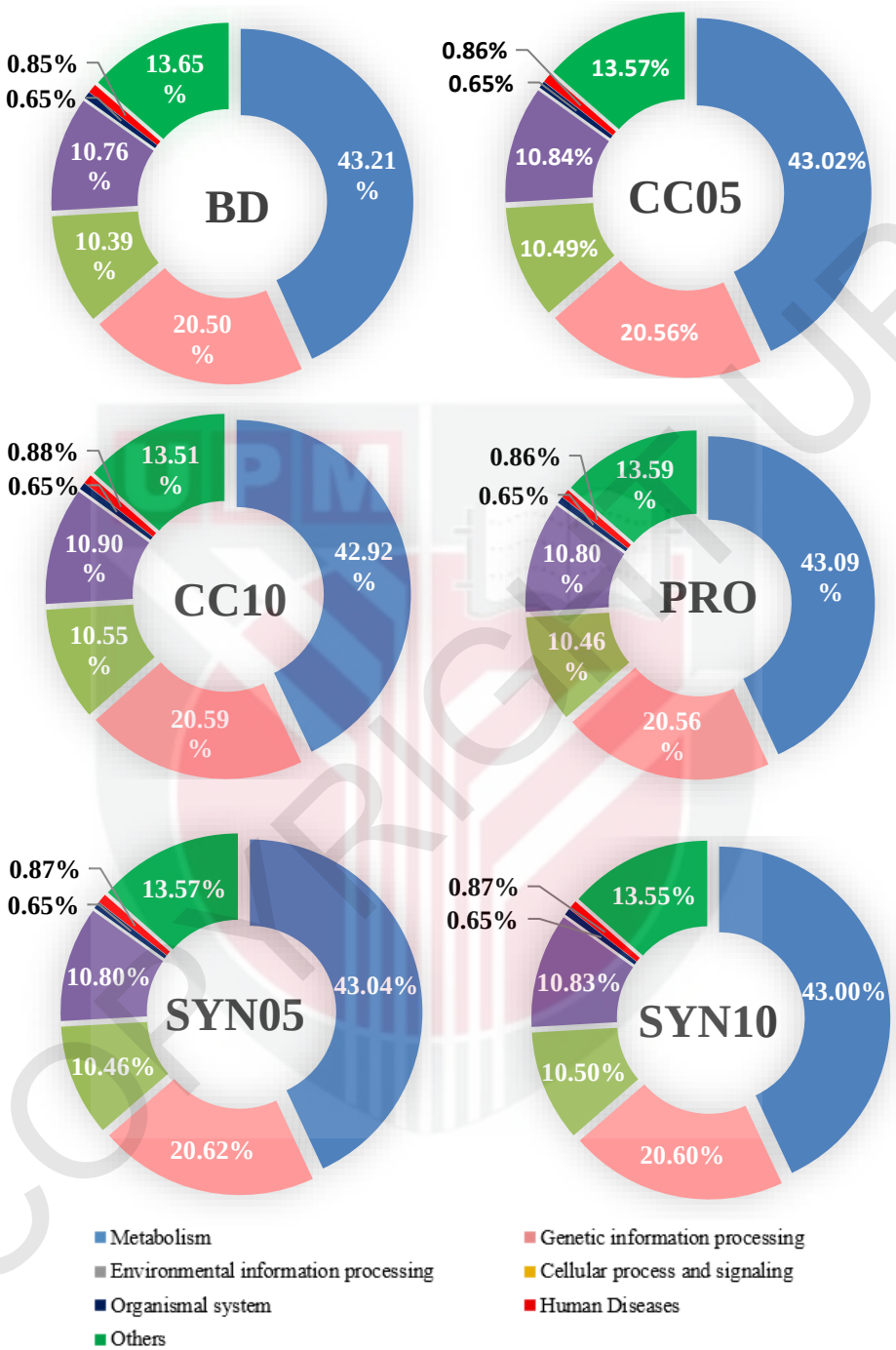


Figure 6.15: Differences in predicted functional metagenomes classified into first level of KEGG orthologs in caeca sample of broilers fed with different dietary treatments at 35 days.

Diet treatment group, diet treatment (BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO).

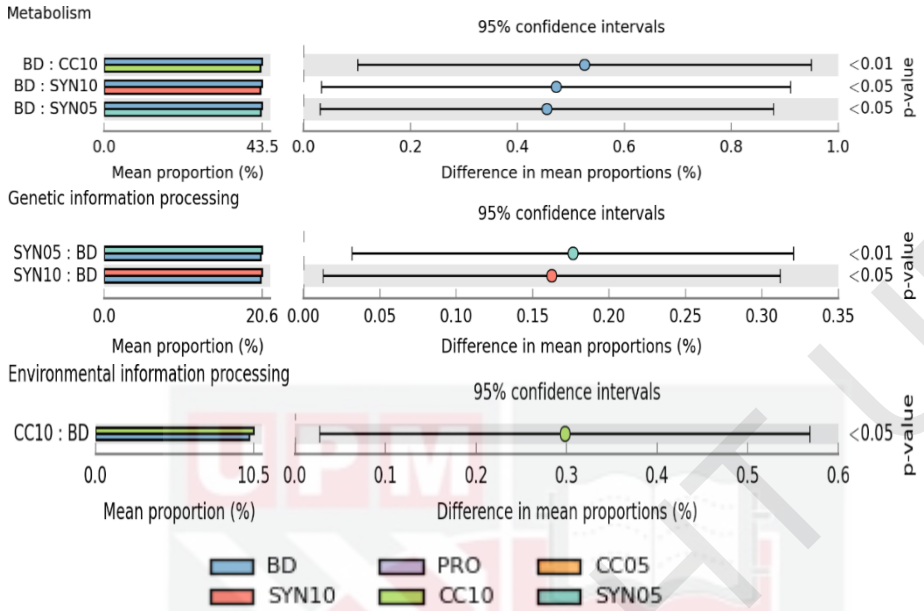


Figure 6.16: Multiple comparison of the diet treatment on caeca predicted functional metagenomes using Storey's FDR multiple test correction methods at 35 days.

BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO.

Others are those unclassified and poorly characterized predicted functional metagenomics that could not be retrieved in Kyoto Encyclopedia of Genes and Genome (KEGG) database.

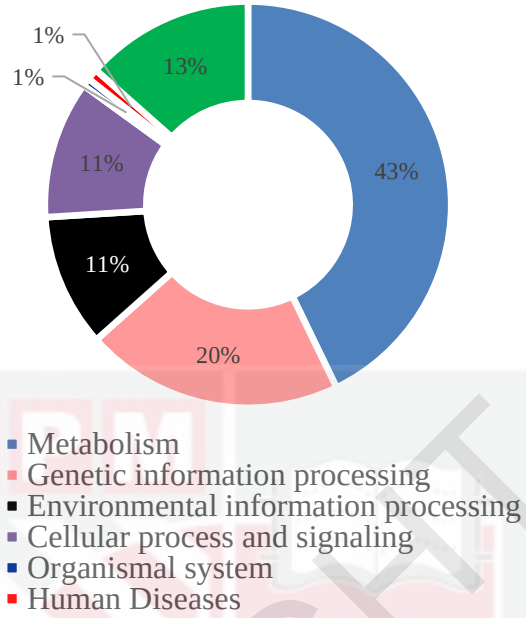


Figure 6.17: The predicted functional metagenomes classified into first level of KEGG orthologs in ilea sample of broilers fed with basal diet at 35 days.

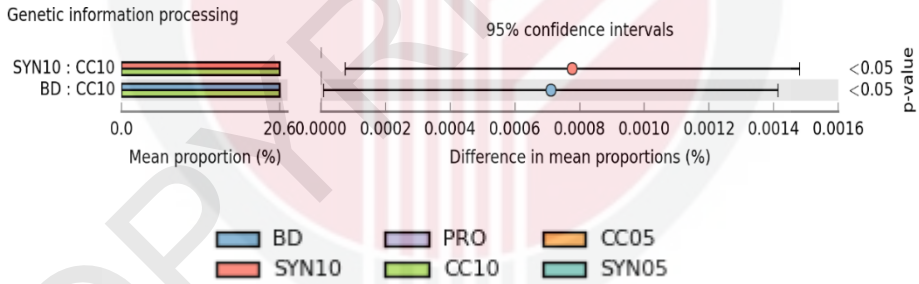


Figure 6.18: Pairwise comparison of the diet treatment on ilea predicted functional metagenomes using Storey's FDR multiple test correction methods at 35 days.

BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO.

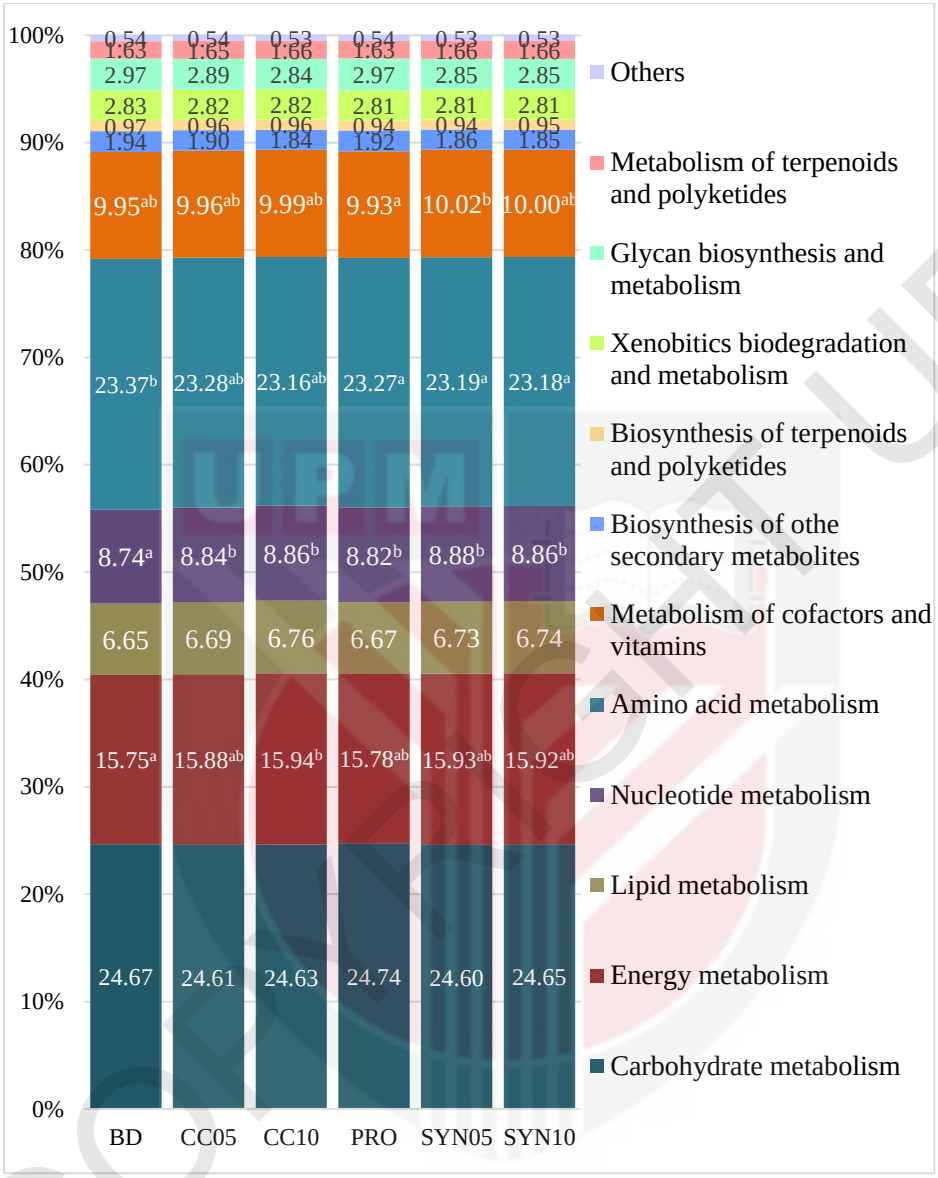


Figure 6.19: Predicted functional metagenomes within the metabolism category in caeca sample of broilers fed with different dietary treatment on day 35.

BD=basal diet; CC05= BD+ 0.5% corn cob; CC10= BD+ 1.0% corn cob; PRO= BD+ 1% probiotic; SYN05= BD+ CC05 + PRO; SYN10= BD+ CC10 + PRO.

6.4 Discussion

With the advancement in biotechnology that makes high-throughput sequencing possible, the effects of the diet on gastrointestinal microbiomes and health can

be investigated. Hence, using the DNA fingerprinting and high throughput sequencing, it was possible to evaluate the changes in the taxonomic composition of the gut microbiota of 21- and 35-days old chickens. According to González-Félix et al., (2018), microbial communities have been found in one-day-old chicken's gastrointestinal tract. The composition of the microbial changes with age as more established bacteria takes root in the digestive tract.

Although both ileum and cecum are parts of the intestinal tracts, there are huge differences in morphology and functions. Ileum is located at the foregut, and its function is more to absorption; cecum, on the other hand, is located at the hindgut, which involved more microbial fermentation. The substantial microbial fermentation yields nutrients, aid in the detoxification of some harmful substances, and prevents the colonization of pathogens (Huang et al., 2018). The study also aligned with this study, where the hindgut microbial diversities were higher than in the foregut compartment. As based on Table 6.5, the microbial richness (ACE, Chao1) in ilea for basal diet is 21.34± and 21.26±8.46 were both significantly ($P < 0.05$) lower than the richness in caeca basal diet, which are 205.68±16.56 and 205.50±16.54. Shannon and Simpson, which represent the microbial diversity in ilea, also lower by 28.71 and 0.74, respectively, fed with basal diet. The diet treatment of SYN05 resulted in the highest microbial richness and diversity among all treatments after 35 days in caeca. The relative abundance profiles of phylum and genus showed distinct microbial features between ilea and caeca samples (Figure 6.8 - Figure 6.10). At the phylum level, *Bacteroidetes* and *Firmicutes* showed significant differences ($P < 0.05$) and were twofold enriched in ilea and forty-eight folds in caeca, respectively. As the previous report (Huang et.al., 2018), *Lactobacillus* is the predominant genus in the ilea but not in the caeca (Figure 6.10). Neijat et al. (2019) discovered that some of the most common microbial communities in the GIT of chicken were *Campylobacter coli*, *L. delbrueckii*, *C. perfringens*, which appeared in most of the young chicks below three days old. The study also found a significant presence of various bacteria in the digestive tract of 21 days old chicken, *Firmicutes* (58.65%) and *Bacteroidetes* (35.98%). *Lactobacillus* can protect the host from colonization of pathogens and provide nutrients by fermentation to broilers (Cross, 2002; LeBlanc et al., 2013). By contrast, various anaerobic genera, such as *Ruminococcus*, *Bacteroides*, *Faecalibacterium*, *Clostridium*, and *Butyricicoccus*, were more abundant in the caeca (Figure 6.10). According to Huang et al. (2018), a negative correlation was due to *Lactobacillus* competitively inhibiting *bacterial* clusters in ilea. The stability of the cluster was formed in caeca due to the positive correlations among some SCFAs producers such as *Clostridium*, *Butyricicoccus*, *and Faecalibacterium*. The beneficial microbes can form a large co-occurrence network by produce metabolites, such as SCFAs by fermentation, thus inhibited the pathogenic bacteria, *Escherichia* and *Enterococcus*. Therefore, the microbial communities are far more complex and diverse in the caeca and ilea.

In the study, real-time PCR quantification (qPCR) resulted, 1.0% corn cob (CC10) supplemented diet has significantly ($P < 0.05$) induced the population of *Lactobacillus* spp. and lower the *E. coli* population than the basal diet (BD) in caeca at 21 days. The corn cob extracts might contain oligosaccharides that alter

the microbiota by enhancing the beneficial bacteria and suppressing the pathogenic bacteria simultaneously, which is known as competitive exclusion. According to Koirala (2018), galactooligosaccharides (GOS) and fructooligosaccharides (FOS) increase the composition of *Lactobacillus* and *Bifidobacterium* in the gastrointestinal tract (GIT). Furthermore, most of the *bifidobacteria* strains often use FOS as a growth and fermentation parameter. According to Quin et al. (2018), it was found that other microorganisms such as the *E. coli* and *C. perfringens* were not able to utilize the FOS as a fermentation carbohydrate source.

Furthermore, most of the *Bifidobacteria* strains use FOS as a growth hormone promoter. Hence, its presence leads to an increase in their colony. The bacteria such as *B. vulgatus, B. ovatus and B. dktasonk* increases their growth through secretion of ß-fructosidase enzyme that readily ferments FOS. Therefore, a dietary increase of the FOS was a significant contributor to the reduction in the *C. perfringens* and *E. coli* while contributing to the rise in the diversity of *Lactobacillus* in the chicken gastrointestinal tract. This theory is also further supported by the significant ($P < 0.05$) enhancement of caeca *Bifidobacterium* spp. population (Figure 6.2) on day 35 and reduction, Log_{10} DNA 0.52 of *E. coli* population compared to basal diet (BD). However, the population of *C. perfringen* and *Salmonella* were not affected compared to the basal diet (BD) group, which is acceptable as a balanced intestinal microbiota consisted of 3 major genera (*Lactobacillus*, *Clostridium*, and *Bacteroides*). This balanced intestinal microbiota with high Lactobacilli diversity are least susceptible to infections (Stanley et al., 2012; Nakphaichit et al., 2011; Patterson et al., 2003).

According to the KEGG functional analysis (Figure 6.14), the microbiota in the ilea was enriched in genetic information processing for transcription, translation, and replication. In contrast, the caeca are enriched for the metabolic functions of amino acids, nucleotides, lipids, energy, carbohydrates, cofactors and vitamins, and secondary metabolites biosynthesis as well as biodegradation. The supplemented CC10 diet significantly promoted microbial energy metabolism in caeca, generating adenosine triphosphate (ATP) from the corn cob extract. The energy was used for microbial growth to ferment the possible oligosaccharides found in corn cob extracts. The end products can benefit the host, especially SCFAs, mainly acetate, propionate, and butyrate fermented by the anaerobic bacteria. Research findings proved that 70% of the energy obtained by intestinal epithelial cells (IECs) is derived from butyrate, mainly produced by commensal bacteria, especially Clostridia species belonging to Firmicutes *Ruminococcus* and *Faecalibacterium* (Serpa et al., 2010). Therefore, the presence of saccharides mixture in corn cob extracts might induce the growth of beneficial bacterial. This finding is aligned with the overall abundance of the microbes with CC10 supplemented diet at 35 days in caeca.

6.5 Conclusion

In general, the microbial function and diversity were significantly different between ilea and caeca. Ilea is of paramount importance for nutrient absorption, whereas caeca are essential for fermentations. As the supplemented diet, corn cob extract consisted of mixtures of saccharides for fermentation, thus enhancing the growth of the beneficial bacteria and suppressing pathogenic bacteria's growth better in caeca but not in ilea. The findings were aligned in both real-time PCR and metagenomic (HT-NGS), where the population of *Lactobacillus* was induced by the supplemented diet compared to basal diet as control. The enhancement of these beneficial bacteria has improved the genetic information processing and metabolism functions in caeca. SYN05 supplemented diet resulted in a significant ($P < 0.05$) higher bacteria richness and diversity reflected compared to the basal group during the finisher period. Besides that, SYN05 significantly ($P < 0.05$) elevated the beneficial bacteria and suppressed the growth of pathogenic bacteria in caeca. In addition, SYN05 also significantly ($P < 0.05$) elevated the metabolism of cofactors of vitamins and nucleotide metabolism compared to BD.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

7.1 General Discussion

The main objective of this study is to screen prebiotics from local plant extracts and investigate its effects on the broiler performance, nutrients absorption, immunity with the combination of commercial probiotic strains, as well as the changes of their gut microbiota. Oligosaccharides are classified as low molecular weight carbohydrates composed of 2-10 sugar units, which are one of the successful substitutes of AGPs (Reyer et al., 2017). According to Rowell et al. (2005), the combination of cellulose and hemicellulose is usually found in wood crops, which have a higher amount of carbohydrates. The combination is full of 40-45% of the cellulose and 12-25% of the hemicellulose. Both are called together holocellulose which covers almost 70% of the content of the carbohydrate. According to Browning (1967), plants consisting polysaccharides are based on the major part of the cellulose (Mansor et al., 2019). The structurally rigid plant cell is known as the lignin. This is aligned with the result of coconut shred, rice bran, and oil palm frond extraction resulted in much lower reducing sugars as well as total carbohydrates compared to fruits' waste extract, which are pineapple peel, banana peel and corn cob. Although the pineapple peel and banana peel consisted of high reducing sugars, the total carbohydrate harvest was lesser than corn cob extract. High sugar content will serve as the substrate for every bacteria, especially pathogenic bacteria, leading to the colonization of pathogenic bacteria in the gut (Garber et al., 2020). Therefore, corn cobs consist of a lower level of lignin and a higher level of hemicellulose and cellulose was the best candidate for further extraction of potential oligosaccharide (Michelin et al., 2018). More sugar monomers consist of hemicelluloses which help in forming more oligosaccharides.

The further optimization of corn cob extract was carried out by further autohydrolysis under non-isothermal conditions (up to experimental temperature). The usage of inorganic chemicals such as acids and ethanol were avoided in the optimization study due to the residues in the extract might affect the health of broilers (Khan et al., 2013). The autohydrolysis via pressure aided to disrupt and remove lignin and hemicellulose that acts as the structural barrier (Ogunbayo et al., 2016). The corn cob extraction with the aids of sonication also enhanced the extraction of oligosaccharides by further disrupt the cell wall and increase the particles' total surface area. The cavitation between the solvent and particles leads to the mass transfer of water-soluble cell content into the solvent (Jovanovic-Malinovska et al., 2015).

The UHPLC-MS/MS analysis revealed the corn cob extracts have consisted of mixtures of mono-,di-as well as polysaccharides. Michelin et al. (2018) also detected the presence of xylooligomers, xylose, glucose, arabinose, acetic acid and furfural in the corn cob extraction. The NMR detected maltotriose in the corn cob extract which is the formation of glucose were possible.

In addition, corn cob crude extract exhibited prebiotic properties by enhancing most of the growth of in vitro relative growth of *Lactobacillus* strains. *L. acidophilus* I16 displayed the highest growth with corn cob extracts as substrate compared to glucose. Extensive studies have shown that each oligosaccharide enhanced the growth and/or the activities of probiotic strains at varying degrees (Kneifel, 2000; Nunpan et al., 2019). This also exhibits the unique prebiotic special characteristic defined by Gibson et al. (2004) selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being.

In vivo feeding trial the supplementation of 1.0% of corn cob extract and 1.0% of probiotics (SYN05) resulted lower FCR and reduction of triglyceride level, and enhanced the antibody production ($P < 0.05$) compared to BD. SYN05 showed the best overall effects that significantly enhanced the beneficial bacteria and suppressed the growth of pathogenic bacteria. This also leads to improved energy and nucleotide metabolism. In overall, *in vivo* broilers fed with 0.1% probiotic and 1.0% corn cob extract (SYN10) resulted in significant ($P < 0.05$) better feed conversion ratio (FCR), body weight gain (BWG), lower serum triglyceride and higher antibody of IgA and IgM compared to BD. SYN10 also significantly enhanced the population of *Lactobacillus* and *Bifidobacterium*, in conjunction with the reduction of *Escherichia coli* and *Clostridium perfringens* population in caeca and ilea during the finisher period compared to BD. On the other hand, 0.1% probiotic and 0.5% corn cob (SYN05) significantly lowered the LDL level ($P < 0.05$) during the finisher period compared to BD.

7.2 Conclusion

It is proven that the extract of the corn cob consisted mixture of mono-, di-, tri- and polysaccharides. Corn cob extract exhibited prebiotic properties by selectively stimulating the growth and/or activity of beneficial intestinal bacteria *Lactobacillus* and exclusive competition suppress the growth of pathogenic bacteria.

From the results of this study, it can be concluded that the diets supplemented with corn cob extracts give positive and promising results on all aspects of the broilers' growth and health performance comparing to the basal diet, especially the body weight gain and feed efficiency of broilers. The combination of corn cob extract with the commercial probiotic mixture (Primalac®) did not always have the synergic effects as expected. With the addition of the 1.0% corn cob extract, it gives the best results on the body weight gain, yet when together with Primalac® (SYN10) the effect remained the same. However, the cholesterol

level was affected with only a synbiotic diet (SYN05 and SYN10) that was significantly lower than BD. The jejuna villus height and ilea crypt depth were significantly thicker in the SYN05 diet. The study of gut microbiota proved that inclusion of the corn cob extract and PrimaLac® alters the gut microbiota, especially *Lactobacillus* species, and leads to the enhancement of microbiome gene functions. Overall, both SYN05 and SYN10 resulted as the potential combination that enhanced the gut microbiota and led to better growth performance with better feed efficiency.

Regardless of the inconclusive evidence in the best corn cob extract dosage to be combined with probiotic mixture Primalac®, the results of this study provide confidence in the potential of corn cob extracts as AGPs replacement and serve as a new framework to a better understanding of the beneficial impact of corn cob extracts as a prebiotic supplement in broiler chickens' diet. Further researches are recommended to be conducted to provide more conclusive results in determining the optimum corn cob extract dosage to be supplemented in the diet.

7.3 Recommendation for Future Research

The above conclusion on the effectiveness of corn cob extracts and its combination with PrimaLac® gives rise to the questions on the best dosage for the combination, which had a positive impact on the performance of the broilers' growth. Thus, future research should include studies on:

(1) Combination of more corn cob extract dosage with different commercial probiotic as supplement in broilers' diet.
(2) The profile of short-chain fatty acids (SCFA), lactic acid produced in broilers' caeca.
(3) The study covers in this thesis only focused on the relative changes in the mRNA levels and the transporters evaluated were just a fragment of the avian's nutrient transporters. Whether or not the expression of mRNA leads to subsequent protein synthesis requires further investigations.
(4) While results of this study showed that mannose might have been absorbed, but it efficacy to replace glucose as an energy source requires further elucidation.
(5) The metagenomics approach employed DNA and not the RNA as the research object; therefore, for these reasons, microbial metatranscriptomic and metabolomics studies of broilers could be carried out in the future to determine its metabolic processes on a large, complex and dynamic scale and also to see if these predicted functions translate to phenotypical changes.

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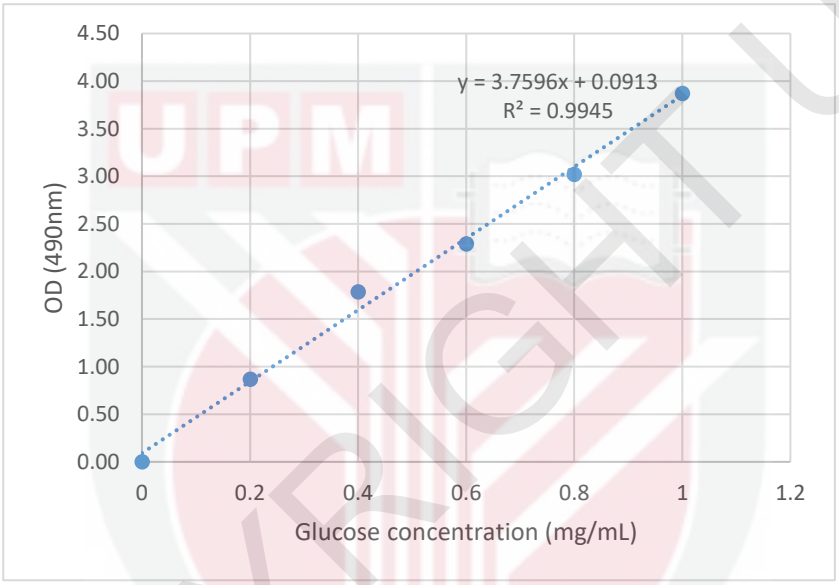
APPENDICES

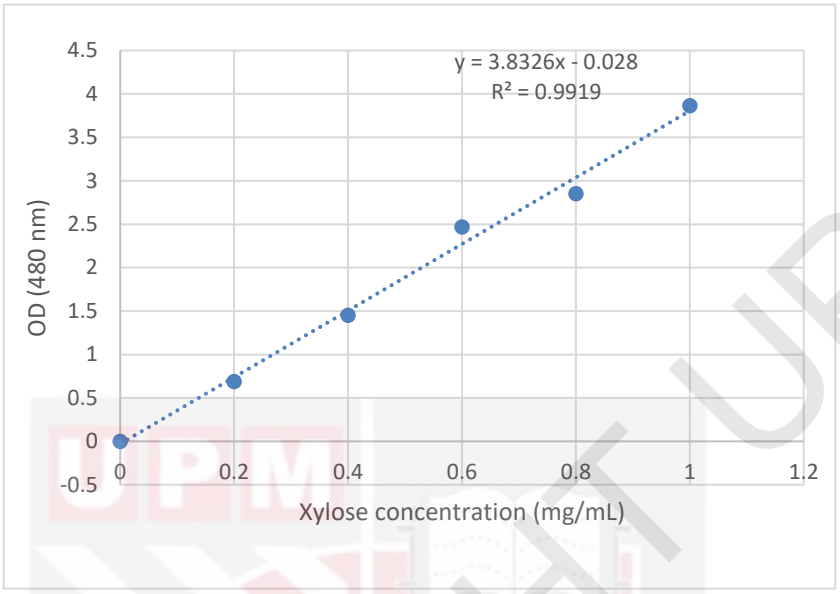
Appendix I

Standard curve of simple sugar concentration (mg/mL) via phenol sulfuric acid method on (a) glucose at 490nm and (b)xylose at 480 nm.

(a)

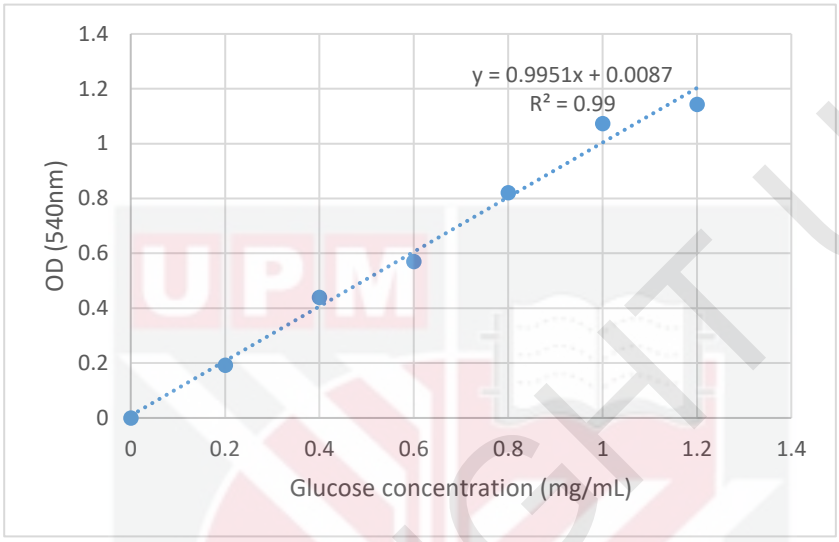


(b)



Appendix II

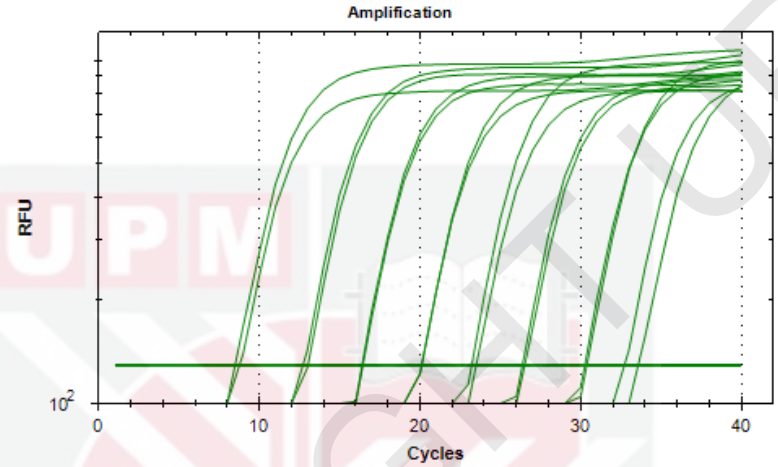
Standard curve of simple sugar concentration (mg/mL) via Dinitrosalicylic Acid (DNS) method on Glucose at 540nm.



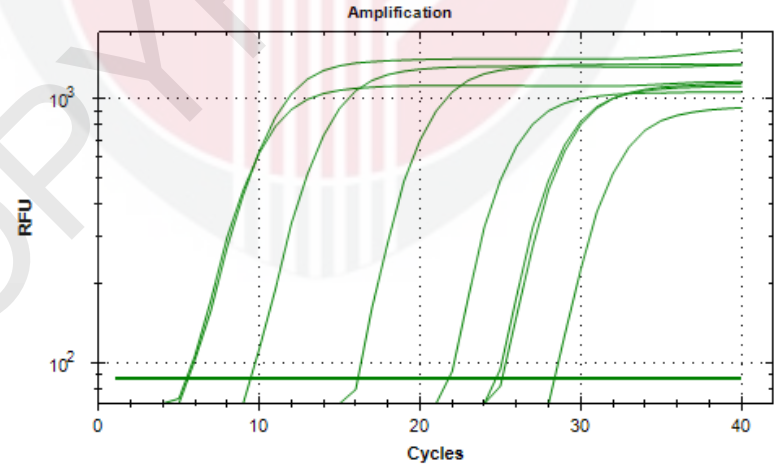
Appendix III

Log View amplification plot from the graph of relative fluorescence unit (RFU) verses number of cycles of (a) *Bifidobacterium* spp. (b) *Lactobacillus* spp., (c) *E. coli*, (d) *C. perfringens* and (e) *Salmonella* spp.

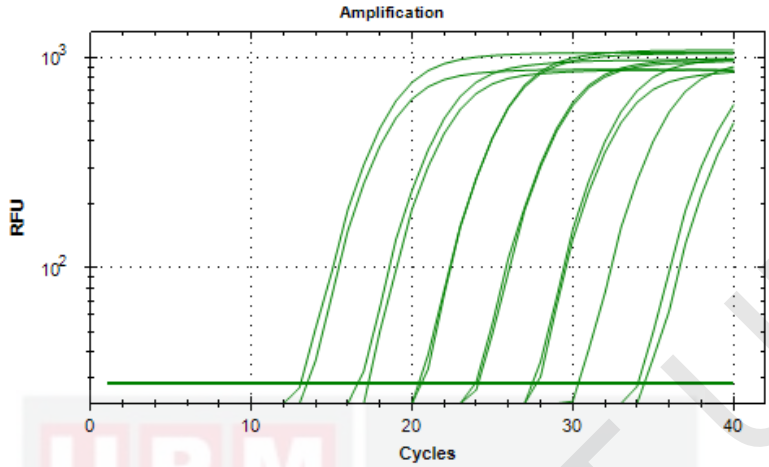
(a)



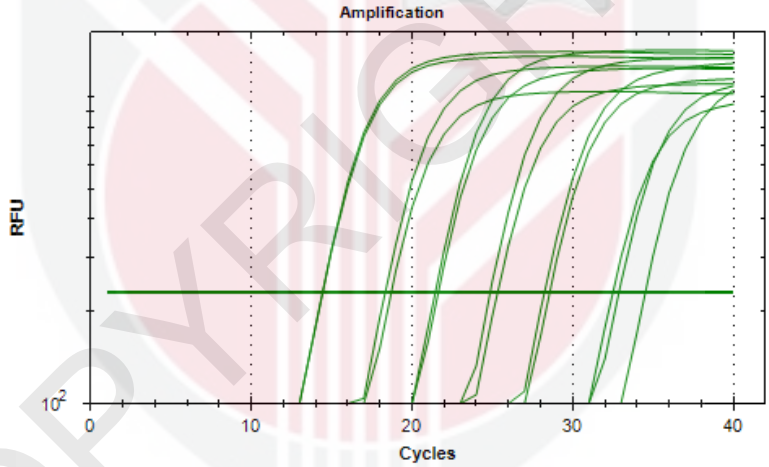
(b)



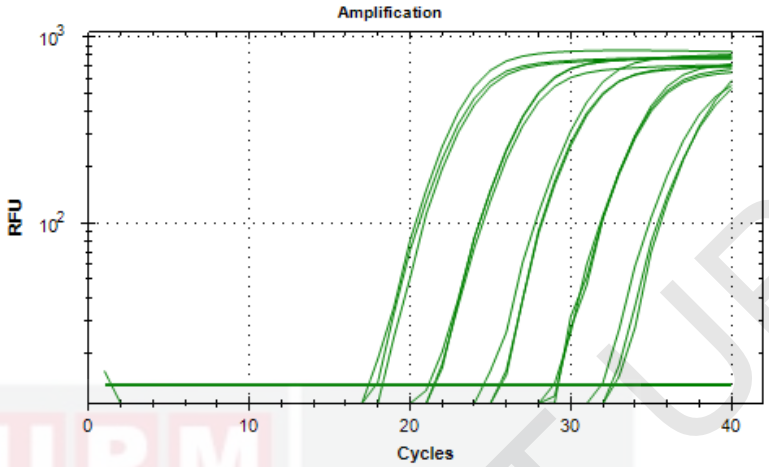
(c)



(d)



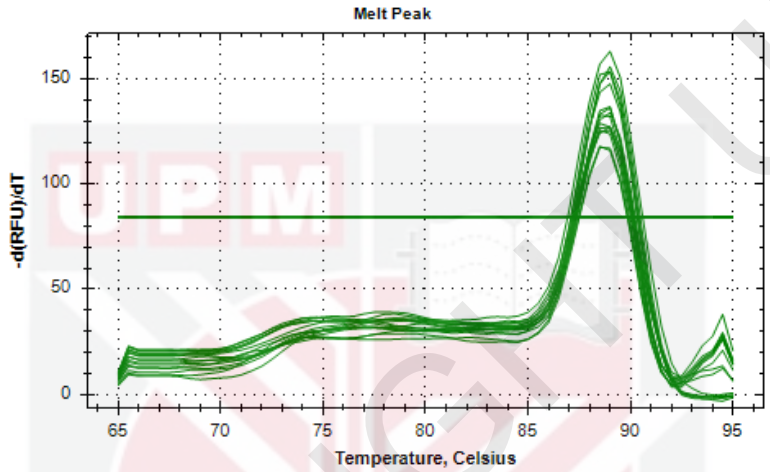
(e)



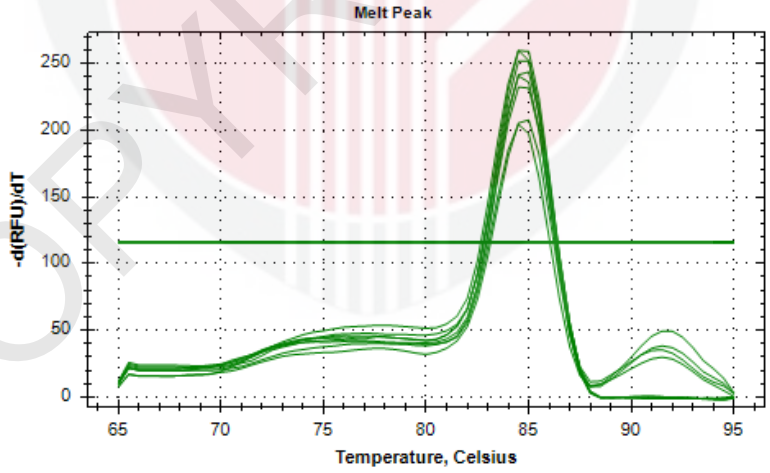
Appendix IV

Melting curves from 16S rRNA gene real-time quantitative PCR of (a) *Bifidobacterium* spp. (b) *Lactobacillus* spp., (c) *E. coli*, (d) *C. perfringens* and (e) *Salmonella* spp.

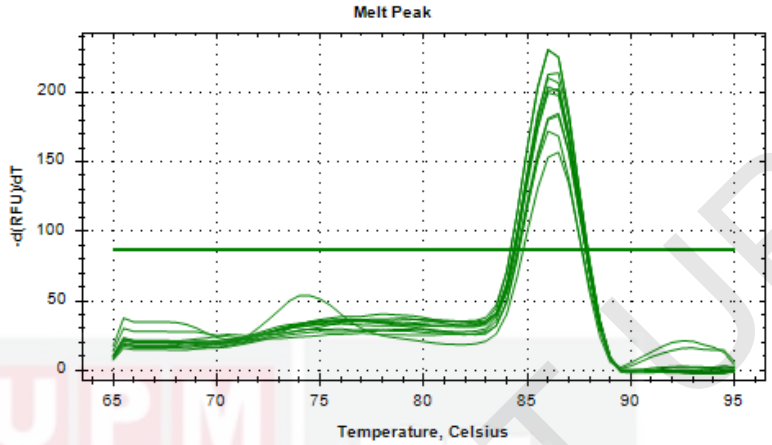
(a)



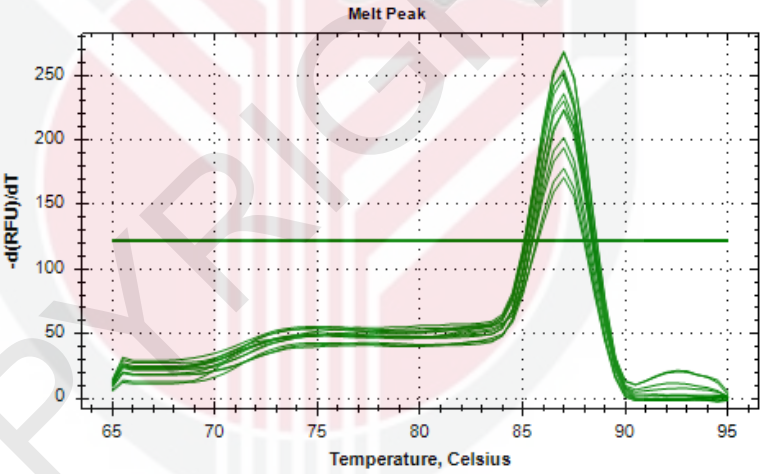
(b)



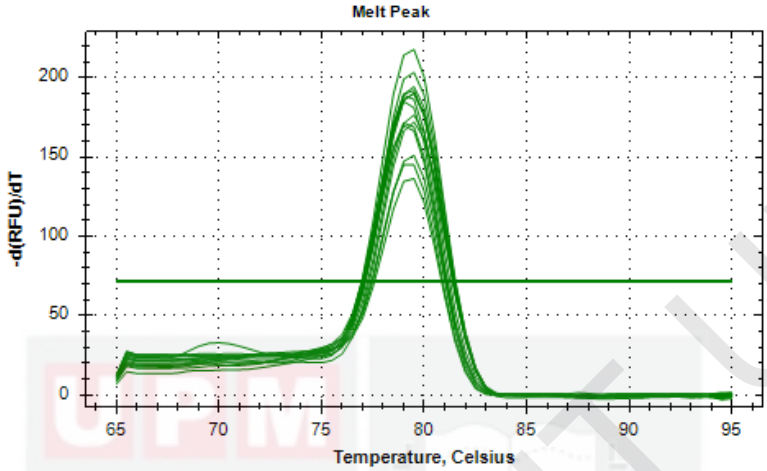
(c)



(d)



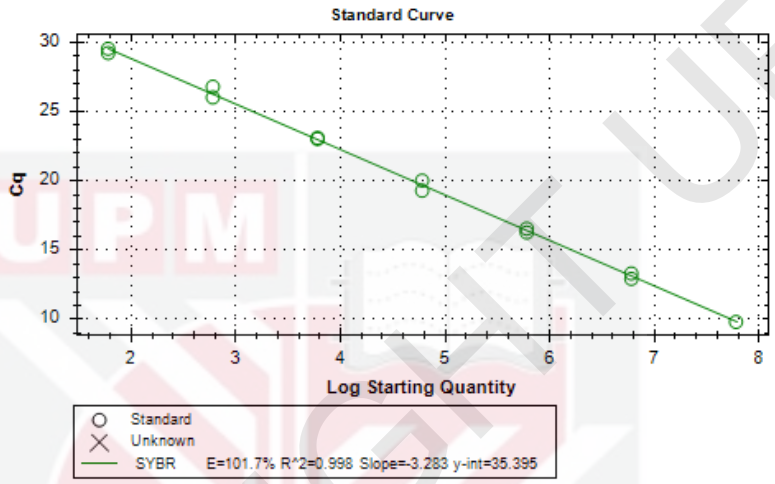
(e)



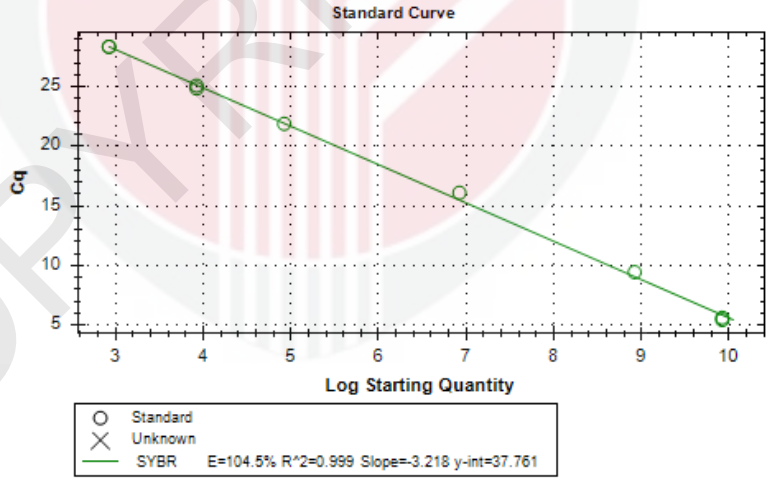
Appendix V

Standard curve plotted from the graph of quantification cycle (Cq) versus 10-fold serial dilutions of log 16S rDNA copy number of (a) *Bifidobacterium* spp. (b) *Lactobacillus* spp., (c) *E. coli*, (d) *C. perfringens* and (e) *Salmonella* spp.

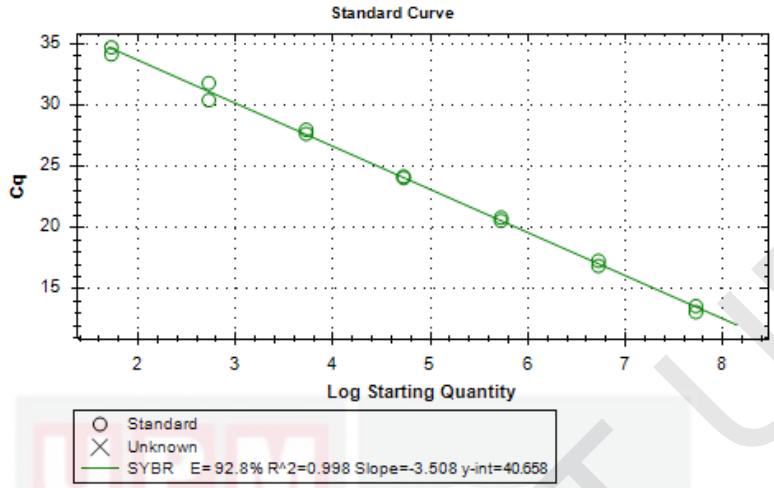
(a)



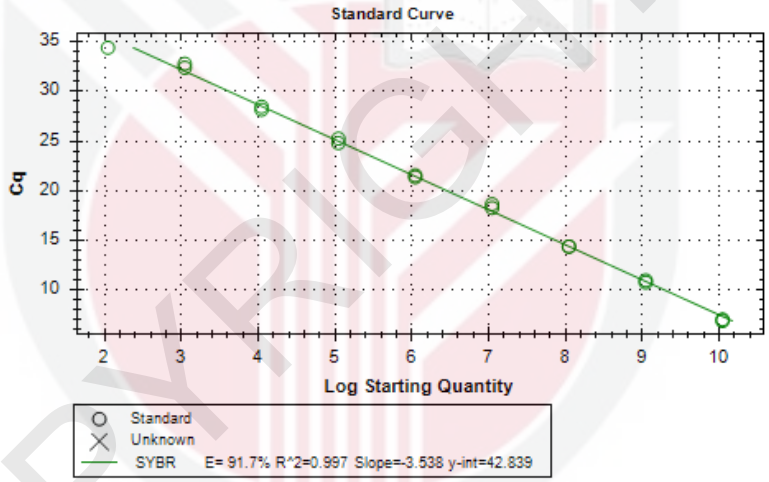
(b)



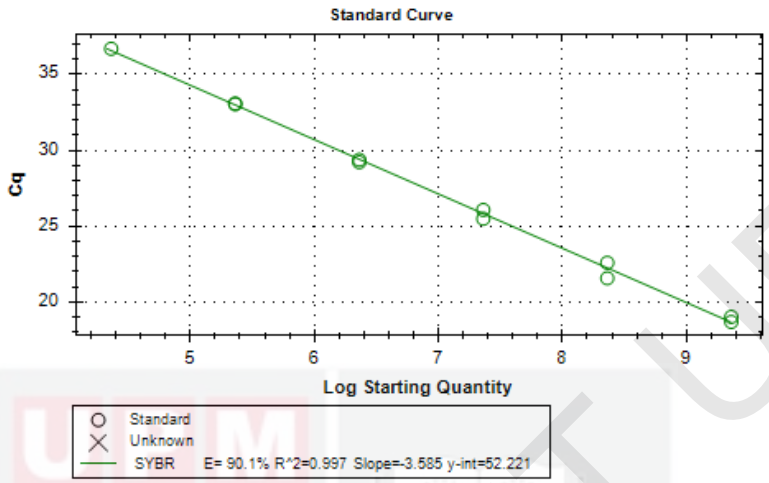
(c)



(d)



(e)



BIODATA OF STUDENT

The author was born on the 5th October 1990 in Besut, Terengganu. She received her primary education at SRJK (C) Chung Hwa Jerteh, Besut and her secondary education at SMK Tengku Mahmud, Besut. During her secondary education, she obtained numerous awards for her participation and contribution in the school's co-curricular activities. She pursued her degree education, majoring in the field of Biology at the Faculty of Science, Universiti Teknologi Malaysia (UTM). During her three-year degree education, she excelled academically and was awarded Dean List Award in all her six study semesters. She graduated with a Bachelor of Science (Hons.) majoring in Biology with First class honors in 2012. In 2013, the author pursued her Doctor of Philosophy in Applied Microbiology at the Institute of Biosciences, UPM under the supervision of her late supervisor, Associate Prof. Dr. Sieo Chin Chin and Prof. Dr. Abdul Rahman Omar, focusing on the studies of applied microbiology, molecular biotechnology and animal nutrition. She has presented her works at several local's conferences. To date, she has published one paper in a journal and three papers in proceedings and obtained two awards for her presentations in conferences and symposia.

LIST OF PUBLICATIONS

Journal

Chan, G. F., Wong, L. L., Noor Aini, A. R., and Fahmi, O. (2012). Novel potential of effective microorganisms as bioattractant of domestic cockroaches in Malaysia. *Insight Microbiology*, (2)1: 1-3.

Proceedings of Paper Presented in Conferences, Symposia, Congress and Seminars

Wong, L. L., Sieo, C. C., Kalavathy, R., and Ho, Y. W. (2013). Effects of commercial oligosaccharides on growth of selected beneficial and non-beneficial gut bacteria of broilers. *World's Poultry Science Association (Malaysia Branch) and World Veterinary Poultry Association (Malaysia Branch) Scientific Conference*, 30 November - 1 December 2013, pp 56-57.

Wong, L. L., Sieo, C. C., Kalavathy, R., and Ho, Y. W. (2013). Growth of beneficial and non-beneficial bacteria in commercial oligosaccharides. *International Congress of The Malaysian Society for Microbiology 2013*, 12 - 15 December 2013, pp 184.

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