DETECTION OF COMMON GUT BACTERIA OF CHICKEN (*GALLUS GALLUS DOMESTICUS*) FED WITH A SUPPLEMENTARY DIET OF *MORINGA OLEIFERA* AND *CURCUMA AERUGINOSA* POWDER

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Detection of common gut bacteria of chicken (*Gallus gallus domesticus*) fed with a supplementary diet of Moringa oleifera and Curcuma aeruginosa powder"** by Eleena Chong, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course DVT 55204 – Research Project.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine in partial requirement for the course DVT 55204- Research Project

The gut microbiome is an important community housed by the digestive tract. Dysbiosis of this community is commonly credited as the source of immunosuppression and disease in the host. Various plant-based supplements have been widely researched to determine the effect of it on this community. This study looks at two common native plants which are *Moringa oleifera* (Moringa) and *Curcuma aeruginosa* (Black ginger) respectively and its effect on an animal model, which is the *Gallus gallus domesticus* (Chicken). This study was conducted to determine the effect that these plants have on the gut microbiome. Established phyla primers were used to identify the presence of these bacteria in the cecal contents of the chicken. The result showed that these common gut bacteria were detected, and these primers were effective in detecting the common gut bacteria phyla-namely *Firmicutes, Bacteroidetes and Proteobacteria*. However, it was inconclusive as to which supplementation was successful in dysbiosis or eubiosis of the gut microbiome. This preliminary successfully identified 3 major phyla of the gut bacteria in all sample groups. Supplementation with 2% moringa and black ginger powder did not yield any difference in the detection using conventional PCR.

Keywords: common gut microbiome, *Moringa oleifera* (moringa), *Curcuma aeruginosa* (black ginger)

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus DVT 55204- Projek Penyelidikan

Mikrobiom usus adalah komuniti penting yang ditempatkan oleh saluran pencernaan. Disbiosis komuniti ini biasanya dikreditkan sebagai sumber imunosupresi dan penyakit dalam perumah. Pelbagai suplemen berasaskan tumbuhan telah dikaji secara meluas untuk menentukan kesannya terhadap komuniti mikrobiam usus ini. Kajian ini melihat dua tumbuhan iaitu *Moringa oleifera* (Kelor) dan *Curcuma aeruginosa* (Halia Hitam/Temu Ireng) serta kesannya terhadap model haiwan, iaitu *Gallus gallus domesticus* (ayam). Kajian ini dijalankan untuk menentukan kesan tumbuhan ini terhadap mikrobiom usus. Primer khusus yang digunakan untuk mengesan phyla bakteria telah dipilih dan digunakan untuk mengenal pasti kewujudan bakteria ini dalam kandungan usus ayam yang diberi suplemen berasaskan Kelor dan Temu Ireng. Hasilnya menunjukkan bahawa bakteria usus biasa ini telah dikesan, dan primer ini berkesan dalam mengesan filum bakteria usus biasa-iaitu *Firmicutes, Bakteroidetes* dan *Proteobakteria*. Walaubagaimanapun, adalah tidak dapat disimpulkan tentang suplemen mana yang berjaya dalam dysbiosis atau eubiosis mikrobiom usus. Pendahuluan ini berjaya mengenal pasti 3 filum utama bakteria usus dalam semua kumpulan sampel. Suplemen dengan 2% serbuk moringa dan halia hitam tidak menghasilkan sebarang perbezaan dalam pengesanan menggunakan PCR konvensional.

Kata kunci: mikrobiom usus, ayam, Temu Ireng, Kelor

1.0 INTRODUCTION

The gut microbiome consists of essential microbial community that plays a crucial role in the health and disease development of the host. It can be considered an 'essential organ' due to its large micro-ecosystem (Ding *et al*.,2019). Some important gut microbes are *Firmicutes, Proteobacteria, Actinomycetes, and Bacteroides*. An imbalance of the microbiome will cause health issues such as obesity, hypertension, cardiovascular disease, diabetes, cancer, inflammatory bowel disease (IBD), gout, and depression (Singh, 2017).

Moringa oleifera is a leafy plant commonly used in Indian cuisines for its ayurvedic benefits. It is native to the Indian subcontinent and relatively easy to cultivate. Dubbed a superfood due to its various health benefits. It has a rich source of multiple vitamins such as A, B, C, D, E, and K. The moringa plant consists of the leaf, pods, seeds, gum, bark, and flowers. All these components are of crucial nutritional value to the usage of the plant in its various therapeutic uses. Moringa has been studied for its different bioactive properties, such as flavonoids, tannins, and saponins (Abdull Razis *et al*., 2014). According to Shang et al. (2018), the gut microbiome can be improved using moringa leaves and can be used as a prebiotic additive in animal feed for the proliferation of beneficial bacteria.

Curcuma aeruginosa, also known as black ginger or wild arrowroot, is a rhizomatous geophyte that grows in the wet tropical biome (Plants of the World Online, 2017). It is also commonly used in Asian medicine for its antibacterial properties (Wahyuni et al., 2017).

C. aeruginosa is also known to be a phytobiotic. A phytobiotic is a natural plant-based substance that has antibiotic properties. Black ginger has an important lipophilic polyphenol compound known as curcumin, responsible for its anti-inflammatory properties. Albeit the pharmacological aspect of curcumin bioavailability remains in question, it has still been widely proposed to be used as a tool for the management of various diseases such as

diabetes and cancer. There have been multiple reports on the interaction of curcumin and the gut microbiome. Interestingly, the concept of bioavailability may not necessarily apply to the gut microbiome as its interaction with functional foods has been proven in various papers. There have been studies that show that it was used as an alternative to antibiotics and "Temu Ireng" was also listed as a growth promoter. It also has certain functions that allow it to be a uterine relaxant. Besides that, it also has anthelmintic properties that was especially effective against ascarides (Hestianah *et al*., 2011).

This project studies the combined effects of both the moringa and black ginger properties on the gut microbiome and the dysbiosis or aerobiosis from applying these supplements in the animal model.

1.1 Research problem

There is a lack of research on the benefits of the combination of the moringa and black ginger supplements on the gut microbiome. It would be interesting to understand the implications of the mixture of moringa and black ginger on the gut microbiome since it plays a vital role in host health for various reasons. Since *M. oleifera* and *C. aeruginosa* supplements are widely available in the commercial market as well as on online platforms, imported from neighboring countries such as Indonesia and Thailand. Their popularity has grown substantially over the course of two years, especially during the pandemic, as people are more aware of the benefits of it. There has been an increased awareness of health implications after the COVID-19 global pandemic in 2019. The usage of curcumin is mainly used for its anti-inflammatory properties, meanwhile moringa is widely used for its effects on gut motility and superfood status (Abdull Razis *et al*., 2014).

1.2 Research questions

1.2.1 Can common gut bacteria be detected after the supplementation of moringa and black ginger powder?

1.2.2 Does moringa and black ginger cause proliferation of beneficial gut bacteria?

1.2.3 What are the effects of moringa and black ginger combination on the common gut bacteria?

1.3 Research hypothesis

1.3.1

Ho: Common gut bacteria cannot be detected after the supplementation of moringa and black ginger.

H_1: Common gut bacteria can be detected after the supplementation of moringa and black ginger.

1.3.2

Ho: There is an effect towards the proliferation of beneficial gut bacteria after the supplementation of moringa and black ginger.

H_1: There is no effect towards the proliferation of beneficial gut bacteria after the supplementation of moringa and black ginger.

1.3.3

Ho: Moringa and black ginger combination affects the common gut bacteria population

H_1: Moringa and black ginger combination does not affect the common gut bacteria population.

1.4 Objectives

This research design aims to identify the effects of *M. oleifera* and *C. aeruginosa* on the gut microbiome portfolio. Specific objectives of this study include:

a) To detect common gut bacteria after supplementation of moringa and black ginger powder.

b) To determine the effect of moringa and black ginger supplement towards the proliferation of beneficial gut bacteria.

c) To measure the effects of moringa and black ginger combination on the common gut bacteria population.

2.0 LITERATURE REVIEW

2.1 Chicken as an experimental model of the gut microbiota

The human colon houses at least 100 trillion microorganisms that make up the gut microbiota, and it makes the colon one of the densest habitats on earth (Rinninella *et al*., 2019). The gut microbiome is the symbiotic component of the gastrointestinal system that consists of various microbial communities. Thus, it makes the colon an independent organ with specific organisms and mechanisms that affect host health and wellbeing (Venema & Van den Abbeele, 2013). According to Wu & Wang (2018), this symbiotic relationship between the gut microbiome and its host is interdependent and coevolved. Two essential and prevalent bacterial phyla of the gastrointestinal tract have been identified: the *Firmicutes* and *Bacteroidetes,* respectively (Venema & Van den Abbeele, 2013).

Domestic chicken, scientifically known as *Gallus gallus domesticus,* is commonly used as a model organism for human biological studies (Oakley *et al*., 2014). Compared to the human microbiome project, there was conservation in metabolic functions despite the taxonomical variation between chickens and humans. Research using metagenomic analysis suggests pattern similarities between the human and chicken gut microbiota, implying that there are correlations in the mechanisms involved in assembling the gut microbiome community (Turnbaugh *et al*., 2009). This can be attributed to lateral gene transfers that occur through selective pressure contributing to pan-genomic functional traits shared by multiple taxa (Qu et al., 2008). Besides that, the cecum of the chicken has the highest microbial density of $1\ x\ 10^{11}\ CFU\ g^{-1}$ thus making it a good organ for microbiome sample collection (Fathima et al., 2022).

Firmicutes and *Bacteroidetes* are essential bacterial markers in determining the condition of the gut microbiome in terms of dysbiosis. Dysbiosis is an imbalance in bacterial composition, distribution, and metabolic activities (DeGruttola *et al*., 2016). *Firmicutes* and

Bacteroides ratio has been cited frequently as obesity biomarkers (Magne *et al*., 2020). The *Firmicute* phyla most commonly break down carbohydrates in the gut, incredibly complex carbohydrates that the host body cannot digest. A typical example of this bacteria is the *Lactobacillus* spp. These bacteria use fermentation to break down carbohydrates to produce beneficial metabolites for the host. Fermented products such as yoghurt, kombucha, and kimchi usually contain this probiotic bacterium. Besides that, this microbe also has metabolic capabilities to make short-chain fatty acids (SFCA) that are crucial in preventing pathogens from disrupting the epithelial barrier function of the intestines (Wang, 2018).

2.2 Application of *16S* rRNA and next-generation sequencing in gut microbiome analysis

The next generation *16S* rRNA sequencing uses high throughput analysis to identify microbial communities (Shin *et al*., 2016). This method also made possible the identification of bacteria that cannot be isolated using traditional culture methods (TCM). That requires specific conditions to proliferate under controlled conditions.

Bacteria, like all prokaryotes, have *16S* rRNA sequences in the conserved region of their DNA. Therefore, using *16S* primers will allow for the identification of a variety of bacterial species in the microbiome (Iyer, 2016). The *16S* rRNA marker is an established gene marker used in gene sequencing to identify bacteria at the species level and assist with differentiation between closely related bacterial species. Application of *16S* rRNA from bacteria requires DNA extraction.

Next, Polymerase Chain Reaction (PCR) is conducted to amplify the *16S* rRNA gene using universal primers. Amplicons are then analyzed using next-generation sequencing (NGS). This provides identification and quantification up to the species level. The

identification of these microbes is done via reference to various databases such as BLAST, SILVA, RDP, and Greengenes (Jovel et al., 2016).

2.3 Use of supplements in promoting host health

Nutrients are the backbone of any living being's existence. The body constantly utilizes nutrients to repair, nourish and sustain life. Dietary guidelines such as Recommended Dietary Allowance (RDA) and Dietary Reference Intakes (DRI) have been frequently used by various countries to formulate appropriate nutrition intake for humans. People who cannot meet the required nutritional requirement usually use dietary supplements to compensate for the inadequacy. When used appropriately, it has the advantage of promoting health and wellbeing. Supplements can be divided into several groups based on their function: bodybuilding, energy-giving, and protection. Gacche (2021), also states that there are various other health claims that these supplements can aid in; this includes cognitive functions, cardiovascular disease, and diabetes prevention, among others.

The Moringa leaves are a good source of dietary selenium and Vitamin A (Lyons *et al.,* 1970). In chickens, selenium is often supplemented to improve meat quality, reducing water loss and prolonging protection from oxidative stressors (Joksimovic-Todorovic *et al*., 2012). Other vitamins such as C and E are essential enhancement of humoral and cellular immune responses due to their role in protecting free radical damage to cells (Nockels, 1988).

Curcumin, an essential lipophilic polyphenol, has very potent anti-inflammatory properties that play a crucial role in the gut microbiota by favouring the proliferation of beneficial strains. This indirectly brings about the improvement in the host's health and immunity. Not only that but curcumin also fortifies the intestinal barrier by restoring disrupted intestinal permeability through the counteracting LPS-induced *IL-1β* that prevents the disruption of regular arrangement in the junction (Ghosh *et al*., 2018).

2.4 Good gut bacteria in the digestive system of chicken and their roles in gut immunity

An imbalance to the microbiome is commonly referred to as dysbiosis. This can be further defined as the quantitative or qualitative change in the normal microbiome composition. Dysbiosis can lead to poor nutrient digestion and poor inflammatory responses (Shang et al., 2018). While the proper balance of the gut microbiota is termed as eubiosis.

Gut bacteria are important in the digestive system. Some important and beneficial gut bacteria in chickens are from the 4 phyla; *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* while the most prevalent genera found was *Clostridium*, *Escherichia coli* *Lactobacillus* and *Bacteroidetes* (Pan & Yu, 2013).

According to Wickramasuriya et al., (2022), gut microbiomes work as barriers in the intestines and ceca and are responsible for the prevention of pathogen proliferation. The gut microbes ferment fiber and digesta producing volatile fatty acid (VFA). These VFA includes acetate, propionate, and butyrate. These essential fatty acids serve as crucial energy sources for intestinal epithelium and directly affects the local immune response. VFA are absorbed through the epithelium via passive diffusion and are used in various metabolic pathways (Pan & Yu, 2013). The direct impact of butyrate demonstrated significant decrease in the production of nitric oxide in macrophages which attributed to the inhibition of pro-inflammatory cytokines such as *IFN-*γ, *IL-6* and *IL-1*β (Fathima et al., 2022).

Gut-associated lymphoid tissue (GALT) is a mucosa-associated component of lymphoid tissue which includes crypt patches, Peyer's patches, and isolated lymphoid follicles (ILF's). These tissues have antigenic capabilities in learning how to differentiate between pathogens and commensal and are one of the key factors in the building of innate immunity (Al-Rashidi, 2022). The presence of commensal bacteria such as the gut bacteria that makes up the gut microbiome aids pattern recognition receptors (PRRs) present in

epithelial and lymphoid cells. These receptors are responsible in ensuring inhibition of inflammatory response and promotion of immunological tolerance of normal microflora. This is an important mechanism to maintain intestinal homeostasis (Rakoff-Nahoum et al., 2004).

2.5 Effect of Moringa on gut microbiome in other animal subjects.

Yasoob et al., (2021), found that the moringa leaf powder was able to relieve oxidative stress and modulate mucosal immune response in rabbits exposed to heat stress and this occurred through the downregulation of mucosal tissue response to inflammation. This is further supported by Khalid et al., (2022), that found supplementation of moringa improved jejunal permeability and digestive functions while positively modulating microbiome composition and mucosal immunity. Another study done by Sugiharto (2018), indicated that when used in combination with garlic powder proved to be useful in the reduction of abdominal fat deposition and coliform bacteria in the ileo-cecum of chickens.

In *Penaeus vannamei* or more commonly known as whiteleg shrimp supplemented with moringa had shown improved growth and immunity in terms of intestinal microbiota modulation. The whiteleg shrimp also showed enhanced resistance against *Vibrio algynolyticus* and *Vibrio parahaemolyticus* which are important primary pathogens that causes economical losses in shrimps (Abidin et al., 2022).

Moringa has frequently been cited for usage as fodder in livestock feeding (Sun et al., 2017). In chicken, it was found to improve layer egg quality and broiler meat production. There was significant increase in daily weight gain due to the increase in muscle fiber diameters indicatior of increased protein deposition (Mahfuz & Piao, 2019). Nathaniel (2021) mentions that moringa leaf powder is a safe alternatives to antibiotic growth promoters due to the significant decrease in coliform growth.

2.6 Effect of Black Ginger on gut microbiome in other animal subjects.

Black ginger is most researched in Indonesia for its benefits. It has many similar beneficial polyphenols as its other ginger cousins. Many researchers have claimed its use for anti-inflammatory properties. According to Wang (2020), temu ireng supplementation in a simulation of in-vitro digestion and fermentation showed an abundance of *bifidobacteria* (phylum: *actinobacteria*) growth, which is a significant bacterium in gut eubiosis.

In humans, it was found that *Pseudomonas aeruginosa* virulence was controlled by supplementing black ginger extract (Pangastuti et al., 2020).

3.0 MATERIALS AND METHODS

3.1 Ethics Statement

The animal research was conducted at the experimental animal laboratory of the Faculty of Veterinary Medicine (FPV), University Malaysia Kelantan (UMK). The research obtained approval of institutional animal care and use committee (IACUC) to conduct research involving animals. The approval code obtained was UMK/FPV/ACUE/FYP/021/2022.

3.2 Animals, Rearing Conditions and Experimental diet

The animal research study was conducted on 25 birds. The local breed used was 'Ayam Kacuk Kampung' (AKK) also known as SASSO broiler chicken. All the chickens were male, brown broilers purchased at the age of 21 days old from a local farmer to ensure their gut microbiome is stable and mature (Kers et al., 2020; Bindari & Gerber, 2022). Male chickens were used to prevent any hormonal interference in the study and to prevent any gender biasness (Cui et al., 2021).

These chickens were randomly separated into 4 groups as listed in ***Table 1.*** Group 1, 2 and 3 had 6 birds respectively, while Group 4 (control) had 7 birds. The extra birds in group 3 were held as spare just in case there were any losses during the experimental period. Moringa and black ginger supplements were administered through oral route mixed with the feed for 21 days until appropriate slaughter age. The dosage given was at 2% per day for each bird (Mahfuz & Piao, 2019). The feed given was Broiler Grower feed with 202P Gold Coin brand (Appendix 1) in pellet form. Before the start of the experimental period, the birds were first acclimatised to the new enclosure/cage for 4 days.

Table 1 Groupings of chickens and the amount of supplements given respectively.

Group	Function	Amount and type of supplement (per bird)
1	Moringa	100g pellet feed + 2g Moringa powder (2%)
2	Black Ginger	100g pellet feed + 2g Curcumin Powder (2%)
3	Moringa and Black Ginger	100g pellet feed + 1g Curcumin Powder (1%) + 1g Moringa Powder (1%)
4	Control	100g pellet feed

The feeding regime was maintained at 98% feed pellet and 2% supplement powder from the age of 21 days old until 42 days old adult. Table 1 shows the average amount of feed required by adult chickens. The feed was increased as the age of the chickens increased.

3.3 Sampling

After 21 days of supplementation, the chickens in all groups were culled. The fresh carcasses were immediately brought to the post-mortem lab of the University where the

cecum was carefully extracted through necropsy. A clear string was tied at both ends of the cecum as shown in **Figure 1** and the whole section was removed from each bird and placed into individual sample storage bags labelled with the ID of each bird. The harvested samples were stored at -20°C.

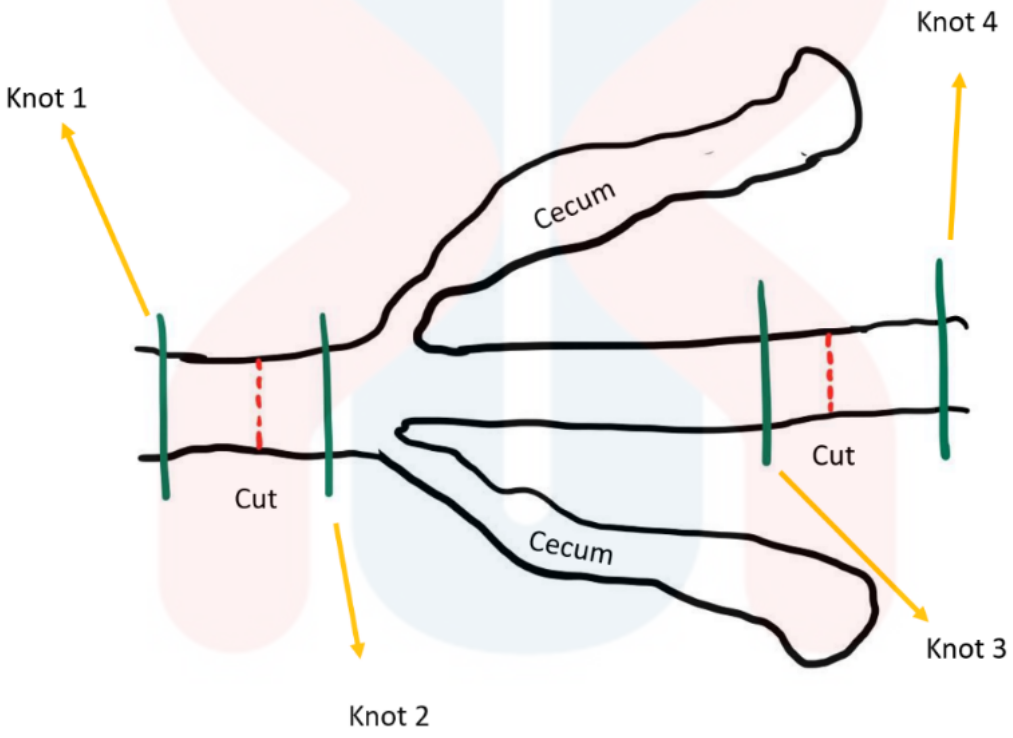


Figure 1: Harvesting of the cecum using necropsy techniques.

3.4 Sample preparation

3.4.1 Caecal contents

The caecal contents were milked from the cecum and the mucosa was also scraped using scalpel blades and placed into bead tubes for DNA extraction.

3.4.2 Extraction and identification

3.4.2.1 Deoxyribonucleic acid (DNA) Extraction

DNA extraction was carried out using Canvax HigherPurity™ Stool DNA Isolation Kit (Canvax Biotech, Spain). The procedure was done following manufacturer's recommendation with several modifications to improve on the yield.

First, the scraped caecal contents samples were weighed and 200mg of sample was transferred into a Bead Tube and placed on ice. 500 µl of Lysis Solution 1 (LS1) and 30 µl of Proteinase K (30mg/ml) and gently vortexed for 5 minutes to ensure that it was completely homogenised. These caecal samples were then incubated at 65℃ for 12 hours. After incubation, the mixture was spun down to remove any water droplets within the lid. The rest of the protocol is the same as indicated in the support protocol. These steps were repeated for each sample that were taken from each bird.

3.4.2.2 Determination of Purity and Concentration of extracted DNA

The extracted DNA was then validated by a Nanophotometer (Nanodrop Technologies, Wilmington, DE) to examine for quality control (QC) and determine the concentration and quality of the DNA extracted using the OD260/280 ratio and 260nm

wavelength (ng/ µl) respectively. 1 µl of AE Buffer was used as a blanking solution to calibrate the Nanophotometer. Next, 1 µl of extracted sample was pipetted and measured. Ideal range for the purity is 1.7-2.0 at OD 260/280. The data was recorded, and the samples were stored at -20˚C freezer.

3.4.2.3 Polymerase Chain Reaction (PCR) Amplification of Phyla specific for identification of common gut bacteria

Polymerase Chain Reaction (PCR) was done to amplify DNA that was extracted. This was done using C1000 Touch TM 96 well Thermal Cycles (Bio-Rad Laboratories, USA)

The primers were diluted to working concentration of 10Mm. Altogether 4 primers were used, the sequences are available in Table 2 respectively. The PCR reaction were prepared by adding 12.5 µL Master Mix (Promega GoTaq® Green Master Mix),1 µL of forward and reverse primer set, 2 µL DNA template and 8.5 µL Nuclease Free Water. The final volume is 25 µL. Negative control containing NFW as replacement for the DNA templates were included for each Primer used. Thermal gradient PCR was first done to obtain appropriate temperatures for primer optimisation of each primer. 4 sets of published phyla-based primers for *Proteobacteria, Bacteroidetes, Firmicutes* and *Actinobacteria* were used in the PCR amplification with the cycling conditions listed in Table 2 below:

Table 2. Oligonucleotide sequence used for PCR identification of common gut bacteria and their respective cyclin conditions.

No.	Targets	Primers (5' – 3')		Reference
1.	**Bacteroidetes**	Forward: Reverse:	CCGGAWTYATTGGGTTTAAAGGG GGTAAGGTTCCTCGCGTA **Cycling condition:** • One cycle for initial denaturation (95˚C for 5 min); • 35 cycles for denaturation, annealing and extension. o 95˚C for 30s denaturation; o 50˚C for 30s annealing; o 72˚C for 30s extension, • One cycle (72˚C for 5 min) for final extension of amplified DNA	(Mühling *et al*., 2008)
2.	**Actinobacteria**	Forward: Reverse:	ACGGGCGGTGTGTACA TCCGAGTTRACCCCGGC **Cycling condition:** • One cycle for initial denaturation (95˚C for 5 min); • 35 cycles for denaturation, annealing and extension. o 95˚C for 30s denaturation; o 50.7˚C for 30s annealing; o 72˚C for 30s extension, • One cycle (72˚C for 5 min) for final extension of amplified DNA	(Blackwood *et al*., 2005)
3.	**Firmicutes**	Forward: Reverse:	GGCAGCAGTRGGGAATCTTC ACACYTAGYACTCATCGTTT **Cycling condition:** • One cycle for initial denaturation (95˚C for 5 min); • 35 cycles for denaturation, annealing and extension. o 95˚C for 30s denaturation; o 50.7˚C for 30s annealing; o 72˚C for 30s extension, • One cycle (72˚C for 5 min) for final extension of amplified DNA	(Mühling *et al*., 2008)
4.	**Proteobacteria**	Forward: Reverse:	CMATGCCGCGTGTGTGAA ACTCCCCAGGCGGTCDACTTA **Cycling condition:** • One cycle for initial denaturation (95˚C for 5 min); • 35 cycles for denaturation, annealing and extension. o 95˚C for 30s denaturation; o 52˚C for 30s annealing; o 72˚C for 30s extension, • One cycle (72˚C for 5 min) for final extension of amplified DNA	(Mühling *et al*., 2008)

3.4.2.4 Gel Electrophoresis

Gel Electrophoresis was conducted using 2% (w/v) agarose gel that was made using 1.4 g of Agarose powder that was mixed in 70 ml of TBE buffer. The mixture was then heated in a microwave for 1 minute and then 1 µL of Midori green (Nippon Genetics, Europe) was added into the agar mixture for better visualisation. The agar was then poured into mould fit with 15 combs.

5 µL of 1kbp DNA Ladder (Promega, USA) was used as a molecular weight marker and it was pipetted into the first well and the negative control was pipetted into the last well. The rest of the samples were pipetted into each well, noting the placement of each sample. Voltage that was used to run the gel was 100 V and 400 mA for 40 minutes. The stained agar was then viewed using a Gel Doc™ EZ Imager (Biorad, USA) UV illuminator to validate the primer products according to the target band size.

4.0 RESULTS

4.1 Molecular identification of common gut microbiome bacteria

25 samples were analysed using molecular technique. Primers used were specific to amplify the *16S* rRNA gene fragments within the phyla of common gut bacteria; *Proteobacteria, Bacteroidetes, Firmicutes* and *Actinobacteria*. Phyla primers were chosen to amplify whole genetic diversity of the bacterial domains thus targeting a larger number of bacteria that fall within the phylogenetic clade of each primer.

In this study, all samples showed positive results for *Proteobacteria, Bacteroidetes* and *Firmicutes* in all groups with exceptional to *Actinobacteria*. This is indicated by the presence of bands on the agar. The intensity of the bands varied among groups. The product sizes for *Bacteroidetes, Firmicutes* and *Proteobacteria* were 500 bp, 800 bp and 750 bp respectively.

Figure 2: The amplification of *Bacteroidetes* showed positive results for Group 1 and 2. (Product Size: 500bp)

Keys: M-100bp Plus DNA Ladder as Marker; 1,2- groups 1 and 2; Blk,Y, W, WB,B,R= Samples

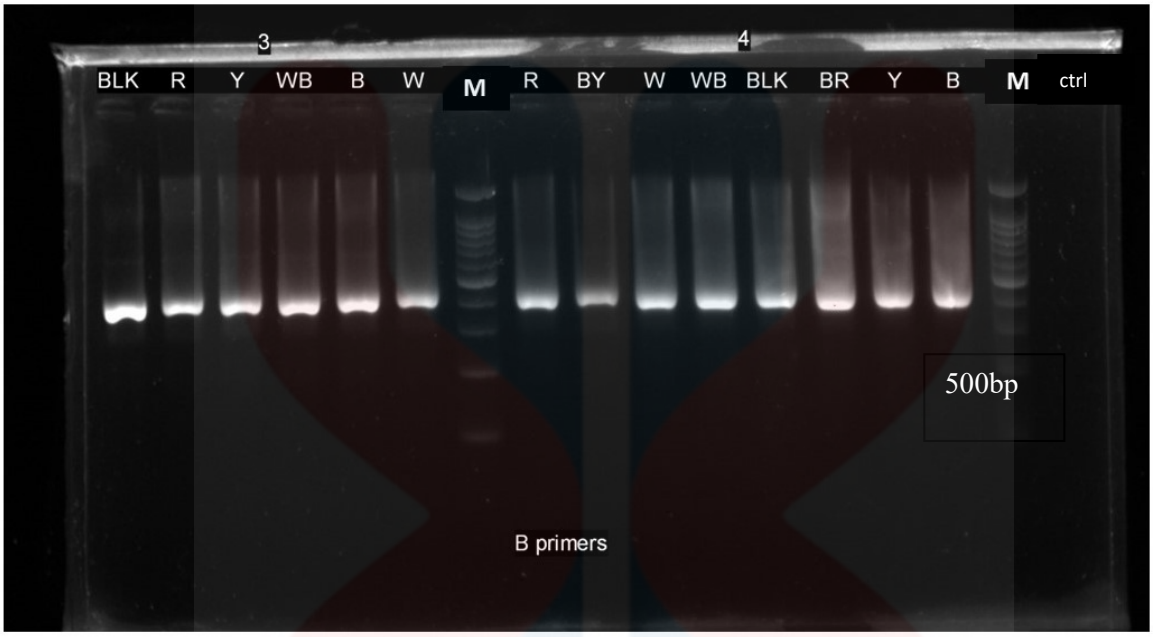
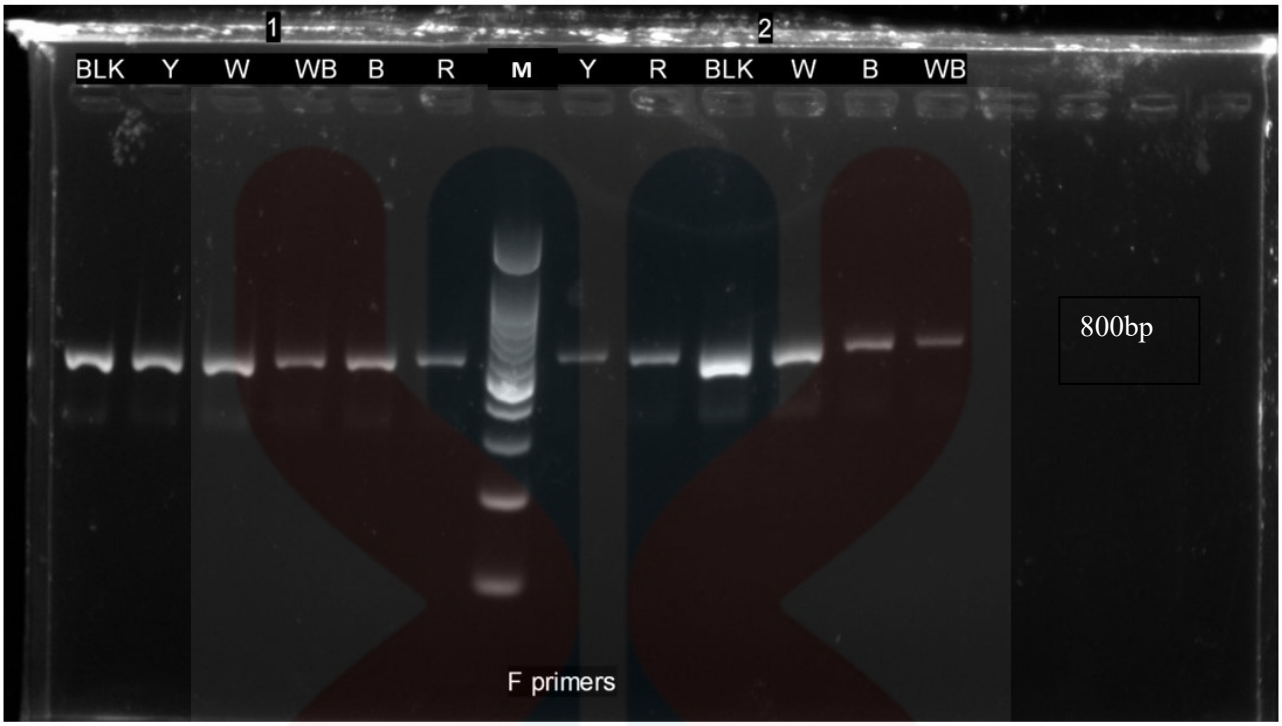


Figure 3: The amplification of *Bacteroidetes* showed positive results for Group 3 and 4.

(Product Size: 500bp)

Keys: M-100bp Plus DNA Ladder as Marker; ctrl- Negative control; 3,4- groups 3 and 4; Blk, Y, W, WB, B, R, BR BY = Samples



1

Figure 4: The amplification of *Firmicutes* showed positive results for Group 1 and 2. (Product Size: 800bp)

Keys: M-100bp Plus DNA Ladder as Marker; 1,2- groups 1 and 2; Blk, Y, W, WB, B, R= Samples

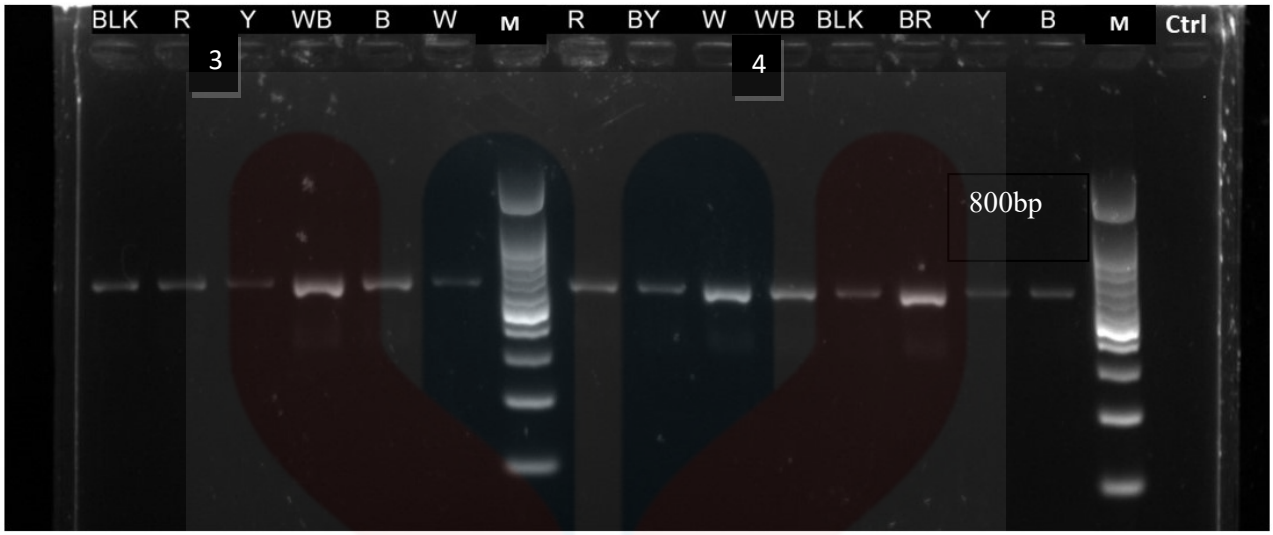


Figure 5: The amplification of Firmicutes showed positive results for Group 3 and 4. (Product Size: 800bp)

Keys: M-100bp Plus DNA Ladder as Marker; 3,4- groups 3 and 4; Blk, Y, W, WB, B, R, BR BY = Samples; ctrl- control

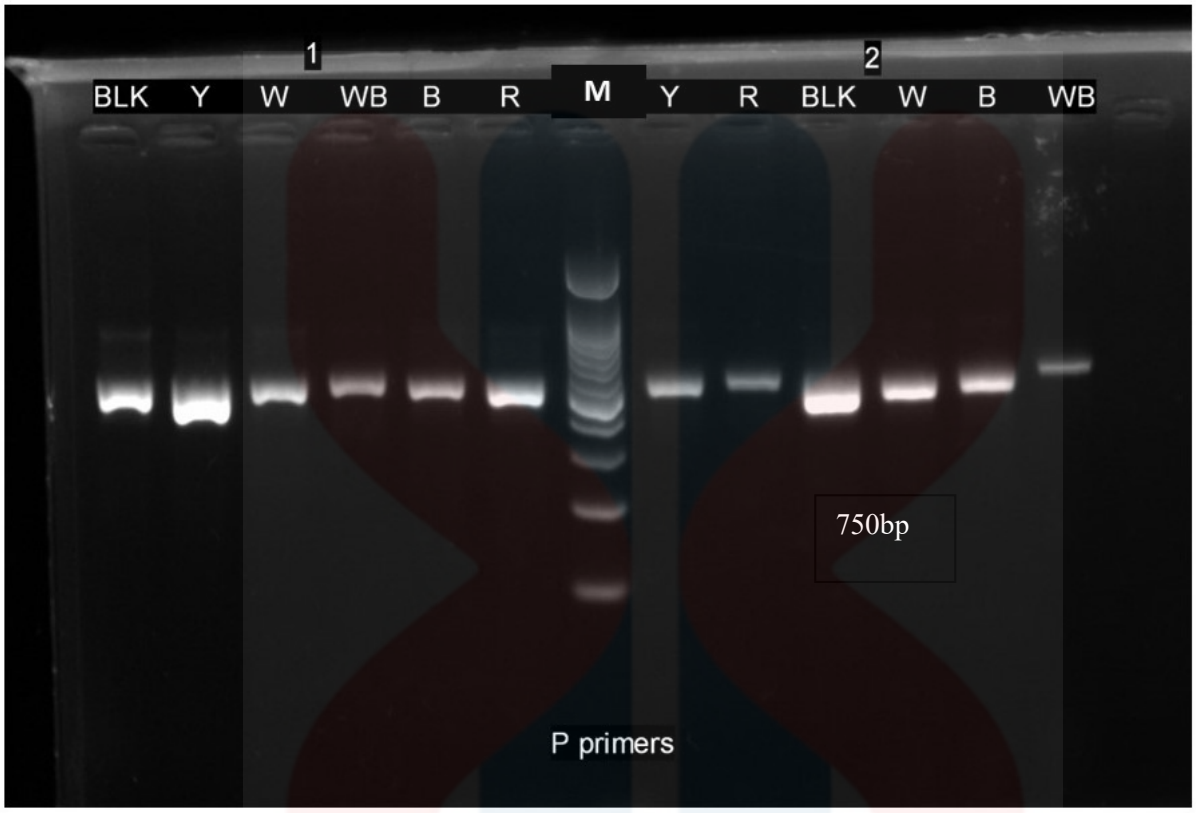


Figure 6: The amplification of *Proteobacteria* showed positive results for Group 1 and 2. (Product Size: 750bp)

Keys: M-100bp Plus DNA Ladder as Marker; 1,2- groups 1 and 2; Blk, Y, W, WB, B, R= Samples

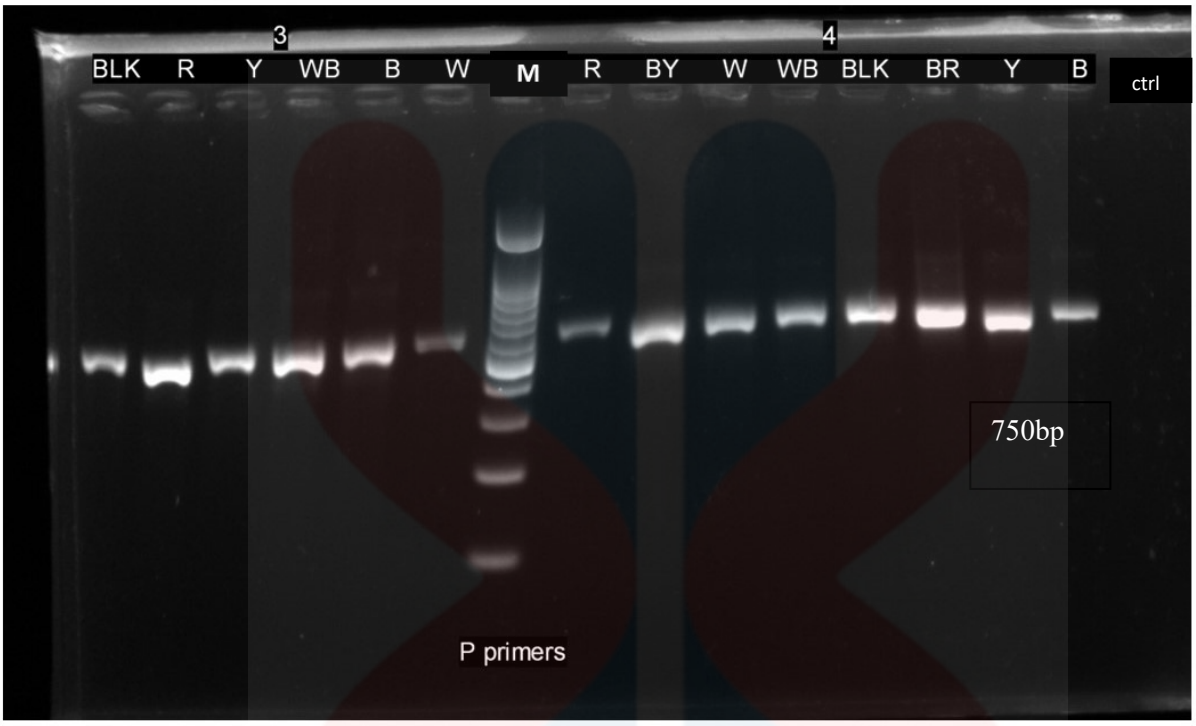


Figure 7: The amplification of *Proteobacteria* showed positive results for Group 3 and 4. (Product Size: 750bp)

Keys: M-100bp Plus DNA Ladder as Marker; ctrl: Negative control; 3,4- groups 3 and 4; Blk, Y, W, WB, B, R, BR BY = Samples

Table 3 Results for PCR ; Key for individual bird identity: Y, R, WB, Blk, B, W, BY, BR; Y: Yellow, R: Red, WB: White Blue, Blk: Black, B: Blue, W: White, BY: Black Yellow

Experimental Groups		**Gut bacteria**			
		Bacteroidetes	**Firmicutes**	**Proteobacteria**	**Actinobacteria**
Group 1 (Moringa) (n=6)	**1Y**	+	+	+	-
	1R	+	+	+	-
	1WB	+	+	+	-
	1Blk	+	+	+	-
	1B	+	+	+	-
	1W	+	+	+	-
Group 2 (Black ginger)	**2Y**	+	+	+	-
	2R	+	+	+	-
	2WB	+	+	+	-
	2Blk	+	+	+	-
	2B	+	+	+	-
	2W	+	+	+	-
Group 3 (Moringa and black ginger)	**3Y**	+	+	+	-
	3R	+	+	+	-
	3WB	+	+	+	-
	3Blk	+	+	+	-
	3B	+	+	+	-
	3W	+	+	+	-
Control	**4Y**	+	+	+	-
	4 R	+	+	+	-
	4WB	+	+	+	-
	4Blk	+	+	+	-
	4B	+	+	+	-
	4BR	+	+	+	-

5.0 DISCUSSION

The intestinal flora also known as the gut microbiome consists of various phyla of bacteria. According to Rinninella *et al*., (2019), it consists of at least 100 trillion microorganisms. Ren *et al*., (2019), mentioned that various phytobiotics can be used as alternatives to antibiotics, and it can aid in the proliferation of good gut bacteria thus improving host intestinal flora. A well-balanced intestinal flora provides better immunomodulation of the host, but it remains understudied if these effects are due to specific strains of bacteria or the modified and specific microbiota of the host (Ren *et al*., 2019). There are various plant extracts and powders that are currently being studied to view potential phytobiotic, prebiotic and probiotic values. The usage of moringa and black ginger in this paper paves way for further research in the usage of these supplements in the proliferation of the gut microbiota of humans and animals. It can be used as additional supplements for both humans and animals.

The samples that were used were harvested from the cecum. The cecum is a pair of tubes that empties every 24 hours (Clavijo & Vives, 2017). It is a major site of cellulose, starch, and polysaccharide digestion (Clench et al., 1995). In comparison to fecal microbiotas, caecal microbiotas have richer Operational Taxanomic Unit (OTU) and is a better representation of microbiota proportions in the chicken. The OTUs are mathematical constructs that is commonly used in the description of bacterial communities. (Lladó Fernández *et al.,* 2018).

Proteobacteria, Firmicutes and *Bacteroidetes* make up 90% of the gut microbiome. According to Clavijo & Flórez (2018), the most predominant phyla is *Firmicutes* followed by *Bacteroidetes, Proteobacteria* and *Actinobacteria* respectively. This is further supported by this paper through the intensity and thickness of the bands produced through PCR that

estimates the relative abundance faint bands indicate a smaller value of molecules and vice versa (Luo et al., 2016). Overall, it was generally noted that for all 3 primers, the combination of moringa and black ginger produced the most vivid bands. Thus, indicating a higher abundance of each phylum.

The extracted DNA was checked for its purity and concentration using Nanophotometry. The parameters which the device detects are the concentration, nanogram per microliter (ng/µL), and the ratio of absorbance at 260 nm and 280 nm respectively. OD260/OD280 ratio of 1.8 to 2.0 general rule of thumb that indicates good purity of the DNA extracted. Meanwhile, the ratio of absorbance at 260 nm and 230 nm (OD260/OD230) of less or equal to 1.6 indicates the presence of proteins, phenol, and other contaminants (Lucena-Aguilar *et al.,* 2016). The principle of this ratio uses the principle of spectrophotometry. 1 µL of the sample is exposed to ultraviolet-visible spectrum (UV-Vis). Nucleic acids will absorb light at 260 nm while purified proteins will absorb light at 280 nm. Thus, this would indicate the purity of the sample (*Thermo Scientific NanoDrop One Product Launch Video*, 2015). The result in this study produced variable results. Most of them samples procured values above 1.7. These results indicated that the extracted product was suitable for sequencing as it had passed the Quality Control test.

The primers, as listed in Table 2 was acquired from research papers. These primers target phyla specific region in the *16S* rRNA region of the bacteria. The primers chosen were run through the BLAST database to pick out appropriate and valuable primers. There were several parameters that were used to narrow down the selection of primers, and a good indicator for good primers. This includes a GC content between 40 – 60%. A length of approximately 19 – 30 bases, melting temperature (TM) of 55 – 75℃ and without intra-primer homology to prevent the forward and reverse primers from complementing sequencing causing it to have primer dimers (The, 2019). The protocol used for PCR is seen

in Table 2. The annealing temperature varies and was determined by thermal gradient that used a temperature range between 50 to 60˚C. The importance of this is for cross checking the primers with the databases before submission for synthesis and to ensure that each sequence belongs to the phyla of interest.

Firmicutes was found positive for all groups. Firmicutes is the most dominant phylum in the cecum. An important bacterium under this phylum is the *Lactobacillus sp*. Other beneficial bacteria include *Eubacterium, Faecalibacterium* and *Anaerostipes* (Edermaniger, 2021). These are all probiotic bacterium that promotes short chain fatty acids and lactates that facilitates the disruption of pathogens. There are pathogenic species such as the *Staphylococcus aureus* and *Clostridium perfringens* in this phylum.

Proteobacteria was also found positive in all groups. *Proteobacteria* have low abundance in the gut of healthy humans, however in chickens, it is the common colonising phyla. Some important bacterium within this phylum includes *Escherichia coli*, *Helicobacter*, *Campylobacter* and *Salmonella*. This phylum specifically uses proteins as its major source of energy. Some bacteria such as the *Desulfovibrio sp*. and *Parasuttuerella sp*. are important in the removal of free hydrogen that forms in the gut due to fermentation (Rychlik, 2020).

Bacteroidetes was also found positive in all groups. *Bacteroidetes* are important in the metabolization of polysaccharides and oligosaccharides that provide important vitamins and nutrition to the host and other microbes residing in the intestines. The *Bacteroides sp*. is one of the most important species in the production of butyrate acid.

For gel electrophoresis, the target bands for all the primers were achieved, except for *Actinobacteria*. Considering that the primer targets an entire phylum and actinobacteria is not an obscure or rare phyla in the gut microbiome, therefore the expectation is that it should have had some result. However, this primer did not have any yield at all indicating that this

pair may have been faulty. Other reasons could be that the primers published may have undergone editorial error which resulted in the primer sequence being wrong.

Since all the groups yielded positive for *proteobacteria, bacteroidetes* and *firmicutes*, it does not clearly indicate which feeding regime causes eubiosis or dysbiosis when compared to the control diet. The suggested dosage was between 1- 10%, perhaps increasing the dose to 7 or 8% may yield clearer results and exert the true potential of the super plant.

However, it was noticed that even at 2% it was difficult for the chicken to eat the pellet that was coated with the powder. A better method of experimenting would be to re-pellet the feed instead of coating the pellets with the powdered supplements to ensure that all of the supplements are digested by the chicken. Another method would be to make the aqueous extract of the supplements can be supplement in the water.

Through conventional PCR, quantification cannot be done hence, better quantification method such as the qPCR can be carried out to determine the significance of each phylum. Results are anticipated to differ if quantification techniques are done. Group 3 is expected to have a significant difference.

Positive control was not included. To include it, the positive samples can be sent for sequencing to confirm the sequence. The sequence can then be compared to BLAST database to ensure the sequence is correct. The sample can then be used as positive control for future uses of the primer.

The information gathered through literature review gives us a foundation of compelling evidence that indicates that these supplements do alter the microbiota and improve the immune system in various animal models and livestock. Supplementation of the powders into the feed improves protein levels and improve gut health in chicken thus making

them more resistant to diseases. Another rough observation made was that the chickens in groups 1, 2 and 3 ate lesser pellet compared to group 4 but had significantly grown in size comparable to group 4. This may be due to the protein levels of the supplements that keep the chickens full for a longer period of time.

The usage of moringa and black ginger in this paper paves way for further research on the effects of these supplements and their differing concentration on both animal and human gut microbiota as there is some unconfirmed evidence in the proliferation of the gut microbiota. This can be further confirmed through sequencing.

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This preliminary study successfully detected *Bacteroidetes, Firmicutes* and *Proteobacteria* from the cecal samples of birds fed with this supplement through phyla-specific primers. Hence the gut microbiome was not affected through the feeding of *Moringa oleifera* and *Curcuma aeruginosa*. In contrast, there was no trace of actinobacteria which may indicate susceptibility to the supplements. Further clarity on the poultry gut microbiota dynamics in response to supplementation requires more sensitive detection and quantification techniques such as the qPCR and *16S* sequencing to determine eubiosis or dysbiosis of each group.

6.2 RECOMMENDATION

For future studies, a different primer for actinobacteria should be chosen from a published paper and the sequence should first run through BLAST before primer synthesis. Next, *16S* sequencing is recommended to further quantify and identify the individual common gut bacteria within each phylum. Thirdly, since thermal gradient obtained good results, these primers can be used for sequencing. Due to budget constraints, this could not be done; however, it can be included in future studies. Lastly, increasing the sample size to at least 50 birds will also give meaningful results that can be used in other research.

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Appendix A

Appendix 1: Feed that was used – Broiler Grower feed (Gold Coin Brand)

Appendix 2: feeder in which the feed and supplements were fed

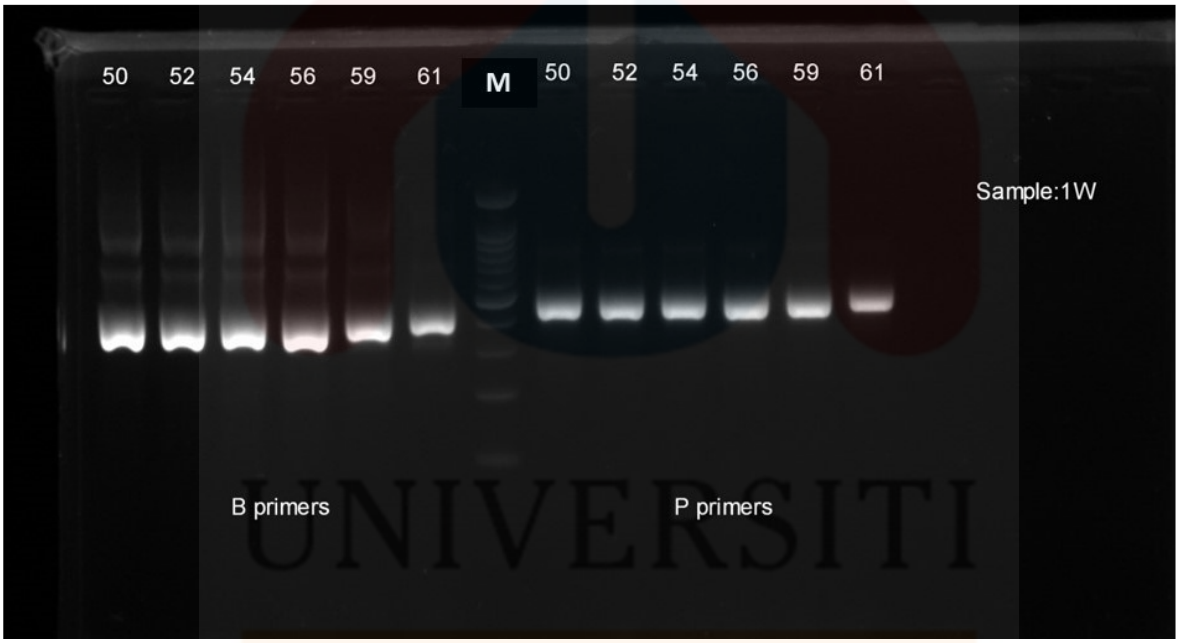
Appendix 3: Cage set up for the broiler chicken (Photo credit: Dr Ali Imran)

Appendix 4: Sasso Broiler Chicken

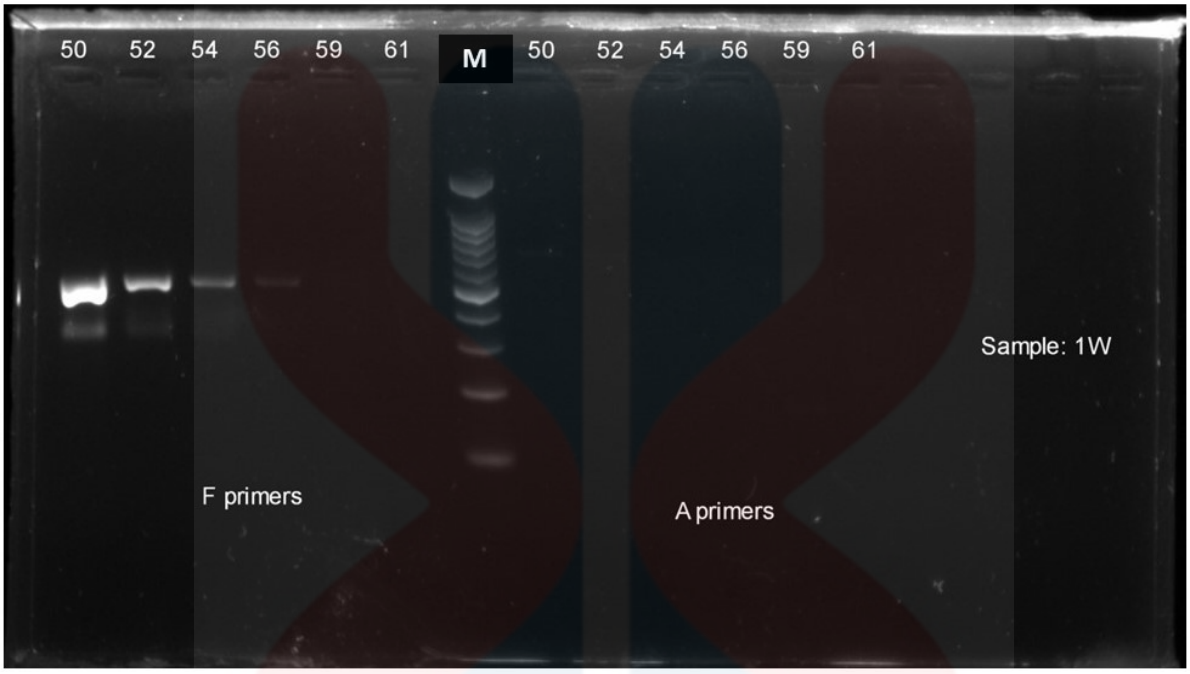
NO.	Sample ID	Nucleic Acid (ng/µl)	OD260/OD280	OD260/OD230
Group 1				
1	1BLK-C	153.2	2.02	1.82
2	1Y-C	236.6	2.01	1.76
3	1W-C	177.2	2	1.8
4	1WB-C	137.6	1.96	1.35
5	1B-C	160.9	1.94	1.61
6	1R-C	194.5	1.85	1.3
Group 2				
7	2Y-C	66.2	2	1.35
8	2R-C	41.7	2	1.38
9	2BLK-C	93	1.94	1.37
10	2W-C	43.2	1.87	1.37
11	2B-C	42.4	1.86	1.02
12	2WB-C	72.6	1.72	1.12
Group 3				
13	3BLK-C	198.7	2.08	2
14	3R-C	187.2	2.03	1.83
15	3Y-C	104.3	1.98	1.63
16	3WB-C	44.8	1.95	1.31
17	3B-C	81.7	1.93	1.29
18	3W-C	329.1	1.91	1.39

Group 4				
19	4R-C	64.8	2.06	1.78
20	4BY-C	219.5	1.99	1.67
21	4WB-C	27.2	1.97	1.35
22	4BLK-C	64.5	1.89	1.44
23	4BR-C	74.6	1.85	1.31
24	4Y-C	28.4	1.83	1.05
25	4B-C	73	1.75	1.25

Appendix 5 Nanophotometer results arranged using 260/280 values (from high to low)



Appendix 6 Thermal Gradient results for Bacteroides and Proteobacteria



Appendix 7: Thermal gradient results for Firmicutes and Actinobacteria

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Fakulti Perubatan Veterinar

RUJ. KAMI *(Our Ref.)* : UMK/FPV/ACUE/FYP/021/2022
TARIKH *(Date)* : 13 SEPTEMBER 2022

DR. ERKIHUN AKLILU WOLDERGIORGIS
Main Supervisor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan

Dear Dr,

APPROVAL OF INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) TO CONDUCT RESEARCH INVOLVING ANIMALS

We are pleased to inform you that your application for approval to conduct research from Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Universiti Malaysia Kelantan has been approved. Please refer the table below for approval code:

APPROVAL CODE	*UMK/FPV/ACUE/FYP/021/2022*
TITLE	*DETECTION OF COMMON GUT BACTERIA OF CHICKEN (GALLUS GALLUS DOMESTICUS)*

2. Please be noted for the Final Year Project, you are responsible to supervise your student to conduct all animal-related procedures as stated during ethic application. The co-supervisor(s) for the project are encouraged to help with the procedures as well.

3. You are advised to always follow "3R" (REDUCE, REFINE, & REPLACE) and all animal ethics and animal welfare principles to reduce suffering in animal.

Thank you.

"RAJA BERDAULAT, RAKYAT MUAFAKAT, NEGERI BERKAT"
"WAWASAN KEMAKMURAN BERSAMA 2030"
"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

(DR. NURSHAHIRAH BINTI SHAHARULNIZIM)
Chairman
Institutional Animal Care and Use Committee
Faculty of Veterinary Medicine

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Appendix 8: Animal Ethics Approval